

**Transcriptomics and gene regulatory network inference to
identify key regulators of *Lactuca sativa* disease resistance**

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Abstract

Plant pests and pathogens are responsible for a large proportion of crop losses. Particularly devastating are generalist necrotrophic fungal pathogens like *Botrytis cinerea* and *Sclerotinia sclerotiorum* which affect many economically crucial crops, including lettuce. Due to the environmental and economic implications of pesticide use and the rise of fungicide-resistant strains, there's an imperative need to develop disease-resistant crop varieties.

In this work, several high-throughput transcriptomic datasets are utilised to identify candidate genes which could be manipulated to develop disease-resistant lettuce cultivars. Firstly, I used data which assessed the pathogen susceptibility and transcriptomes of 114 lettuce samples from 27 diverse accessions post-infection with *S. sclerotiorum* or *B. cinerea*. This revealed over 5,000 lettuce genes whose expression correlated with *S. sclerotiorum* resistance across the diversity panel.

In addition, two high-resolution time-series datasets of the transcriptomic response to *B. cinerea* and *S. sclerotiorum* infection in lettuce leaves, identifying a core set of 4,362 genes which are differentially expressed in the same direction in response to both pathogens.

Utilising all four transcriptomic datasets, I inferred a causal gene regulatory network (GRN), highlighting “hub genes”, key transcription factors which are integral to transcriptional reprogramming upon infection. We selected six of these hub genes to validate their *in planta* defence function. Four of these lettuce hubs altered *B. cinerea* resistance when constitutively expressed in Arabidopsis or lettuce. Furthermore, the predicted GRN targets genes regulated downstream of a hub gene *in planta* with higher accuracy than either random-guessing or co-expression modules.

This work, therefore, demonstrates a significant advancement in our understanding of defence-induced transcription reprogramming in a crop species. We have been able to successfully predict hub genes and validate their role in the defence response. These results demonstrate that GRNs can be used to identify key regulators of the response to plant stresses in non-model species.

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Author's Declaration

I declare that this thesis is a presentation of original work and I am the sole author (unless stated otherwise, see co-author contributions). This work has not previously been presented for a degree or other qualification at this University or elsewhere. All sources are acknowledged as references.

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Co-author Contributions

Chapter 1 and 5 are all my own work.

Chapter 2 has been peer-reviewed and published in Theoretical Applied Genetics (Pink et al. 2022). I performed all bioinformatic and data analysis for the manuscript (except initial read mapping and mapping population genotyping), which includes polytunnel analysis (Figure 2.5), gene expression-lesion size correlations (Figure 2.9, 2.11, 2.12), GO-term enrichment (Figure 2.10), QTL mapping (Figure 2.14, 2.16) and differential expression between mapping population parents (Figure 2.17, 2.18). I also generated all figures for the manuscript and wrote the full manuscript with editing and input from Katherine Denby. Experimental work by co-authors prior to my PhD included generating lettuce-diversity panel RNAseq reads, phenotyping a 96-accession lettuce diversity panel and 230 RIL mapping population for *B. cinerea* and *S. sclerotiorum* susceptibility.

Chapter 3 has been uploaded to bioRxiv preprint server (Pink et al. 2023), and has been submitted to Plant Cell. Work by co-authors prior to my PhD generated the lettuce-*B. cinerea* time series RNAseq data, identified differentially expressed genes, their time of first differential expression (TOFDE) and overlap between *B. cinerea* and *S. sclerotiorum* induced transcriptional reprogramming. For this manuscript, I performed the co-expression modelling (Figure 3.9), functional analysis of co-expression modules (DNA-binding elements and GO-enrichment- Figures 3.10, 3.11), gene regulatory network inference (Figure 3.13) and all analysis of the gene regulatory network (Figures 3.14, 3.15, 3.16), as well as the generation and phenotyping of transgenic Arabidopsis lines (Figures 3.19, 3.20). Under my supervision, undergraduate students, Oliver Cooper and Rebecca Law, assisted with the generation and phenotyping of LsBOS1 transgenic Arabidopsis lines. I also generated all figures for the manuscript and wrote the full manuscript with editing and input from Katherine Denby.

Chapter 4 will be submitted for publication a later date. LsERF1 was cloned by Elspeth Ransom and LsERF1 transgenic lettuce lines were generated at UC Davis transformation facility. I performed all other work for this manuscript including *B. cinerea* phenotyping and RNAseq analysis of the LsERF1 lettuce transgenic plants as well as the generation and phenotyping of Arabidopsis transgenic lines used. Under my supervision, Oliver Cooper and Rebecca Law, assisted with the generation of LsWRKY7B transgenic lines. I also generated all figures for the manuscript and wrote the full manuscript with editing and input from Katherine Denby.

Abbreviations

ABA	Absciscic Acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AOC	allene oxide cyclases
AOS	allene oxide synthases
AUDPC	Area under the disease progression curve
bHLH	basic helix-loop-helix
BOA	botcinic acid
BOT	botrydial
bZIP	basic-leucine zipper
CaM	calmodulin
CAMTA	calmodulin-binding transcription activator
CaMV	Cauliflower Mosaic Virus
CBP60g	CALMODULIN-BINDING PROTEIN 60-LIKE g
CDPKs	calcium-dependent protein kinases
CE	Carbohydrate esterase
CERK1	Chitin Elicitor Receptor Kinase 1
ChIP-seq	chromatin-immunoprecipitation sequencing
COI1	Coronatine insensitive 1

CPM	counts per million
CRE	cis-regulatory element
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CWDE	cell wall degrading enzyme
DAMP	damage associated molecular pattern
DAP-seq	DNA Affinity Purification sequencing
DBD	DNA binding domain
DCL	Dicer-like
DEG	Differentially expressed gene
DMR6	Downy Mildew Resistant 6
DRB	double-stranded RNA binding protein
EDS1	Enhanced Disease susceptibility 1
EIL	ethylene insensitive 3-like
EIN3	ethylene insensitive 3
EREBP	Ethylene Response Element Binding Protein
ERF	ethylene response factor
ET	ethylene
ETI	Effector-triggered immunity
FN	false negative
FP	false positive
GE	genome editing
GFP	green fluorescent protein
GH	Glycoside Hydrolases
GLM	generalised linear model
GM	genetic modification
GO	Gene ontology

GRN	Gene Regulatory Network
GWAS	Genome Wide Association Studies
HPI	hours post infection
HR	Hypersensitive Response
ICS	isochorismate synthase
IRE1	Inositol-requiring enzyme 1
JA	jasmonic acid
JAZ	jasmonate-zim domain
LOD	logarithm of the odds
log ₂ FC	log2 fold change
LOX	lipoxygenases
LPMO	lytic polysaccharide monooxygenase
LRR	leucine rich repeat
LysM	lysine motif
MAMPs	microbe associated molecular patterns
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MeJA	methyl-jasmonate
miRNAs	microRNAs
MYB	myeloblastosis-related
NAC	NAM-ATAF1-CUC2
NBS	nucleotide-binding site
NEP1	Necrosis and ethylene-inducible peptide 1
NLPs	NEP1-like proteins
NLRs	NBS-LRR receptors

NPR1	NONEXPRESSER OF PR GENES 1
OA	Oxalic Acid
OPDA	12-oxophytodienoic acid
ORF	open reading frame
p35S	35S promotor
PAD3	Phytoalexin deficient 3
PAL	Phenylalanine Ammonia Lyases
PCA	principal component analysis
PCD	programmed cell death
PDF	Plant Defensin
PG	polygalacturonase
PL	pectin lyase
PME	pectin methyl-esterase
PMEI	PME inhibitors
PPR	pentatricopeptide repeat
PRR	pathogen recognition receptor
Pst DC3000	<i>Pseudomonas syringae</i> pv. tomato DC3000
PTGS	Post-transcriptional gene silencing
PTI	pattern triggered immunity
QDR	quantitative disease resistance
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait loci
REML	Restricted Maximum Likelihood
RIL	recombinant inbred line
RISC	RNA-induced silencing complex
RLCK	Receptor-like cytoplasmic kinases

RLK	receptor-like kinase
RLP	receptor-like protein
RNAi	RNA interference
RNAseq	RNA sequencing
ROS	reactive oxygen species
SA	salicylic acid
SCF	Skp/Cullin/F-box
sgRNA	single guide RNA
SGS3	Suppressor of gene silencing 3
siRNAs	Small interfering RNA
sRNAs	small RNAs
STL	sesquiterpene lactone
TF	transcription factor
TGAs	TGACG-binding transcription factors
TIR	TOLL/interleukin-1 receptor
TOFDE	time of first differential expression
UPR	unfolded protein response

Chapter 1

Introduction

1.1 Plant pathogens pose a risk to global food security

The challenge of feeding a rapidly growing global population, expected to reach 9.7 billion by 2050, while ensuring sustainable agricultural practices and reducing greenhouse gas emissions, is a pressing concern. The effects of climate change, such as more frequent extreme weather events, including droughts, heatwaves, and flooding, further compound the issue (Juroszek et al. 2020). Climate warming may also result in a latitudinal shift of plant pathogens' geographical ranges, which may result in plants being infected by novel pathogens that they are unable to defend themselves against (Bebber 2015; Chaloner et al. 2021). Therefore, to achieve global food security, it is crucial to reduce agricultural losses and increase yields simultaneously.

Plants have to overcome a large number of biotic stresses from viruses, bacteria, fungi, oomycetes, insects and even parasitic plants. The Food and Agriculture Organization of the United Nations (FAO) reported that plant pests and pathogens cause a loss of over 20% of crop yields, resulting in a global economic loss of US\$220 billion annually (Food and Agriculture Organization 2021a). Savary et al. 2019 performed global estimations for yield loss caused by pests and pathogens in five key crop species (wheat, rice, maize, potato and soybean) which ranged between 17.2-30%. They reported that crop losses were higher in more food-insecure regions such as West Asia, North Africa and Sub-Saharan Africa.

Several strategies can mitigate crop losses from plant diseases, including phyto-sanitation, crop rotation, biological control, chemical pesticides, fungicides, and planting disease-resistant cultivars. Chemical options have been effective, but they can negatively impact the environment and human health, and pathogens can develop resistance (Rupp et al. 2016; Bass et al. 2015; Powles and Yu 2010; Hawkins et al. 2019). Therefore, disease-resistant cultivars provide a sustainable and more enduring solution for reducing crop losses. However, genetic sources of resistance may have been lost from elite cultivars during selection, or crop species may be

grown in different regions from where they originated and may be exposed to pathogens that they do not have evolved resistance against. Therefore, we must identify novel sources of genetic resistance which can be introduced into elite breeding populations.

1.2 Classifications of plant pathogens: lifestyle and host-range

Plant pathogens can generally be categorised into two pathogen lifestyles; biotrophs, those which extract nutrients from living host tissue, and necrotrophs those which extract nutrients from decaying host tissue. As a result, these classes of plant pathogens have differing infection strategies, similarly, the plant has opposing defence mechanisms. However, the distinction between these two pathogens lifestyles is blurred as pathogens are able to switch lifestyles during their infection. Hemibiotrophs start their infection as a biotroph and make a necrotrophic switch during the later stages of the infection. But even pathogens such as *Botrytis cinerea* that were considered "true" necrotrophs, are now thought to possess an early biotrophic phase to their infection (Van Kan et al. 2014).

Biotrophic pathogens tend to be highly specialised having evolved to infect a specific host, for example *Bremia lactucae*, the lettuce downy mildew pathogen is only able to infect lettuce, while other downy mildews evolved to infect other hosts such as Arabidopsis (*Hyaloperonospora arabidopsidis*). Biotrophic pathogens often co-evolve with their host, developing an arsenal of secreted effector proteins that can manipulate host cells to create a favourable environment for the pathogen (Rausher 2001). These effector proteins can suppress host defence responses and alter host metabolism to facilitate nutrient acquisition (Mapuranga et al. 2022). As biotrophs need to keep the host tissue alive they often have more subtle symptoms than necrotrophs, but they will utilise the plants resources leading to stunted growth, and blemishes/lesions render the plant unmarketable. Notable biotrophic pathogens of high economic importance include; wheat yellow stripe rust (*Puccinia striiformis*) (Hovmøller et al. 2011), grape downy mildew (*Plasmopara viticola*) (Gessler et al. 2011) and maize smut (*Ustilago maydis*) (Steinberg and Perez-Martin 2008). Numerous hemibiotrophic pathogens are highly devastating for crop growth and food security such as rice blast (*Magnaporthe oryzae*) (Ou 1980), *Fusarium spp.* wilt (Gordon 2017) and potato late-blight (*Phytophthora infestans*) (Grünwald and Flier 2005).

Necrotrophic pathogens can be categorized based on their host range into two groups: broad-host range necrotrophs and host species-specific necrotrophs. One such example of a host species-specific necrotroph is *Cochliobolus carbonum*, which causes northern leaf spot disease in maize (Panaccione et al. 1992). On the other hand, many major necrotrophic pathogens of high economic importance are considered "generalists" with broad host ranges, including fungi (*Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Alternaria brassicicola*), bacteria (*Pectobacterium carotovorum*, *Ralstonia solanacearum*) and oomycetes (*Pythium irregulare*, *Pythium ultimum*) (Laluk and Mengiste 2010). In contrast to biotrophs, necrotrophs are highly destructive pathogens that cause tissue maceration, rotting, and necrosis. They secrete an array of cell wall degrading enzymes (CWDEs) to gain entry into their host (Kubicek et al. 2014). Once inside, necrotrophs release toxic secondary metabolites to aid in colonizing host tissues (Pusztahelyi et al. 2015). These toxins can have a range of effects on the host, for example, alternariol 9-methyl ether produced by

Alternaria spp. reduces electron transport chain efficiency and inhibits photosynthesis (Demuner et al. 2013), whereas Botrydial toxin produced by *B. cinerea* induces necrosis and cell death (Lindner and Gross 1974; Colmenares et al. 2002). Similar to biotrophs, necrotrophs also secrete protein effectors that can dampen the host defence response or manipulate the plant into inducing cell death (Shao et al. 2021). However, unlike biotrophs, the effectors of broad-host range necrotrophs must be able to manipulate many host species.

1.3 Nutritional and economic significance of *Lactuca sativa* (lettuce)

Lactuca sativa (lettuce) is a member of the Asteraceae family consisting of over 23,000 species (Gao et al. 2010), including chicory (*Cichorium intybus*), sunflower (*Helianthus annuus*) and the anti-malarial plant *Artemisia annua*. Lettuce is an economically valuable leafy vegetable crop, with the US lettuce market worth 2.4 billion in 2019 (USDA-NASS, 2019). In FAO's global agricultural statistics, lettuce and chicory are grouped, estimating their 2020 total global "Gross Production Value" at US\$20 billion, with the largest producer being; China (\$12bn), USA (\$2.9bn), Japan (\$639 million), Netherlands (\$ 613 million), Korea (\$441 million), Germany (\$440 million) and France (\$296 million)(Food and Agriculture Organization 2021b). In 2021, the UK lettuce industry was valued at \$187 million by DEFRA (Department for Environment Food and Rural Affairs 2022).

Lettuce has many nutritional qualities, which can make up part of a well-balanced diet, having a high content of many crucial vitamins, minerals and bioactive compounds (Shi et al. 2022; Mou 2009). Bioactive secondary metabolites in lettuce such as flavonoids, sesquiterpene lactones, carotenoids and tocopherols have been shown to have antioxidant, anti-inflammatory and anti-cancer properties (Yang et al. 2022b). Qin et al. 2018 demonstrated that extracts from red-leaf lettuce inhibited the growth of multiple cancer cell lines.

1.3.1 Horticultural Types of lettuce

Many varieties of cultivated lettuce (*Lactuca sativa*) exist which have been selected for different qualities, and have resulted in distinct morphologies (Rodenburg and Basse 1960). Many lettuce varieties form "heads", densely packed rosette leaves which form a compact structure.

Crisphead/Batavian lettuce form tight heads with crispy textured leaves, which is popular in sandwiches and burgers. Butterhead lettuce (*Lactuca sativa* var. capitata) forms loose heads with soft-tender pale leaves which have a pale yellow-green colour, making them a popular choice in salads. Cos/Romaine lettuce (*Lactuca sativa* var. longifolia) forms tall, loose heads with crispy leaves which have a slight bitter taste and have a darker green colour, popular in Mediterranean regions. Cutting lettuce (*Lactuca sativa* var. acephala) is a non-heading type, with open rosette leaves. Latin lettuce forms loose-heads with thick leathery leaves. Oilseed lettuce are primitive varieties which were used for the oil content of their seeds. Also a non-heading type, these plants have a "stalky" architecture that is morphologically similar to wild relatives such as *L. serriola*. Leaves have a bitter taste and are not consumed. Stem lettuce (*Lactuca sativa* var. augustana) is a type of non-heading lettuce, primarily cultivated in China. The pale-green leaves can be eaten in salads, but the stem is the most widely consumed tissue which is stir-fried.

1.3.2 Lettuce is susceptible to a wide range of pests and pathogens

Lettuce crops are susceptible to a large number of plant diseases which can lead to significant yield losses. These include downy mildew (*Bremia lactucae* - oomycete), lettuce drop (*S. sclerotiorum* and *Sclerotinia. minor* - fungi), grey mould (*B. cinerea* - fungi), root rot (*R. solani* - fungi), corky root (*Rhizomonas suberifaciens* - bacteria), lettuce mosaic virus, and the currant-lettuce aphid (*Nasonovia ribisnigri* - insect).

Bremia lactucae is the causative agent of downy mildew in *Lactuca sativa*, a highly damaging disease that can have significant economic consequences for lettuce growers (Patterson et al. 1986). This is especially true in the Salinas Valley of California, which produces over 70% of the lettuce grown in the United States (Wu et al. 2001). Symptoms of lettuce downy mildew include pale yellow blemishes on the upper surface of leaves which renders the crop unmarketable.

S. sclerotiorum is a causative agent for lettuce drop disease which leads to a complete collapse of the lettuce head, white mycelial growth is seen in the later stages of the infection. In field grown lettuce yield losses as a result of *S. sclerotiorum* have been reported at 50% in the UK (Young et al. 2004). Average lettuce yield losses to lettuce drop are approximated to be 15% (Subbarao 1998), which equate to \$28 million and \$360 million in the UK and USA respectively based on DEFRA and USDA statistics.

B. cinerea is the causative agent of grey mould disease in lettuce, which leads to water-soaked lesions and the growth of dark brownish-grey mycelium on the leaf. In advanced stages of disease progression black sclerotia form and the entire plant will eventually rot and wilt. Humidity pockets that occur within lettuce heads greatly increase the chances of infection.

Significant efforts have been made to understand resistance against downy mildew caused by *Bremia lactucae*, with many genes conferring resistance already discovered (Parra et al. 2016; Parra et al. 2021). However, far fewer lettuce genes or even loci are known which contribute to necrotrophic resistance (Mamo et al. 2019). As a result, this work will aim to identify genes and their underlying mechanisms which contribute to *B. cinerea* and *S. sclerotiorum* resistance in lettuce.

1.4 Generalist necrotrophic pathogens *B. cinerea* and *S. sclerotiorum*

1.4.1 Sclerotiniaceae plant pathogens

B. cinerea, first described by Christiaan Hendrik Persoon in 1794 (Persoon 1794) and *S. sclerotiorum*, first described by Heinrich Anton de Bary (deBary 1886) are two closely related ascomycete fungi in the Sclerotiniaceae family. *Sclerotiniaceae spp* are noted for their formation of sclerotia, long-term storage bodies that remain in the soil for prolonged periods, *Sclerotinia trifoliorum* sclerotia have been shown to re-infect after 5 years buried in the soil (Halkilahti 1962; Willetts 1971). This facilitates repeated infections in successive years, particularly if infected material is not swiftly removed, making disease control challenging.

Species within *Sclerotiniaceae* show a very large diversity in pathogen lifestyle and host-species specificity. *B. cinerea* and *S. sclerotiorum* are both broad-host range necrotrophs that can infect over 200 species, but *Sclerotinia glacialis* shows host specificity to glacier buttercup (*Ranunculus glacialis*) (Graf and Schumacher 1995) while *Myriosclerotinia spp* and *Ciborinia whetzeli* are biotrophic species (Andrew et al. 2012). Even within the *Botrytis* genus, *B. deweyae* exists mostly as an endophyte within daylilies *Hemerocallis spp*, but can switch to a necrotrophic lifestyle causing "spring sickness" (Grant-Downton et al. 2014). *Botrytis elliptica* is a fungal necrotroph but shows host specificity to lily plants (Van Baarlen et al. 2004). Comparative genomic and secretome analysis was carried out across nine *Botrytis* species (*B. calthae*, *B. convoluta*, *B. elliptica*, *B. galanthina*, *B. hyacinthi*, *B. narcissicola*, *B. paeoniae*, *B. porri* and *B. tulipae*) (Valero-Jiménez et al. 2019). However, this was unable to detect a "silver bullet" which allows *B. cinerea* to infect such a wide number of host species.

Despite the diversity across the Sclerotiniaceae family, both *B. cinerea* and *S. sclerotiorum* are both notorious necrotrophic pathogens which employ highly similar virulence mechanisms in order to infect a large number of host plants (Amselem et al. 2011; Mbengue et al. 2016). These virulence mechanisms will be discussed in depth below.

1.4.2 Infection strategies of *B. cinerea* and *S. sclerotiorum*

B. cinerea and *S. sclerotiorum* employ a large arsenal of virulence factors and infection strategies to establish infections, colonise host tissue and subvert the host defence response. These will be reviewed within this section.

1.4.2.1 *S. sclerotiorum* phytotoxin: Oxalic Acid (OA)

The production and secretion of phytotoxic metabolites are a major infection strategy for broad-host range necrotrophic pathogens such as *B. cinerea* and *S. sclerotiorum*. Oxalic Acid (OA) is a key phytotoxin produced by *S. sclerotiorum*.

Godoy et al. 1990 isolated UV-irradiated strains of *S. sclerotiorum* that were deficient for OA production and showed dramatically reduced pathogenicity on bean (*Phaseolus vulgaris*) leaves, stems and pods. Mutants of an OA biosynthetic enzyme, oxaloacetate acetylhydrolase $\Delta Ssoah1$, have completely abolished OA accumulation and reduced virulence in multiple hosts (Liang et al. 2015). Transgenic *Arabidopsis thaliana* (*Arabidopsis*) expressing *Ssoah1*-siRNA, which silences *Ssoah1* transcripts during infection, exhibits increased resistance to *S. sclerotiorum* infection (Rana et al. 2022). OA oxidase enzymes have been identified in wheat and barley (Chiriboga 1966) which catalyse the breakdown of OA (Thompson et al. 1995). Transgenic expression of OA oxidases increases resistance to *S. sclerotiorum* in several species including; peanut (Livingstone et al. 2005), tomato (Walz et al. 2008), soybean (Yang et al. 2019) and brassica (Verma and Kaur 2021). Together, these findings provide robust evidence for OA as a vital *S. sclerotiorum* virulence factor.

The mechanism by which OA promotes virulence in *S. sclerotiorum* is still debated, the proposed mechanisms are summarised in Figure 1.1. One school of thought is that OA modulates pH during infection to provide optimal conditions for CWDEs. By the 1960s, a link between the acidification of host tissues by OA and increased polygalacturonase activity during *Sclerotinia spp* infection had been established (Bateman and Beer 1965; Hancock 1966). To support this idea, Marciano et al. 1983 showed that CWDEs secreted upon infection of sunflower were pH sensitive, highly active at pH4.0, but severely inhibited at pH6.0, suggesting a role of OA in providing optimal conditions for CWDEs.

More recent work has suggested that induction of programmed cell death (PCD) is the mechanism behind OA pathogenicity. Treatment of tobacco leaf discs with exogenous OA was able to induce PCD and DNA laddering, which could be replicated with potassium oxalate at pH7.0 but could be inhibited with the addition of antioxidants - suggesting a role for reactive oxygen species (ROS). Manipulation of the hosts' redox state appears to be a critical mechanism of virulence for *S. sclerotiorum*, repressing the plants' oxidative burst in the early stages of infection and initiating a ROS burst in later stages of infection, with this ROS manipulation abolished in OA-deficient non-pathogenic mutants (Williams et al. 2011; Cessna et al. 2000). Additional OA virulence mechanisms have also been proposed such as deregulation of the guard cells (Guimaraes and Stotz 2004) and chelating calcium into calcium oxalate crystals (Heller and Witt-Geiges 2013).

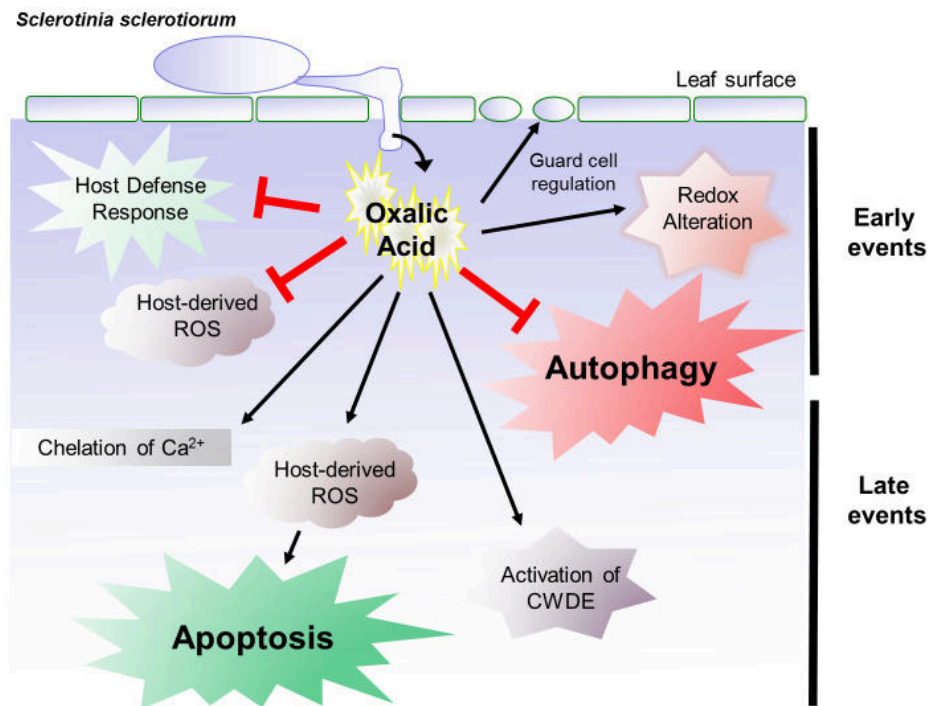


Figure 1.1: Summary of the proposed *S. sclerotiorum* oxalic acid virulence mechanisms. Figure from Kabbage et al. 2013

1.4.2.2 *B. cinerea* phytotoxins: Botrydial and Botcinic Acid

The sesquiterpene botrydial (Lindner and Gross 1974; Colmenares et al. 2002) and polyketide botcinic acid (Tani et al. 2006) are two key secondary metabolites from *B. cinerea* with phytotoxic effects on hosts. Over 90% of putative secondary metabolite genes in *B. cinerea* and *S. sclerotiorum* are located in biosynthetic gene clusters. Over 30% of these clusters contain a Zn(II)2Cys6 transcription factor which may regulate the expression of the co-located biosynthetic enzymes (Amselem et al. 2011). The botrydial biosynthetic gene cluster contains 7 genes (BcBOT1-7): three P450 monooxygenases (BcBOT1/3/4), a sesquiterpene synthase (BcBOT2), an acetyl-transferase (BcBOT5), a Zn(II)2Cys6 transcription factor (BcBOT6) and a dehydrogenase (BcBOT7) (Pinedo et al. 2008; Porquier et al. 2016). The botcinic acid biosynthetic cluster contains 13 genes (BcBOA1-13), BcBOA6/9 have been characterised as polyketide synthases (Dalmais et al. 2011) and BcBOA13 is a Zn(II)2Cys6 transcription factor. Both transcription factors positively regulate the expression of all other genes in their respective clusters and the accumulation of the respective metabolite (Porquier et al. 2016; Porquier et al. 2019).

Unexpectedly, neither $\Delta BcBOT1$ nor $\Delta BcBOT6$ single mutants had any effect on virulence in *B. cinerea* B.05 background, despite reduced botrydial accumulation in these mutants (Porquier et al. 2016; Siewers et al. 2005). However, $\Delta BcBOT1$ and $\Delta BcBOT2$ mutants in *B. cinerea* T4 background had dramatically reduced lesion size on multiple hosts, showing isolate-specificity in the virulence effect of botrydial. A possible explanation for this is that the wild-type T4 isolate accumulates very low levels of botcinic acid, and

therefore may have a higher reliance on botrydial for virulence, suggesting a possible functional redundancy between the two toxins (Siewers et al. 2005; Pinedo et al. 2008). Dalmais et al. 2011 further explore this redundancy, showing that $\Delta BcBOT1/BcBOA6$ double mutants in B.05 background are completely deficient for accumulation of both botrydial and botcinic acid, and have dramatically reduced virulence. All seven BcBOT genes were identified in the 20 highest virulence-associated transcripts across 96 *B. cinerea* isolates and 3 *Arabidopsis* genotypes (Zhang et al. 2019). This provides further evidence for the importance of these phytotoxins in *B. cinerea* virulence.

The phytotoxic mechanisms underlying how botrydial and botcinic acid promote pathogen virulence are poorly understood. However, exogenous application of botrydial has been shown to induce chlorosis and collapse of host tissue via induction of the hypersensitive response in a salicylic acid (SA) dependent manner (Colmenares et al. 2002; Rossi et al. 2011).

1.4.2.3 Cell Wall Degrading Enzymes (CWDEs)

Cell wall degrading enzymes (CWDEs) are a diverse class of enzymes capable of breaking down the plant cell wall, which are utilised by both biotrophic and necrotrophic fungi for initial host penetration (Kubicek et al. 2014). Plant cell walls are heterologous structures consisting of cellulose, xylan, pectin, lignins, cutin and proteins. Hence pathogens require a large arsenal of CWDEs to hydrolyse the various cell wall polymers to facilitate penetration, including; Glycoside Hydrolases (GH), Carbohydrate esterases (CEs), pectin lyases (PLs), polygalacturonases (PGs), lignocellulases and lytic polysaccharide monooxygenases (LPMOs) (Zhao et al. 2014).

The version 1 genomes of *B. cinerea* and *S. sclerotiorum* contained 346 and 367 genes encoding putative carbohydrate-active enzymes (CAZymes) respectively, of which 118 and 106 had predicted CWDE-activity (Amselem et al. 2011). However, new classes of CAZyme are being identified all the time, with 535 *B. cinerea* and 438 *S. sclerotiorum* CAZymes now recognised by the CAZy database (Drula et al. 2022). As expected these CAZymes are readily secreted upon pathogen infection with 20% and 21% of the *B. cinerea* and *S. sclerotiorum* secretomes being made up of glycoside hydrolase families (Heard et al. 2015).

CWDEs are essential virulence factors for a successful necrotrophic infection, with many published examples of single CWDE knock-out pathogen strains displaying reduced pathogenicity. Endo-1,4- β -xylanases are a GH class, which mainly catalyse the hydrolysis of β -1,4-d-xylosidic linkage in xylan. BcXyn11a and SsXyl1 are both endo-1,4- β -xylanases that are required for full virulence, with deletion mutants displaying reduced lesion area (Brito et al. 2006; Yu et al. 2016). SsCut1, a *S. sclerotiorum* cutinase was shown to be upregulated

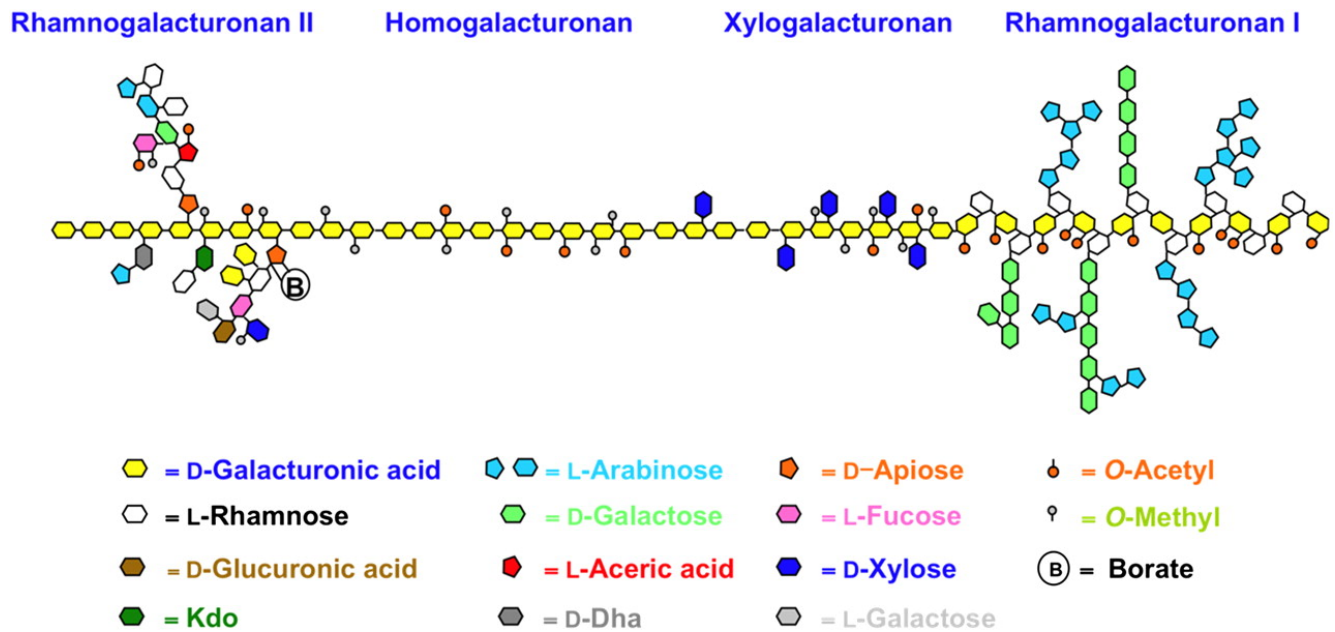


Figure 1.2: Schematic for the structure of 4 pectin sub-types with varying degrees of complexity, from Harholt et al. 2010

upon *Arabidopsis* infection, and $\Delta SsCut1-3$ mutants show reduced virulence in *Arabidopsis* and oilseed rape detached leaf assays (Gong et al. 2022)

B. cinerea and *S. sclerotiorum* had a high proportion of CWDEs with predicted activity against pectin, 37% and 31% respectively, suggesting that the degradation of pectin has high importance for virulence (Amselem et al. 2011). Pectin is a complex polysaccharide with a galacturonic acid main-chain, which can be methylated, and variable side chains including arabinose Figure 1.2 (Harholt et al. 2010). PGs hydrolyse α -1,4 glycosidic bonds between galacturonic acid residues of pectin (Zhao et al. 2014). *Bcpg1* mutants show reduced virulence on tomato leaves (Have et al. 1998). BcPG2 was sufficient to induce necrosis in infiltrated *N. benthamiana* leaves (Joubert et al. 2007). Hydrolysis of pectin sub-units is also linked to virulence, as mutants of an α -1,5-L-endo-arabinanase, $\Delta BcAra1$ were shown to have reduced virulence on *Arabidopsis*, but not on *N. benthamiana* (Nafisi et al. 2014). N-methyl-N'-nitroso-guanidine mutagenised strains of *Sclerotinia trifoliorum* screened for deficient arabinofuranosidase activity showed reduced virulence on pea, but not alfalfa (Rehnstrom et al. 1994). These data show that arabinose hydrolysis associated virulence may have a degree of host specificity. Nafisi et al. 2014 additionally demonstrated that crude protein extracts in *B. cinerea*-inoculated *Arabidopsis* contained greater arabinanase activity than *N. benthamiana*. Hence, suggesting that *B. cinerea* can fine-tune the expression of its CWDEs upon the infection of different host species. Espino et al. 2010 additionally performed *B. cinerea* secretome analysis in the presence of glucose media, tomato extracts and kiwi extracts and identified media-specific secretion of many CWDEs.

To minimise pectin degradation and maintain cell wall integrity, many host species contain PG-inhibiting proteins (PGIPs), and PGIP overexpression leads to increased resistance against *B. cinerea* and *S. sclerotiorum*

(Joubert et al. 2006; Bashir et al. 2013). To retain PG activity, pathogens contain PGIP-INactivating Effectors (PINEs). SsPINE1 physically binds AtPGIP1, outcompeting binding of SsPG1, both SsPINE1 and BcPINE1 promote increased virulence against *Arabidopsis* (Wei et al. 2022). Further still, to ensure fungal PGs are able to degrade pectin, pathogens express pectin methyl-esterases (PMEs) which demethylate pectin, ensuring it is accessible to CWDEs (Kan 2006). *Bcpme1* mutants showed reduced growth on high pectin medium and reduced virulence on apple fruits, grapevine, and *Arabidopsis* leaves (Valette-Collet et al. 2003). Host plants again combat this by expressing PME inhibitors (PMEIs), AtPMEIs are rapidly upregulated after *B. cinerea* inoculation (Windram et al. 2012) and PMEI overexpression reduces pathogen virulence (Lionetti et al. 2007; Lionetti et al. 2017).

1.4.2.4 Protein Effectors

Plant pathogens secrete a multitude of proteins, collectively known as “effectors”, that modulate host plant physiology to facilitate colonisation of host tissue (Lo Presti et al. 2015). Any fungal protein that is secreted during infection and contributes to the colonisation of host tissue can be classified as an effector (Shao et al. 2021). These effectors can evade host immune responses, affect host metabolism to acquire nutrients, or directly contribute to virulence mechanisms. Bioinformatic identification of fungal effectors is challenging, as they show little sequence conservation apart from an N-terminal signal peptide and the lack of a transmembrane domain. Guyon et al. 2014 and Derbyshire et al. 2017 identified 78 and 70 candidate *S. sclerotiorum* effectors respectively using distinct predictive methods, however only 9 effectors were identified by both studies. Guyon et al. 2014 identified putative effectors using a combination of domain presence/absence, *in planta* microarray expression data and secretomic, whereas Derbyshire et al. 2017 used a machine-learning approach to identify effectors. The incorporation of artificial intelligence may be able to improve effector predictions, for example, AlphaFold Multimer has been used to predict interaction between secreted fungal proteins and host proteins, identifying four novel effectors (Homma et al. 2023).

The direct interaction between effectors and host proteins has been extensively studied in biotrophic pathogens for their ability to facilitate the colonisation of a single host plant species. For instance, Pep1 secreted by *Ustilago maydis* directly interacts with a maize peroxidase (ZmPOX12), thereby preventing a reactive oxygen species (ROS) burst and promoting fungal colonization (Doehlemann et al. 2009; Hemetsberger et al. 2012). However, effectors from broad-host range necrotrophs such as *B. cinerea* and *S. sclerotiorum* need to interact with and successfully modulate the activity of their target proteins in multiple hosts to promote virulence. BcXyl1, a *B. cinerea* effector, encodes a xylanase CWDE that is required for full virulence and induces cell death in multiple species such as tobacco, soybean, tomato, and cotton (Yang et al. 2018). Interestingly, the cell death activity of BcXyl1 is independent of its xylanase activity but dependent on host immune receptors NbBAK1 and NbSOBIR1 (discussed later in section 1.5.2), indicating that BcXyl1 may manipulate the host's

biotrophic defence response to trigger cell death and promote virulence (Yang et al. 2018). BcSpl1 is another effector that is highly abundant in the *B. cinerea* secretome, inducing cell death in a BAK1 dependent-manner and $\Delta Bcsp1$ mutants show reduced virulence (Frias et al. 2011). A *S. sclerotiorum* integrin-like secreted effector (SsITL) is also required for full virulence, with SsITL-silenced strains exhibiting smaller lesions (Zhu et al. 2013).

Necrosis and ethylene-inducible peptide 1 (NEP1)-like proteins (NLPs) are a conserved family of peptides secreted by plant pathogenic fungi, bacteria and oomycetes, first identified in *Fusarium oxysporum* (Bailey 1995; Oome et al. 2014). NLP peptides have been identified in both *B. cinerea* and *S. sclerotiorum*, which are capable of inducing cell death (Schouten et al. 2008; Dallal Bashi et al. 2010). Dallal Bashi et al. 2010 reported that SsNEP2 was rapidly upregulated during *Brassica napus* infection but SsNEP1 was barely detectable although SsNEP1 could induce cell death when transiently expressed in *N. benthamiana*. $\Delta Ssnep2$ deletion mutants show reduced virulence (Yang et al. 2022a), however, $\Delta Bcnep1$ and $\Delta Bcnep2$ single mutants had no defects in virulence, which may suggest there is functional redundancy between the BcNEP paralogs.

Bccrh1 is a transglycosylase enzyme which is secreted into the host cytoplasm and induces cell death independently of its enzymatic activity when transiently expressed in *N. benthamiana*. However, the virulence of $\Delta bccrh1$ mutant strains was unaffected and p35S::Bccrh1 transgenic Arabidopsis lines had increased resistance to *B. cinerea* and increased expression of defence genes, suggesting that the presence of the effector activates a plant defence response (Bi et al. 2021). Effector-triggered immunity (ETI) is a well-established plant defence response in which secreted biotrophic effectors are detected by intracellular host immune receptors and used to mount a defence response (Cui et al. 2015a). The role of ETI in necrotrophic pathogen defence is unclear, however Bccrh1 may be activating an ETI-like response in *B. cinerea* infection.

Together, these observations support that secreted effectors from fungal necrotrophs have important roles in pathogen virulence, and host-recognition of the invader. However, knowledge of direct interaction between the fungal effectors and host proteins is limited, particularly whether the effectors are able to manipulate protein function in multiple hosts. Proteomic approaches have identified 89 secreted *B. cinerea* proteins during plant infection (Shah et al. 2009), suggesting there could be many more effectors with currently unknown functions/targets.

1.4.2.5 Small RNA effectors

In addition to the secretion of protein effectors, it is now well-established that extracellular vesicles containing small RNAs (sRNAs) are secreted by plant pathogens during infection which can also act as effectors (Qiao et al. 2021a; Dunker et al. 2020; He et al. 2023a). Weiberg et al. 2013 demonstrated the first example

of cross-kingdom gene silencing, demonstrating that *B. cinerea* sRNAs were able to target the degradation of host transcripts in Arabidopsis and tomato. Pathogen sRNAs are processed by host Argonaute (AGO) proteins and hijack the plant RNA-induced silencing complex (RISC) in order to perform cross-kingdom RNA interference (RNAi) (Weiberg et al. 2013; Dunker et al. 2020). The expression of several *B. cinerea* small RNAs (*Bc-siR3.1*, *Bc-siR3.2*, *Bc-siR5* and *Bc-siR37*) in transgenic Arabidopsis has been shown to increase pathogen resistance, and demonstrated the silencing of host defence genes such as MPK1/2 and WRKY7 (Weiberg et al. 2013; Wang et al. 2017c).

It has also been shown that *S. sclerotiorum* expresses sRNAs during infection, which contain sequence complementary to Arabidopsis defence regulators which are downregulated during infection (Derbyshire et al. 2019). However, it remains to be elucidated whether these *S. sclerotiorum* sRNAs directly silence host targets, and whether this requires host AGO.

A complex sRNA battleground takes place during plant-pathogen interactions with fungal sRNAs manipulating the expression of host genes (Weiberg et al. 2013), host sRNAs are also able to manipulate expression of pathogen genes (Cai et al. 2018), and hosts use sRNAs to manipulate expression of their own genes during pathogen infection (Ronemus et al. 2006; Borges and Martienssen 2015). These complex interactions are discussed in more detail in Section 1.5.6.1.

1.5 Plant Defence Responses

Due to their sessile nature, plants are extremely vulnerable to biotic stresses, such as microbe infection and insect attack. As a result, plants have developed a sophisticated immune system to rapidly detect an invading pathogen and mount an appropriate defence response. Plants must also mount the “correct” immune response as pathogens of different lifestyles have different virulence strategies, requiring a specialised immune response for different classes of pathogens.

1.5.1 Classical models of biotrophic pathogen perception: gene-for-gene to zig-zag

Harold H. Flor conducted seminal research, investigating the disease resistance of Flax varieties to an obligate biotroph rust fungus (*Melampsora lini*), discovering that F_2 crosses of resistant x susceptible Flax varieties and virulent x avirulent *M. lini* races segregated in a 3:1 mendelian ratio (Flor 1942). This was a key observation which led him to propose the gene-for-gene model, hypothesising that a single dominant plant resistance gene (R-gene) and a single pathogen avirulence gene (Avr) must be present together for the plant to exhibit full resistance, thus preventing a successful infection by the pathogen (Flor 1955, 1971). Numerous single dominant R-genes were subsequently identified in other plant species, conferring complete resistance to biotrophic pathogens in a race-specific manner (Hammond-Kosack and Jones 1997). Many R-genes were found to encode nucleotide-binding site (NBS)-leucine-rich repeat (LRR) receptors (NLRs) (Whitham et al. 1994; Bent et al. 1994), which can activate a defence response via directly interacting with Avr proteins (Jia et al. 2000) (Mackey et al. 2002; Kim et al. 2005). Many Avr genes are now known to encode secreted effectors that are important for virulence in plant species without a corresponding R-gene (De Wit et al. 2009), thus the immune response activated by NLRs is termed effector-triggered immunity (ETI).

NLRs are intracellular receptors lacking a transmembrane domain, therefore are only able to detect effectors that have gained entry into the plant cell. However a number of extracellular molecular “patterns” exist which signal the presence of an invading microbe; microbe associated molecular patterns (MAMPs) and damage associated molecular patterns (DAMPs). MAMPs are directly associated with the pathogen itself, including; bacterial flagellum, bacterial elongation factor Tu (EF-Tu) and chitin (Felix et al. 1999; Kunze et al. 2004; Shibuya and Minami 2001). DAMPs are released from plant components following damage inflicted by the pathogen, such as CWDE degraded oligogalacturonides (Zarattini et al. 2021; Bellincampi et al. 2000) and endogenous plant signalling peptides, e.g. Pep1 (Huffaker et al. 2006). Treatment of lettuce with Cellobiose (degraded cellulose) has been shown to activate defence responses, increasing resistance to *B. cinerea* (He et al. 2023b).

MAMPs/DAMPs are detected by transmembrane pathogen recognition receptors (PRRs), containing an extra-cellular ligand-binding domain, typically an LRR, a transmembrane domain and an intracellular domain which activates pattern triggered immunity (PTI), often a serine/threonine protein kinase. PRRs are broadly classified as receptor-like kinases (RLKs) or receptor-like proteins (RLPs) based on the presence or absence of an intracellular kinase domain. The defence response activated downstream of PRRs is termed pattern triggered immunity (PTI) (DeFalco and Zipfel 2021). Flagellin-sensitive 2 (FLS2) is a well-characterised example of a PRR, FLS2 is an LRR-RLK which directly binds flg22, a 22 amino-acid epitope of bacterial flagellin, activating downstream kinases and promoting increased resistance to numerous bacterial species (Zipfel et al. 2004; Chinchilla et al. 2006; Lu et al. 2010). Many PTI responses, such as FLS2-induced immunity, act as an initial defence mechanism and provide partial resistance to many pathogens. However, several R-genes have been identified which encode for PRRs that bind extracellular Avr, conferring full resistance in a race-specific manner, such as; Xa21 a rice LRR-RLK (Song et al. 1995) and Cf-9 a tomato LRR-RLP (Jones et al. 1994).

Jones and Dangl 2006 proposed the zig-zag model, summarising the respective roles of PTI and ETI in plant defence against biotrophic pathogens. MAMPs/DAMPs are initially detected by PRRs triggering a PTI defence response, this is followed by the secretion of pathogen effectors to overcome the hosts' PTI response. Some of the effectors may then be recognised by cytoplasmic NLRs, triggering an amplified defence response, ETI. Localised cell death, known as the Hypersensitive Response (HR) and an oxidative burst will follow (Stakman 1915; Morel and Dangl 1997). Pathogen races that avoid R-gene detection via mutation or complete loss of the detected effector can evade ETI and therefore successfully colonise the host tissue.

1.5.2 Pathogen perception of necrotrophic pathogens

While the zig-zag model provides an accurate summary of the defence response against biotrophic pathogens, it is not sufficient to describe the complexities of the necrotrophic pathogen defence response. NLR-mediated ETI is a core component of the zigzag model, however its role in necrotrophic pathogen defence remains unclear. R-genes, often encoded by NLRs provide full resistance to biotrophic pathogens via the detection of an Avr gene alerting the plant to the presence of an invading pathogen. HR (localised cell death) is triggered which is sufficient to prevent colonisation of biotrophic pathogens that require living host cells. By contrast, generalist necrotrophic pathogens promote cell death and tissue collapse in their hosts, therefore activation of HR cannot prevent colonisation. Some pathogens even secrete effectors that promote HR, such as hypersensitive response-inducing protein 1 (Hip1) in *B. cinerea* (Jeblick et al. 2023).

R-genes conferring full pathogen resistance have been identified against host-specific necrotrophic fungi, however they do not encode NLRs and are not expected to be involved in ETI. For example, *Cochliobolus carbonum*, the causal agent of northern leaf spot in maize requires HC toxin for virulence, and the R-gene

providing full resistance, HM1, encodes a NADPH-dependent HC toxin reductase that inactivates the toxin (Johal and Briggs 1992; Panaccione et al. 1992). Broad host range necrotrophs have an arsenal of virulence mechanisms to induce tissue necrosis, hence the inactivation of a single toxin or induction HR does not provide full resistance. As a result, resistance to broad host range necrotrophs is a polygenic trait, with many genes contributing a small effect to the overall quantitative disease resistance (QDR) (Corwin and Kliebenstein 2017). Quantitative trait loci (QTL) studies which aim to map the genomic regions associated with variation in a specific trait (discussed in detail in section 1.7.1) have often identified many loci with minor effects on QDR (such as Denby et al. 2004).

While no single dominant R-genes have been identified that provide full resistance against *B. cinerea* or *S. sclerotiorum*, Arabidopsis NLRs RLM3 and LAZ5 have been shown to be involved in defence against these pathogens. RLM3, promotes partially increased resistance to *B. cinerea* infection (Staal et al. 2008), whereas LAZ5 promotes increased susceptibility against *S. sclerotiorum* (Barbacci et al. 2020). However, it remains to be elucidated whether RLM3 is involved in a *B. cinerea* ETI response, as currently no interactions with fungal effectors have been reported. As mentioned above, Bcch1 is a secreted *B. cinerea* effector with cytoplasmic localisation that increases plant resistance when transgenically expressed in Arabidopsis (Bi et al. 2021), suggesting it may be detected by an NLR but no host interactors have been identified.

PTI, however, is well-established as a critical component of the defence response against generalist necrotrophic fungi such as *B. cinerea* and *S. sclerotiorum*. Chitin, a polysaccharide component of the fungal cell wall is a key MAMP recognised during PTI, detected by chitin elicitor receptor kinase 1 (CERK1), a non-LRR RLK, with chitin binding mediated via its extracellular Lysin Motif (LysM) domain (Miya et al. 2007). CERK1, a pair of closely-related chitin-binding LysM-RLKs (LYK4/LYK5), an LRR-RLK that interacts with CERK1 (LIK1) and a Receptor-like cytoplasmic kinase (RLCK) which is phosphorylated by CERK1 (PBL27) are all required for full resistance to generalist necrotrophic fungi, suggesting that recognition of chitin via PTI is essential for defence (Liu et al. 2018; Cao et al. 2014; Le et al. 2014; Shinya et al. 2014).

Another PRR with a vital role in fungal necrotroph defence is Brassinosteroid(BR)-insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1), an LRR-RLK which is required for full resistance to *B. cinerea* and *S. sclerotiorum*, with T-DNA mutants showing hyper-susceptibility (Kemmerling et al. 2007; Zhang et al. 2013). BAK1 is not thought to directly bind elicitor molecules but instead functions as a co-receptor. BAK1 LRR domains have been shown to interact with other LRR-RLKs/LRR-RLPs in their ligand-bound conformation (Sun et al. 2013). BAK1 forms a receptor complex with BRI1 once it has bound brassinolide, activating BR growth signalling (Li et al. 2002), and is also able to form a co-receptor complex with FLS2 once bound to flg22, activating downstream PTI defence signalling (Chinchilla et al. 2007). BAK1 is also able to form tripartite complexes with SOBIR1 (an LRR-RLK) and RLPs. RLP23 constitutively interacts

with SOBIR1, RLP23 then directly binds NLP peptides, promoting formation of an RLP23-SOBIR1-BAK1 complex, which activates ethylene production (Albert et al. 2015; Albert et al. 2019). In addition, a purified *S. sclerotiorum* elicitor of unknown sequence, SCLEROTINIA CULTURE FILTRATE ELICITOR1 (SCFE1), induces ethylene and ROS accumulation in a manner dependent on RLP30, BAK1 and SOBIR1 (Zhang et al. 2013). However, it remains to be elucidated whether an RLP-SOBIR1-BAK1 tripartite complex forms after SCFE1 recognition. In addition to *bak1*, Arabidopsis mutants of *sobir1-12* and *rlp30-2* are hypersusceptible to both *B. cinerea* and *S. sclerotiorum* (Zhang et al. 2013) and *rlp23-1* mutants are hypersusceptible to *B. cinerea* (Ono et al. 2020), suggesting that PTI-activation by RLP23/30-SOBIR1-BAK1 complexes is vital for generalist-necrotroph defence.

In addition to pathogen-derived MAMPs, molecular “patterns” of damage to the host cell caused by the pathogen (DAMPs) can also be detected by PRRs to activate a PTI response. Cello-oligosaccharides (COS) which have been oxidised by LPMOs during cell wall degradation act as DAMPs, as Arabidopsis treated with LPMO-oxidised COS showed increased resistance to *B. cinerea*, triggering a MAP kinase cascade, upregulating defence genes such as WRKY33 and increasing camalexin secretion (Zarattini et al. 2021). This PTI response was abolished in several single LRR-RLK mutants; *bak1-3*, *sif2*, *sif4* and *the1*, suggesting all 4 PRRs are required for LPMO COS-induced immunity. Given the known co-receptor function of BAK1, these results suggest some of these RLKs could interact in a complex to detect COS.

1.5.3 Signal Transduction

Following the perception of an invading pathogen via either a PRR or an NLR, the host plant must activate a complex signalling network to coordinate an appropriate defence response, including mitogen-activated protein kinases (MAPK) cascades and a calcium burst to trigger a rapid response.

1.5.3.1 MAPK cascade

MAPK phosphorylation cascades are conserved signalling modules, present across all eukaryotes, which transduce an extracellular signal detected by a transmembrane receptor-kinase and trigger an intracellular response to the stimulus (Widmann et al. 1999). MAPK cascades typically involve three kinases: a MAP kinase kinase kinase (MAPKKK) which when activated phosphorylates Ser/Thr residues in the Ser/Thr- X_{3-5} -Ser/Thr activation loop of a MAP kinase kinase (MAPKK), which is then in-turn able to phosphorylate Thr/Tyr residues in the Thr-X-Tyr MAPK activation loop. In plants, MAPK nomenclature differs slightly, with, MAPKs being referred to as MPKs, and MAPKKs/MAPKKKs named MKKs and MKKKs respectively (Ichimura et al. 2002).

The activation of MAPK cascades is a critical component of the initial rapid PTI response (Meng and Zhang 2013), with full chitin-induced MAPK cascade activation occurring within 5 minutes after chitin treatment (Wan et al. 2004; Cao et al. 2014). Following the perception of chitin by CERK1, a cytoplasmic kinase PBL27 is activated via phosphorylation (Shinya et al. 2014). PBL27 directly activates MKKK5, triggering a chitin-induced MKKK5-MKK4/5-MPK3/6 cascade (Yamada et al. 2016; Asai et al. 2002). Activated MPK3/6 then directly phosphorylates defence transcription factor, WRKY33, which is required for a full *B. cinerea* PTI response. Arabidopsis expressing WRKY33 with mutated MPK3/6 phosphosites (*wrky33-2*/WRKY33^{Ser→Ala}) showed reduced *B. cinerea*-induced camalexin production (Mao et al. 2011). Further demonstrating the importance of this signalling module, mutants of *pbl27-1*, *mkkk5-1*, *mpk3-1* and *wrky33-1* all display increased susceptibility to fungal necrotrophic pathogens (Shinya et al. 2014; Yamada et al. 2016; Ren et al. 2008; Zheng et al. 2006).

1.5.3.2 Calcium burst

Calcium ions (Ca^{2+}) are secondary messenger signalling molecules across all eukaryotes (Clapham 2007), forming an integral component of the signal transduction in response to almost all environmental stresses and developmental stimuli, including pathogen infection (Kudla et al. 2010). Ca^{2+} is maintained at very low cytosolic levels ($\sim 10^{-8}$ M), generating huge Ca^{2+} gradients across plasma and organellar membranes (Pirayesh et al. 2021). Ca^{2+} -permeable channels are then able to facilitate rapid Ca^{2+} -influxes into the cytoplasm, known

as “calcium burst”. Cyclic nucleotide-gated channel 2 (CNGC2) and CNGC4 form a Ca^{2+} -permeable channel which is activated by Botrytis-induced kinase 1 (BIK1), an RLCK, facilitating a PTI-activated Ca^{2+} -influx (Tian et al. 2019).

Following Ca^{2+} -influx, calcium-sensing proteins such as calmodulin (CaM), calreticulin B and calcium-dependent protein kinases (CDPKs) are activated (Ikura 1996; Köster et al. 2022). Arabidopsis triple CDPK mutants *cdpk5/6/11* show hyper-susceptibility and reduced ethylene response to *B. cinerea*. *cdpk5/6/11* plants also have completely abolished flg22 and oligogalacturonide-induced immunity against *B. cinerea*, suggesting these CDPKs are redundant regulators of the PTI response which is triggered by MAMPs/DAMPs (Gravino et al. 2015). CDPK5/6 were demonstrated to phosphorylate WRKY33, increasing its DNA-binding activity, and activating camalexin biosynthesis (Zhou et al. 2020).

Pathogen effectors may also target the Ca^{2+} -influx to dampen the hosts' PTI response. SsITL, which promotes increased *S. sclerotiorum* virulence, directly interacts with a chloroplast-localised Ca^{2+} sensing-receptor (CAS). Although CAS does not have any Ca^{2+} binding domains, it has been reported to regulate Ca^{2+} flux in guard cells and *cas-1* mutants are hyper-susceptible to *S. sclerotiorum*, suggesting it acts as a positive defence regulator (Weinl et al. 2008; Tang et al. 2020).

CAMTA3, also known as signal responsive 1 (SR1), is a calmodulin-binding transcription factor which directs transcriptional reprogramming downstream of Ca^{2+} signalling (Yuan et al. 2022). CAMTA3 has been shown to repress SA accumulation, rendering plants more tolerant to drought but more susceptible to pathogens (Zeng et al. 2022; Du et al. 2009). *camta3-1* mutants show increased SA accumulation and quantitatively increased resistance to *B. cinerea*, *S. sclerotiorum* and *Pseudomonas syringae* pv. tomato (*Pst*) DC3000, showing that CAMTA3 negatively regulates defence against both biotrophic and necrotrophic pathogens (Du et al. 2009; Galon et al. 2008; Rahman et al. 2016). Furthermore, Ca^{2+} -signalling appears to be required for CAMTA3-induced immune suppression, as plants expressing a mutated isoform unable to bind calmodulin (*camta3-1/p35S::CAMTA3^{K907E}*) were also *Pst* DC3000 resistant (Du et al. 2009). However, to limit the effects of its immune suppression, CAMTA3 is degraded and exported from the nucleus upon flg22 treatment MPK3/6 phosphorylation and degraded by a ubiquitin E3 ligase SR1-interacting protein 1 (SR1IP1) after *Pst* DC3000 *avrRps4* infection (Jiang et al. 2020; Zhang et al. 2014a). It has not yet been demonstrated whether CAMTA3 is degraded upon *B. cinerea* infection.

These results show that Ca^{2+} -signalling is a crucial component of the PTI defence response, with CDPK5/6/11 and CAS providing increased disease resistance to necrotrophs. However, Ca^{2+} is a universal signal used to respond to multiple stresses (e.g. drought stress), therefore, not all downstream Ca^{2+} signalling

events will be applicable to all stresses, requiring fine-tuning for the specific stress, such as degradation and nuclear export of CAMTA3 upon pathogen infection.

1.5.4 Defence Phytohormones

Phytohormones control almost every aspect of plant physiology, including the defence response to pathogens. Key defence hormones including jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) coordinate the response to pathogen infection. In this section, I will summarise the discovery, biosynthesis, perception, signalling and role in necrotrophic pathogen defence for each of these defence hormones.

1.5.4.1 Jasmonic Acid

Jasmonic acid (JA) is an oxylipin plant hormone, which was first isolated from jasmine oil (*Jasminum grandiflorum*) (Demole et al. 1962), which functions in various physiological processes such as; inhibition of root growth (Dathe et al. 1981), degradation of Rubisco (Weidhase et al. 1987), pollen development (McConn and Browse 1996), wounding (Creelman et al. 1992) and necrotrophic pathogen defence (Thomma et al. 1998).

JA biosynthesis via the octadecanoid pathway has been well characterised, starting with an 18-carbon poly-unsaturated fatty acid, α -linolenic acid (α LA) (Vick and Zimmerman 1984; Wasternack 2007). α LA is released from chloroplast membranes by phospholipase A₁ (PLA1) and is then oxidised by lipoxygenases (LOXs) to 13S-hydroperoxyoctadecatrienoic acid (13-HPOT) (Ishiguro et al. 2001; Liavonchanka and Feussner 2006). Plastid-localised allene oxide synthases (AOS) and allene oxide cyclases (AOC), catalyse the conversion of 13-HPOT to 12-oxophytodienoic acid (OPDA) via an unstable 12,13-epoxyoctadecatrienoic acid (12,13-EOT) intermediate (Song et al. 1993; Stumpe et al. 2006; Ziegler et al. 2000; Stenzel et al. 2012). An unknown transporter then shuttles OPDA from the chloroplast to the peroxisome, where it is reduced to 3-oxo-2-(2'-[Z]-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0) by 12-oxophytodienoate reductase 3 (OPR3) (Schaller et al. 2000; Scalschi et al. 2015). OPC-8:0 undergoes coA ligated by OPC-8:0 CoA Ligase1 (OPCL1) (Koo et al. 2006) and three rounds of β oxidation to form JA, which is carried out by enzymes such as acyl-CoA oxidase (ACX) and L-3-ketoacyl CoA thiolase (KAT) are required to produce JA (Koo et al. 2006; Li et al. 2005; Schillmiller et al. 2007; Castillo et al. 2004).

Following biosynthesis, JA can be further metabolised into a number of derivative compounds, which modulate its activity (Wasternack and Song 2017). JA can be conjugated to amino acids such as isoleucine or valine by GH3 family amino acid conjugate synthases GH3.10 and GH3.11 (also known as JASMONATE RESISTANT1; JAR1) (Staswick and Tiryaki 2004; Delfin et al. 2022). Wounding-induced transcriptional

activation of JA-responsive genes JAZ1 and OPR3 was dramatically reduced in *gh3.10-1/jar1-11* double mutants, demonstrating the importance of amino acid conjugation in JA signalling (Delfin et al. 2022). JA can also be methylated by jasmonic acid carboxyl methyltransferase (JMT), which when over-expressed in *Arabidopsis* increased accumulation of methyl-jasmonate (MeJA) and increased resistance to *B. cinerea* (Seo et al. 2001).

Coronatine insensitive 1 (COI1) is the jasmonate receptor, with *coi1-1* mutants insensitive to MeJA induced root inhibition (Feys et al. 1994) and displaying hyper-susceptibility to *B. cinerea* (Thomma et al. 1998). COI1 encodes an F-box protein (Xie et al. 1998) which forms a Skp/Cullin/F-box (SCF) E3 ubiquitin ligase complex with CUL1 (Cullin 1) and ASK1 (*Arabidopsis* Skp1 homologue) called *SCF^{COI1}* (Xu et al. 2002). In the presence of JA-Ile, but not MeJA, COI1 could bind jasmonate-zim domain (JAZ) proteins targeting them for proteasome degradation (Thines et al. 2007). JAZs act as transcriptional co-repressors preventing expression of defence genes in normal growth conditions, hence JA-Ile mediated degradation by *SCF^{COI1}* activates the expression of these genes. A decuple mutant, *jazD*, defective in 10 out of 13 *Arabidopsis* JAZs shows constitutive activation of JA-responsive defence genes, and increased *B. cinerea* resistance (Guo et al. 2018).

Many core components of the JA biosynthesis pathway including, LOX3, LOX4, AOC1, AOC3, ACX1, KAT2 and JMT, were all identified as being transcriptionally upregulated in response to *B. cinerea* infection (Windram et al. 2012). In the same study, genes assigned to GO-term "response to jasmonic acid" significantly enriched in genes upregulated at 16 hours post *B. cinerea* infection, demonstrating that the JA signalling is a critical component of the response to necrotrophic infection. Further many JA biosynthesis and signalling mutants have been demonstrated to show increased *B. cinerea* susceptibility including *coi1-1* (Thomma et al. 1998), *jar1-1* (Ferrari et al. 2003), *aos* (Rowe et al. 2010) and tomato *acx1* (AbuQamar et al. 2008).

1.5.4.2 Ethylene

Ethylene (ET) gas (C₂H₄) was first identified as a plant hormone through observations that leaking illumination gas (fuel for gas lamps which contains ET) affected plant physiology, such as horizontal epicotyl growth (Neljubow 1901). ET was also observed to promote abscission (Doubt 1917) and fruit ripening (Denny 1924; Kidd and West 1933). Later, a role for ET in pathogen defence was proposed, with ET being rapidly synthesised upon pathogen infection (Paradies et al. 1979; Mauch et al. 1984), driving the expression of defence genes (Ecker and Davis 1987).

ET has a relatively simple biosynthetic pathway consisting of just two enzymatic steps (Kende 1993). S-adenosyl-L-methionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5'-methylthioadenosine (MTA) by an ACC synthase (ACS) (Boller et al. 1979). An ACC oxidase (ACO)

then converts ACC to ET, CO₂ and cyanide (Hamilton et al. 1991). Cyanide is then rapidly detoxified by conversion to β -cyanoalanine (Yip and Yang 1988). The MTA by-product of the ACS reaction is recycled back to methionine via the Yang Cycle (Murr and Yang 1975), this is needed to maintain SAM levels required to rapidly synthesise ET. Rice plants overexpressing Acireductone dioxygenase (OsARD1), a key component of the Yang cycle, had higher levels of ET accumulation and improved submergence tolerance (Liang et al. 2019).

Arabidopsis has five known ET receptors localised to the ER membrane; ETR (ethylene response)1, ETR2, ERS (ethylene response sensor)1, ERS2 and EIN4 (ethylene insensitive 4) (Bleecker et al. 1988; Chang et al. 1993; Hua et al. 1998; Hua et al. 1995; Sakai et al. 1998). Unexpectedly all five ET receptors function to repress ET signalling; upon binding with ET the receptor function is inhibited, which results in the activation of downstream ET signalling (Binder 2020). Constitutive triple response 1 (CTR1) is an ER-localised Ser/Thr kinase that directly interacts with ET receptors and also negatively regulates ET signalling (Kieber et al. 1993; Ju et al. 2012). In the absence of ET CTR1 phosphorylates EIN2, promoting proteasomal degradation of EIN2 (Alonso et al. 1999; Ju et al. 2012; Qiao et al. 2009). EIN2 encodes a membrane-localised protein with homology to metal transporters but shows no transporter activity. In the presence of ET, proteolytic cleavage releases the EIN2 C-terminus (EIN2-C) which is translocated to the nucleus (Thomine et al. 2000; Ju et al. 2012). EIN2-C interacts with EIN2 nuclear-associated protein (ENAP1) in the nucleus, promoting histone acetylation and increasing chromatin accessibility at EIN3 targets (Zhang et al. 2017a). EIN3 and ethylene insensitive3-like (EIL)1/2 encode transcription factors which act downstream of EIN2-C. EIN3 binds the promotor of ethylene response factor 1 (ERF1) which in turn activates the expression of ET-responsive genes such as PDF1.2 (Chao et al. 1997; Solano et al. 1998).

Upon infection by the necrotrophic fungus *B. cinerea*, ET biosynthesis is rapidly activated, with elevated ET accumulation detectable as early as 6 hours post infection (hpi) and peaking at 36 hpi (Han et al. 2010). A MAPK cascade results in the rapid activation of ACS proteins via phosphorylation, with *mpk3/mpk6* double mutants and *ACS6^{AAA}* phospho-mutants both showing dramatically reduced *B. cinerea*-induced ET biosynthesis (Han et al. 2010). Treatment with a *S. sclerotiorum* elicitor (SCFE1) also induces ethylene biosynthesis, and *rlp30-2* mutants which show no SCFE1 response show increased susceptibility to *S. sclerotiorum* (Zhang et al. 2013). Further emphasising the critical role of ET biosynthesis in defence, an octuple *Arabidopsis* ACS mutant (*acs2-1/acs4-1/acs5-2/acs6-1/acs7-1/acs9-1/amiR-acs8/amiR-acs11*) displays extreme susceptibility to *B. cinerea* (Tsuchisaka et al. 2009). These results demonstrate that the ability to synthesise ET in response to necrotrophic pathogen infection is vital for defence. EIN2, EIN3, EIL1 and ERF1 all having been identified as positive regulators of *B. cinerea* resistance, demonstrating that downstream ET signalling components are also critical in *Arabidopsis*' defence against necrotrophic fungi (Thomma et al. 1999; Alonso et al. 2003b; Berrocal-Lobo et al. 2002).

1.5.4.3 Salicylic Acid

In 1763, an Oxfordshire clergyman Rev Edward Stone reported that extracts from the bark of willow trees (*Salix spp.*) could relieve symptoms of fever and shivering in patients (Stone 1763). By the early 1800s salicylic acid (SA) was identified as the active component of willow bark (Leroux 1830; Desborough and Keeling 2017), with early clinical trials showing an anti-inflammatory effect (MacLagan 1879). Chemists at Bayer synthesised an acetylated version of SA (acetylsalicylic acid) which had fewer side effects and was subsequently marketed as Aspirin (Dreser 1899).

For many years the physiological role of SA was unknown, until the discovery that Aspirin treatment induces Tobacco Mosaic Virus resistance in *Nicotiana tabacum* (White 1979), identifying a now well-established role for SA in the plant defence response particularly against biotrophic pathogens (Peng et al. 2021).

SA biosynthesis can occur in plants via the isochorismate synthase (ICS) or Phenylalanine Ammonia Lyases (PAL) pathway, with ICS being the predominant pathway in Arabidopsis. A chloroplast-localised ICS catalyses the conversion of chorismate to isochorismate (IC) (Wildermuth et al. 2001), IC is then exported to the cytosol by enhanced disease susceptibility 5 (EDS5), which encodes a MATE transporter (Serrano et al. 2013; Rekhter et al. 2019). AvrPphB Susceptible 3 (PBS3), also known as GH3.12 (the same family as JAR1 which converts JA to JA-Ile), then catalyses the conjugation of glutamate to IC in the cytosol forming isochorismate-9-glutamate (IC-9-Glu) (Rekhter et al. 2019). IC-9-Glu can spontaneously decay into SA and N-pyruvoyl-L-glutamate, but may also be catalysed by an BAHD acyltransferase, enhanced pseudomonas susceptibility 1 (EPS1) (Zheng et al. 2009; Torrens-Spence et al. 2019).

In Arabidopsis, ICS is the predominant pathway of SA biosynthesis, as *ics1/ics2* double mutants have dramatically reduced SA levels (Garcion et al. 2008). However, plants contain a second SA biosynthetic pathway which occurs via PALs with quadruple PAL mutants also showing reduced pathogen-induced SA accumulation, although PAL mutants still had far higher SA levels than ICS mutants (Huang et al. 2010). The relative importance of the two SA biosynthetic pathways is not consistent across species, as SA accumulation in rice and soybean is heavily dependent on the PAL pathway (Shine et al. 2016; Xu et al. 2017; He et al. 2020a).

A rapid accumulation of SA in has been observed upon infection with biotrophic pathogens, providing systemic acquired resistance to repeat infections (Métraux et al. 1990; Malamy et al. 1990; Rasmussen et al. 1991). However, ICS1 and EPS1 were both downregulated in response to *B. cinerea* infection in Arabidopsis, suggesting SA biosynthesis is not activated under necrotrophic infection. Despite this exogenous treatment with SA or its analogue benzothiadiazole (BTH) increases Arabidopsis resistance to both *B. cinerea* and *S. sclerotiorum* (Ferrari et al. 2003; Guo and Stotz 2007).

NONEXPRESSER OF PR GENES 1 (NPR1) is a central regulator of SA-induced gene expression, such as pathogenesis-related 1 (PR1) (Cao et al. 1994; Cao et al. 1997). However, NPR1 does not contain a DNA-binding domain, instead interacting with TGACG-binding transcription factors (TGAs) in the presence of SA to induce its transcriptional response (Zhang et al. 2003; Jin et al. 2018). TGAs then activate the expression of further transcription factors, such as SAR-DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60-LIKE g (CBP60g) which target SA biosynthetic genes such as ICS1 and PBS3 in a positive feedback loop (Sun et al. 2020).

A classical model of phytohormone-induced defence suggests that two mutually-exclusive signalling networks exist which exert reciprocal repression on each other, JA/ET promoting resistance against necrotrophs, and SA promoting resistance against biotrophs (Spoel et al. 2007; Pieterse et al. 2012; Aerts et al. 2021). Although this model is now considered an oversimplification with complex JA-SA cross-talk known (Caarls et al. 2015), however the effect of SA signalling in necrotroph defence is inconsistent and dependent on the mutant. For example, expressing a bacterial enzyme which degrades SA (*nahG*) renders *Arabidopsis* more susceptible to *B. cinerea*, but *sid2-1* (SALICYLIC ACID INDUCTION DEFICIENT 2, which encodes ICS1) and *npr1-1* mutants had no changes in susceptibility to *B. cinerea* (Ferrari et al. 2003), while *npr1-1* showed increased susceptibility to *S. sclerotiorum* (Guo and Stotz 2007). Mutants of TGA3 (a NPR1-interacting transcription factor) were more susceptible to *B. cinerea* (Windram et al. 2012). Adding even further complexity, NPR1 is a negative regulator of *B. cinerea* resistance in tomato (Rahman et al. 2012; Li et al. 2020) and a positive regulator of *S. sclerotiorum* resistance in *Brassica napus* (Wang et al. 2020b), demonstrating that the role of SA in necrotroph defence differs between host species.

1.5.5 Transcriptional control of the defence response

Transcriptional reprogramming is mediated by transcription factors (TFs), proteins able to bind DNA in a sequence-specific manner via a DNA binding domain (DBD) to positively or negatively modulate the rate of transcription. TFs bind DNA elements, often in a 1-2Kb promotor region upstream of a gene, recruiting proteins to modify chromatin accessibility or influence the assembly of a transcription initiation complex (Strader et al. 2022). A single TF may co-regulate several target genes involved in a single biological process or function with a common cis-regulatory element, allowing an entire genetic program to be turned on or off simultaneously. For example PAX6/eyeless is a conserved TF which controls eye development, and ectopically expressing the TF in *Drosophila* wings or legs is sufficient to induce the formation of eye-like structures in those tissues (Halder et al. 1995). Similarly, expression of Arabidopsis PLT2 in the shoot meristem can induce the formation of ectopic roots and constitutive expression of ERF1 in Arabidopsis is sufficient to activate an ethylene response in the absence of hormone or pathogen treatment (Galinha et al. 2007; Solano et al. 1998).

Once an invading pathogen has been successfully detected, and a PTI response has been activated via MAPK cascades and Ca^{2+} bursts, phytohormones play pivotal roles in fine-tuning and amplifying the transcriptional response required to coordinate a larger defence against the pathogen. In the complex phytohormone signalling network, TFs act as “nodes of convergence”, able to integrate many input signals generating a specific response for a specific pathogen. For example, JA is involved in the defence against both necrotrophic pathogens and insects. JA signalling is fine-tuned via cross-talk with other phytohormones, including its integration with ET by the ERF1 transcription factor, which enhances defence against necrotrophic pathogens (Lorenzo et al. 2003; Cheng et al. 2013). Whereas the integration of JA and abscisic acid (ABA) signals, largely by the MYC2 transcription factor, increases insect defence (Verhage et al. 2011; Kazan and Manners 2013).

WRKY33 is a key example of an Arabidopsis TF that integrates signals of pathogen detection, initiates transcriptional reprogramming events which amplify the defence response. WRKY33 was first identified as a gene upregulated during *B. cinerea* infection (AbuQamar et al. 2006). Further studies revealed that *wrky33-1* mutants were hypersusceptible to *B. cinerea*, establishing its crucial role in plant defence (Zheng et al. 2006).

WRKY33 is activated by phosphorylation through distinct kinases: MPK3/6 and CDPK5/6. These phosphorylations occur at specific sites and have different consequences for WRKY33's function. For example, CDPK5 phosphorylation enhances WRKY33 binding at the PAD3 promoter, while MPK phosphorylation increases the transcriptional activation activity of WRKY33 (Li et al. 2012; Zhou et al. 2020).

CDPK5/6 and MPK3 have an additive effect on *B. cinerea* defence, suggesting the integration of MAPK and Ca^{2+} signalling is required for full WRKY33 activation. When activated, WRKY33 orchestrates various

transcriptional reprogramming events, including camalexin biosynthesis activation, ethylene biosynthesis activation, and ABA biosynthesis repression (Liu et al. 2015). The simultaneous activation of ET, and repression of ABA will activate the JA-ET signalling network driven by ERF1 (Lorenzo et al. 2003; Cheng et al. 2013).

Key TF families participating in plant defence response include WRKY, Ethylene Response Factor (ERFs), MYBs, basic helix-loop-helix (bHLH), basic-leucine zipper (bZIP), NAM-ATAF1-CUC2 (NAC), ethylene insensitive 3-like (EIL), and calmodulin-binding transcription activator (CAMTA). Many of these TFs (e.g., WRKY33, CAMTA3, EIN3, ERF1) have been identified as key regulators of necrotroph defence, demonstrating the importance of these transcriptional reprogramming events to the defence response.

This section will further elaborate on the roles and mechanisms of these various TF families in the necrotrophic defence response.

1.5.5.1 WRKYs

WRKYs are a super-family of plant TFs, first identified in sweet potato (Ishiguro and Nakamura 1994), characterised by the presence of a 60 amino acid N-terminal DBD with a conserved Trp(W)-Arg(R)-Lys(K)-Tyr(Y) motif and a C-terminal zinc-finger (Chen et al. 2019). WRKYs bind a conserved W-box (T)TGAC(C/T), with binding specificity within the family determined by the sequences flanking this consensus (Ciolkowski et al. 2008). WRKYs are specific to plants and algae with 2 present in *Klebsormidium flaccidum* (a multi-cellular filamentous algae which produces several plant hormones; Hori et al. 2014), 14 in *Marchantia polymorpha*, 72 in *Arabidopsis*, 76 in Lettuce, 81 in *Solanum lycopersicum* (tomato), 130 in *Zea mays* (maize) (Zheng et al. 2016).

WRKYs can be classified into three groups based on their domain structure (Eulgem et al. 2000). Group I WRKYs have two WRKY DBDs and a C2H2 zinc finger domain, Group II WRKYs have one WRKY DBD and a C2H2 zinc finger, whereas Group III have one WRKY DBD and C2HC zinc finger. X-ray crystallography has revealed that a group II WRKY DBD (from AtWRKY18) is able to deform the W-box double helix structure, widening the major-groove to facilitate binding (Grzechowiak et al. 2022).

WRKYs have been identified as regulators of many biotic and abiotic stress responses including pathogen defence in numerous plant species, which has been widely reviewed (Banerjee and Roychoudhury 2015; Phukan et al. 2016; Wani et al. 2021). As previously discussed, AtWRKY33 (group I) is a well-characterised positive regulator of *B. cinerea* defence activated by MAPK cascade, and regulates genes involved JA/ET signalling

(Zheng et al. 2006; Mao et al. 2011; Li et al. 2012; Birkenbihl et al. 2012; Liu et al. 2015).

AtWRKY54/70 are two closely-related, and functionally redundant group III WRKYs which regulate SA biosynthesis through SARD1/CBP60g, and are required for the auto-immunity phenotype of Arabidopsis expressing a constitutively active NLR, *snc2-1D* (Chen et al. 2021). *wrky54/wrky70* double mutants show increased susceptibility to *Pst* DC3000 and increased resistance to *B. cinerea* (Li et al. 2017). *Pst* DC3000 susceptibility was further increased in SA biosynthetic mutant background (*wrky54/wrky70/sid2*), beyond the level of either *sid2* or *wrky54/wrky70*, suggesting SA biosynthesis independent functions. However, increased *B. cinerea* resistance was dependent on SA biosynthesis, as (*wrky54/wrky70/sid2*) has comparable lesion sizes to wild-type and *sid2* (Li et al. 2017).

A *Gossypium hirsutum* (Cotton) orthologue of AtWRKY70, GhWRKY70D13 (also a group III WRKY), has been demonstrated as a negative regulator of defence against necrotrophic fungal pathogen, *Verticillium dahliae*, as RNAi-GhWRKY70D13 lines show increased resistance and upregulation of JA/ET genes such as GhERF1 and GhAOS2 (Xiong et al. 2020). A group I WRKY from *Fragaria X ananassa* (strawberry), FaWRKY25 has been identified as a negative regulator of JA biosynthesis with RNAi-FaWRKY25 promoting increased *B. cinerea* resistance (Jia et al. 2020). An Amur grape (*Vitis amurensis*) WRKY (VaWRKY10, group I) was also demonstrated to increase resistance to *B. cinerea* when overexpressed in Arabidopsis or *Vitis vinifera* (Wan et al. 2021).

These results demonstrate that WRKY TFs act as both positive and negative regulators of necrotrophic pathogen resistance in plant species, even within a single group.

1.5.5.2 AP2/ERFs

APETALA 2 (AP2)/Ethylene Response Factor (ERF) also known as Ethylene Response Element Binding Proteins (EREBPs) are a super-family of plant-specific transcription factors, with well characterised roles in plant defence (Nakano et al. 2006). The first AP2/ERF domain identified was in Arabidopsis AP2 (Jofuku et al. 1994). Shortly afterwards, 4 closely related tobacco AP2/ERF domain TFs were identified and shown to bind GCC-box elements (TAAGAGCCGCC) in ethylene-inducible promoters (Ohme-Takagi and Shinshi 1995). AP2/ERF domains are 60-70 amino acids, containing three anti-parallel β -strands and a single α -helix (Nakano et al. 2006; Allen et al. 1998), with β -strands directly interacting with GCC-box nucleotides (Chen et al. 2020a). Three sub-families of AP2/ERFs exists, AP2-type, RAV-type and ERF-type EREBPs (ERFs, hereafter), ERFs are by far the largest sub-family and are involved in pathogen defence (Nakano et al. 2006). Lettuce has an expanded ERF family containing 190 predicted ERFs, compared to 4 ERFs in *K. flaccidum*, 26 in *M. polymorpha*, 128 in Arabidopsis, 140 in tomato and 194 in maize.

Numerous ERFs have been identified as regulators of the necrotroph defence response, such as ERF1 (Berrocal-Lobo et al. 2002) which has been previously discussed. OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59) has been demonstrated as a positive regulator of *B. cinerea* defence, upregulating JA/ET downstream genes such as PDF1.2 (Penninckx et al. 1996; Pré et al. 2008). Tomato (*Solanum lycopersicum*) ERFs have also been shown to promote *B. cinerea* resistance, with silencing of SIERF.A1, SIERF.B4, SIERF.C3 and SIERF.A3 increasing susceptibility (Ouyang et al. 2016).

1.5.5.3 NACs

NACs are another large TF family in plants, named after three early TFs that were identified a conserved domain; **NAM** (NO APICAL MERISTEM)-**ATAF1**(*Arabidopsis thaliana* Activation Factor 1)-**CUC2** (CUP-SHAPED COTYLEDON2) (Olsen et al. 2005). Lettuce has 101 NACs, compared to 2, 9, 96, 112 and 135 found in *K. flaccidum*, *M. polymorpha*, tomato, *Arabidopsis* and maize respectively (Zheng et al. 2016). NACs have been shown to regulate a number of plant physiological process such as development (Souer et al. 1996), senescence (Kim et al. 2016), abiotic stress including drought (Nakashima et al. 2012; Mao et al. 2016) and pathogen defence response (Yuan et al. 2019). X-ray crystallography of DNA-bound NAC19 revealed that the NAC-DBD utilises 7 β -strands for DNA binding flanked by α -helices, with the β -strands directly binding the DNA major groove without DNA helix disruption (Welner et al. 2012). Some NACs show additional regulation via a C-terminal transmembrane domain which prevents nuclear localisation, in response to a stimulus, proteolytic cleavage allows translocation of the N-terminal NAC DBD to the nucleus facilitating transcriptional regulation (Kim et al. 2007a).

Many NACs have shown to act as both positive and negative regulators of defence against necrotrophic pathogens. *Arabidopsis* NAC19/55 show redundant negative regulation of *B. cinerea* defence, with roles in fine-tuning JA signalling downstream of MYC2 (a bHLH-family TF which regulates JA-ABA signalling and will be discussed below) (Bu et al. 2008). ATAF1 has been demonstrated as a conserved negative regulator of defence response, with overexpression of AtATAF1 promoting increased susceptibility to *B. cinerea*, *A. brassicicola* and *Pst* DC3000 (Wang et al. 2009a). Similarly, overexpression of cotton ATAF1 orthologue (GhATAF1) in cotton plants suppressed JA signalling, increased susceptibility to *B. cinerea* and *V. dahliae* (He et al. 2016). However, SISRN1, a tomato transmembrane-domain containing NAC which positively regulates defence, as *B. cinerea* resistance is increased when transiently overexpressed, and decreased when silenced (Liu et al. 2014).

1.5.5.4 MYBs

Unlike the TF families introduced thus far, myeloblastosis-related (MYB) TFs are not plant-specific, with their name originating from an avian myeloblastosis virus TF which promotes myeloid leukemia in chickens (Klempnauer et al. 1984). COLORED1 was the first MYB identified in plants, which regulates anthocyanin biosynthesis in maize (Paz-Ares et al. 1987). Human c-myc contains three adjacent helix-turn-helix (HTH) repeats (R1, R2 and R3), and plant MYBs are classified by the presence of these repeats. R2R3-MYBs, containing two adjacent HTH repeats with similarity to c-myc R2 and R3 respectively are the most abundant sub-class of plant MYB with 126 in Arabidopsis, 3R-MYBs (R1-R2-R3) and 4R-MYBs (R1-R2-R3-R1/2) also exist in plants (Dubos et al. 2010). This HTH architecture shows a clear difference between animal and plant MYB TFs. The lettuce genome contains 155 R2R3 MYBs (MYBs hereafter) and 68 additional non-R2R3 MYBs (MYB-related).

Many plant MYBs have been characterised as regulators of secondary metabolite biosynthesis, as well as maize colored, MdMYBA in apple *Malus x domestica* directly activates anthocyanin biosynthesis resulting in red pigmentation (Ban et al. 2007). MYBs have also been well-characterised as regulators of flavonoid biosynthesis, a diverse class of compounds which have anti-microbial properties and give desirable flavour-profiles in many fruits (Czemmel et al. 2012; Xu et al. 2015).

MYBs have also been identified as regulators of the pathogen defence response. SIMYB1 has recently been shown to promote *B. cinerea* resistance in tomato fruits by increasing flavonoid and carotenoid content (Yin et al. 2023). AtMYB108 was originally suggested to be a positive regulator of defence against *B. cinerea*, as T-DNA insertion mutants were hypersusceptible to *B. cinerea*, hence was named Botrytis Susceptible 1 (BOS1) (Mengiste et al. 2003). However, re-analysis of the original mutants by Cui et al. 2022 recently demonstrated that *bos1-1* was a gain-of-function mutation, and the AtMYB108 is a negative regulator of *B. cinerea* defence. Supporting its role as a negative defence regulator, Hickman et al. 2013 showed that AtMYB108 activates the expression of negative defence regulators, NAC19/55 (Bu et al. 2008), during *B. cinerea* infection. However, a highly similar orthologue in cotton, GhMYB108 promotes increased resistance to *V. dahliae* in cotton plants and increased resistance to both *V. dahliae* and *B. cinerea* in transgenic Arabidopsis, showing species-specific differences in MYB108 function. It remains unclear how the highly similar AtMYB108 and GhMYB108 are able to have opposite effects on the defence response to fungal necrotrophs.

1.5.5.5 bHLHs and bZIPs

Basic helix-loop-helix (bHLH) and basic leucine zipper (bZIP) are two non-plant specific TF super-families with 137, 72 and 158, 91 members in Arabidopsis and Lettuce respectively (Toledo-Ortiz et al. 2003; Jakoby

et al. 2002). While neither family are considered to have major roles in stress response, members of both families have been identified as key defence regulators.

bHLH transcription factors MYC2, MYC3 and MYC4 function redundantly to integrate JA-Abscisic Acid (ABA) signalling for herbivore defence, a contrasting branch of JA response to JA/ET integration via ERF1 and ORA59 which promotes necrotrophic pathogen defence (Kazan and Manners 2013). As a result, MYC2 represses the ERF1/ORA59-branch (PDF1.2 expression) promoting susceptibility to *B. cinerea* (Lorenzo et al. 2004; Zhang et al. 2014b) and increased insect resistance (Verhage et al. 2011).

TGACG SEQUENCE-SPECIFIC BINDING PROTEINs (TGAs) are a subgroup of bZIPs, which interact with NPR1 to promote SA-induced gene expression (Zhang et al. 2003; Jin et al. 2018). However TGA mutants *tga3-2* and *tga2/5/6* display increased susceptibility, suggesting they promote increased resistance against necrotrophic pathogens (Windram et al. 2012; Zander et al. 2014). Other bZIPs not classified within the TGA subgroup have also been demonstrated as defence regulators, overexpression of soybean GmbZIP15 promotes resistance to *S. sclerotiorum* (Zhang et al. 2021). Furthermore, bZIP17, bZIP28 and bZIP60 are well characterised regulators of the unfolded protein response (UPR), a stress response conserved across eukaryotes which is triggered by the accumulation of misfolded and aggregated proteins in the ER (Bao and Howell 2017). Silencing of bZIP60 in *Nicotiana attenuata* lead to increased susceptibility to a fungal necrotroph (*Alternaria alternata*) and downregulation of protein folding chaperones calnexin (CNX), BiP and protein disulphide isomerase (PDI) (Xu et al. 2019).

1.5.5.6 Identifying TF targets

Once a potential TF of interest has been identified, determining its downstream target genes can shed light on the molecular processes underlying the TF phenotype. Many of the DNA binding motifs described (such as the WRKY W-box and the ERF GCC-box) are core consensus motifs for an entire TF super-family. However, such motifs are highly abundant within genomes, with 12,200 W-box motifs being identified in the promoters of 7670 Arabidopsis genes, and many of the 72 Arabidopsis WRKYs could bind each of them (Dhatterwal et al. 2019).

Direct TF-DNA interactions can be identified via DNA Affinity Purification sequencing (DAP-seq, *in vitro*) or chromatin-immunoprecipitation sequencing (ChIP-seq, *in vivo*). These methods may be able to reveal subtle differences in DNA-binding specificities between members of expanded TF families. DAP-seq has been performed on 529 Arabidopsis TFs *in vitro*, identifying > 2.7 million TF binding sites (O'Malley et al. 2016a). However, DNA-binding may not be sufficient as the recruitment of additional proteins may be required for transcriptional activators such as Mediator complex sub-units that induce looping between cis-regulatory

elements, contributing to the formation of the transcriptional pre-initiation complex under JA signalling (Kidd et al. 2009; Wang et al. 2019a). In addition transient TF-DNA interactions may be challenging to detect with existing assays (Swift and Coruzzi 2017).

In the case of WRKY33, DAP-seq identified 4472 putative targets, but a coupled ChIP-seq and RNA-seq experiment after *B. cinerea* infection in *wrky33-1/P_{WRKY33}*:WRKY33-HA plants identified 1576 ChIP-targets, only 318 of which were DEGs (Liu et al. 2015). Only 169 of the high-confidence direct WRKY33 regulated *B. cinerea* targets were identified as a DAP-seq target of WRKY33. This data suggests that DAP-seq may struggle to identify context-specific DNA-binding events, such as those which only occur in response to pathogen infection.

Alvarez et al. 2020 used an innovative approach to identify the direct targets of a nitrogen-responsive Arabidopsis transcription factor, NIN-LIKE PROTEIN 7 (NLP7). They expressed an NLP7-GR (glucocorticoid receptor) fusion protein, which allowed conditional nuclear import of NLP7 after DEX treatment. To ensure observed changes in gene expression were direct effects of NLP7 and not due to downstream TFs, cells were pre-treated with cycloheximide (CHX), a translation inhibitor. Following DEX treatment (and nuclear import of NLP7-GR), time series ChIP-seq experiments were performed, and RNA-seq was conducted 180 minutes after +DEX or -DEX treatment. Of the 492 differentially expressed genes (+DEX +CHX vs -DEX +CHX) identified, only 12% (n=61) were stable NLP7 targets in ChIP-seq, and 33% (n=161) were transient. 55% (n= 270) of DEGs weren't NLP7-bound in any ChIP time-point. DamID, a technique that uses a DNA adenine methyltransferase fused to a TF, was utilised to mark regions of the genome where NLP7 interacts with DNA (Gutierrez-Triana et al. 2016; Aughey and Southall 2016). This identified 191 targets that were directly differentially expressed by NLP7, including 92 not detected by ChIP. TFs within these 92 targets with highly-transient NLP7-binding only detectable using DamID were demonstrated to drive a significant proportion of the indirect NLP7-induced transcriptional reprogramming. This work highlights that many biologically relevant TF-DNA interactions are highly-transient and are difficult to detect with ChIP, even in a time series with 5 minute resolution.

This demonstrates why it may be challenging to experimentally or bioinformatically identify direct TF targets (also reviewed in depth by Alvarez et al. 2021). Therefore, many efforts to computationally predict transcriptional regulation don't discriminate between direct and indirect downstream target genes, and instead ask which TF gene expression is best able to explain gene expression of a given target (Geng et al. 2021). This will be discussed in more detail in section 1.6.

1.5.6 Post-transcriptional control of the defence response

In the previous section, “gene expression” is analysed using mRNA abundance as a proxy for protein activity, largely due to the emergence of microarrays and RNA sequencing as revolutionary cost-effective and high-throughput tools for gene discovery (Wang et al. 2009b). However, it is well established that gene expression changes in response to pathogen infection aren't fully encapsulated by total mRNA abundance, due to differing rates of degradation and active translation amongst mRNA populations (Maldonado-Bonilla 2014). One mechanism of translation inhibition or degradation of specific mRNAs, is the sequence-specific binding of small RNAs (sRNA) (Ronemus et al. 2006).

Actively translated mRNAs can be isolated via the pull-down of a tagged ribosomal sub-unit and sequenced using RNA sequencing, giving a “translatome” (Zanetti et al. 2005). This methodology has been utilised to demonstrate large differences between defence-induced reprogramming transcriptome and translatome; with many transcriptome DEGs showing no difference in the translatome and vice-versa (Meteignier et al. 2017; Tabassum et al. 2020).

1.5.6.1 Small RNAs: a battle for post-transcriptional control

In the 1990s a novel form of gene regulation was discovered in *Caenorhabditis elegans* (nematode worm), whereby short 20-24nt fragments of RNA can post-transcriptionally silence a gene in a sequence-specific manner, termed RNA interference (RNAi) (Fire et al. 1991; Fire et al. 1998). Subsequently, RNAi was recognised as an evolutionarily conserved mechanism for gene regulation with discoveries of short RNA based gene silencing quickly following in: fungi (Romano and Macino 1992), insects (Pal-Bhadra et al. 1997), protozoa (Ruiz et al. 1998), plants (Hamilton and Baulcombe 1999; Baulcombe 2004) and mammals (Elbashir et al. 2001).

Plants encode numerous classes of small RNAs (sRNAs). These are produced through distinct, yet overlapping, processing steps which are involved in both epigenetic and post-transcriptional silencing (reviewed in Axtell 2013; Borges and Martienssen 2015). Dicer-like (DCL) proteins are specialised endoribonucleases which cleave double-stranded RNA (dsRNA) or stem-loop RNA into short-fragments are common to all sRNA biogenesis pathways (Fukudome and Fukuhara 2017). Once processed, sRNAs bind to ARGONAUTE (AGO) proteins, forming the RNA-induced silencing complex (RISC) that can induce mRNA cleavage, translation inhibition or epigenetic modification (Zhang et al. 2015a).

Micro RNAs (miRNAs) are transcribed from their own loci, precursor transcripts contain stem-loops that are cleaved by DCL1 to produce 20–22 nt mature miRNAs. Small interfering RNAs (siRNAs) processed from

dsRNA transcripts that are processed by DCLs, sub-classifications of siRNA exist depending on how the dsRNA transcript is generated. Natural antisense siRNAs (natsiRNAs) are produced from RNA transcripts with regions of perfect complementarity forming dsRNA, such as overlapping sense and anti-sense transcription (Borsani et al. 2005). Heterochromatic siRNAs (hetsiRNAs) are 24nt that direct epigenetic silencing of transposable elements via RNA-dependent DNA Methylation (RdDM). Processing of hetsiRNAs requires dsRNA synthesis by an RNA-dependent RNA polymerase (RDR) and subsequent cleavage by DCL3 (Matzke and Mosher 2014a). Secondary siRNAs can be produced from the fragments of an mRNA that has been cleaved by RISC. RDR6 produces a dsRNA copy of the cleaved fragment, which is subsequently cleaved by DCL4 at "phased" 21nt intervals producing phased siRNAs (phasiRNAs) (Fei et al. 2013; Xie et al. 2005). Suppressor of gene silencing 3 (SGS3) is required for phasiRNA biosynthesis, and has been suggested to stabilise the 3' end of RISC cleaved fragment, preventing degradation and allowing RDR6 to synthesise a dsRNA (Peragine et al. 2004; Yoshikawa et al. 2013). Protein-coding mRNA transcripts are a major source of phasiRNAs, such as those which encode pentatricopeptide repeats (PPRs) (Howell et al. 2007). A subset of phased siRNAs that are produced from non-coding (TAS) transcripts which have been demonstrated to be trans-acting, hence trans-acting siRNAs (tasiRNAs) (Allen et al. 2005).

Small RNAs perform critical plant-pathogen interactions, determining infection outcomes in a "battle for post-transcriptional control" (Qiao et al. 2021a). As previously discussed in Section 1.4.2.5, *B. cinerea* produces sRNAs using their own endogenous DCLs, secrete sRNAs in vesicles which enter plant cells, hijack host AGO to degrade host targets and promote pathogen virulence (Weiberg et al. 2013; He et al. 2023a; Wang et al. 2017c). Additionally Weiberg et al. 2013 demonstrated that Arabidopsis *dcl1-7* mutants showed increased *B. cinerea* susceptibility, while AGO1 directly bound Bc-siRNAs and *ago1-27* showed increased resistance. Thereby suggesting that DCL1-dependent miRNA biogenesis positively regulates *B. cinerea* defence but AGO1 negatively regulates defence as it is hijacked by fungal small RNAs.

However, AGO positively regulates *S. sclerotiorum* defence, with *ago1-27*, *ago1-32*, *ago2-1* and *ago9-1* showing increased susceptibility (Cao et al. 2016b; Cao et al. 2016a), suggesting that in a *S. sclerotiorum* infection host AGO is positively regulating defence. This is despite *S. sclerotiorum* producing sRNAs during infection, that have sequence complementary to Arabidopsis defence regulators which are downregulated during infection (Derbyshire et al. 2019). It remains to be elucidated whether these *S. sclerotiorum* sRNAs directly silence host targets, and whether this requires host AGO.

Plant miRNAs are able to fine-tune their own gene expression in response to pathogen infection, enhancing resistance. Arabidopsis miR156 targets and silences the mRNA of a TF, SPL7, which itself is a negative regulator of JA signalling, hence miR156 activates JA signalling and is a positive regulator of *B. cinerea* resistance (Sun et al. 2022; Mao et al. 2017). *Capsicum annuum* (chilli pepper) miRn37a is a positive regulator

of *Colletotrichum truncatum* defence, silencing three ERF TFs, leading to the upregulation of CaPDF1.2 (Mishra et al. 2018).

Arabidopsis mutants that are deficient in phasiRNA and tasiRNA biosynthesis (*dcl2/3/4*, *dcl4-2*, *rdr6* and *sgs3-1*) all show increased susceptibility to necrotrophic fungal pathogens (either *B. cinerea*, *S. sclerotiorum* or *V. dahliae*) (Cai et al. 2018; Cao et al. 2016a; Ellendorff et al. 2009). Arabidopsis secretes vesicles that contain tasiRNAs during *B. cinerea* infection, which are taken up by fungal cells and silence virulence genes (Cai et al. 2018). The effector *Phytophthora* suppressor of RNAi 2 (PSR2) has been shown to repress biosynthesis of phasiRNAs, specifically those generated from PPR transcripts (PPR-siRNAs), and promoting Arabidopsis susceptibility to *Phytophthora capsici* (Hou et al. 2019).

Together, these findings clearly demonstrate that post-transcriptional control of gene expression is vital in determining the outcome of plant-pathogen interactions. Both the host and attacker utilise small RNAs to facilitate a “successful outcome”.

1.5.7 Downstream anti-fungal responses

As outlined above, after the detection of an invading necrotrophic fungus via PRRs, a downstream PTI response is activated, triggering biosynthesis of defence phytohormones (JA/ET) and transcriptional reprogramming. After all these signal transduction events, a number of “anti-fungal” responses must finally be activated to restrict colonisation of the pathogen. These include strengthening of the cell wall to limit degradation by fungal CWDEs, synthesis of secondary metabolites which are toxic to the invading pathogen, and the accumulation of pathogenesis-related (PR) proteins.

1.5.7.1 Cell wall reinforcement

The plant cell wall is the primary barrier to microbe infection. Fungal necrotrophs actively destroy the cell wall through CWDE degradation to induce tissue maceration (section 1.4.2.3). Hence plants must respond and activate defence responses to maintain cell wall integrity. One approach is to activate the expression of pectin methylesterase inhibitors (PMEIs), protecting pectin from CWDE degradation (Lionetti et al. 2017). Overexpression of AtPMEI1 and AtPMEI2 increases *B. cinerea* resistance in Arabidopsis (Lionetti et al. 2007).

Papillae, which contain callose, a β -1,3-glucan polymer, are deposited at the cell wall at the site of microbe infection (Aist 1976; Voigt 2014). Callose deposition is activated by the chitin PTI response (Yamada et al. 2016). Mutants of powdery mildew resistant 4 (*pmr4-1*) also known as glucan synthase like 5, do not deposit callose upon infection and show increased susceptibility to the fungal necrotroph *A. brassicicola*. However, there may be some callose-independent functions of PMR4/GSL5, as double SA-callose biosynthetic mutants (*sid2-1/pmr4-1*) also lack callose deposition, but have similar *A. brassicicola* resistance to *sid2-1* (Flors et al. 2008). Callose deposition in tomato also appears to be dependent on OPDA (JA precursor), as RNAi silencing of SLOPR3 leads to *B. cinerea* susceptibility and reduced callose deposition, however both phenotypes could be rescued with OPDA treatment, but not JA treatment (Scalschi et al. 2015). It is unclear how OPDA may regulate callose independently of JA.

1.5.7.2 Anti-fungal metabolites

Plants produce vast numbers of complex secondary metabolites, with >10,000 compounds detected within a single species and estimates of up to 1 million specialised metabolites being produced across the plant kingdom (Fang et al. 2019). Many secondary metabolites have been utilised for their medicinal properties, such as a sesquiterpene lactone (STL) isolated from *Artemisia annua* with anti-malarial activity artemisinin, and morphine which is derived from opium poppy (*Papaver somniferum*) (Rai et al. 2017).

A major *in planta* function for many secondary metabolites is thought to be defence against pathogens, with many classes having been reported with anti-fungal activity (Kliebenstein 2004). A Brassicaceae specific indole-alkaloid, camalexin has been demonstrated as an essential component of *B. cinerea* resistance in Arabidopsis. Phytoalexin deficient 3 (PAD3) encodes a cytochrome P450 that catalyses the final stage of camalexin biosynthesis (Zhou et al. 1999), with *pad3-1* and camalexin secretion mutants (*pen3-3/pdr12-2*) exhibiting hypersusceptibility to *B. cinerea* (Ferrari et al. 2003; He et al. 2019). Sensitivity to metabolites can vary between closely-related fungal species, for example *pad3-1* only shows minor susceptibility to *S. sclerotiorum* due to its ability to detoxify camalexin (Stotz et al. 2011; Pedras and Ahiahonu 2002). Sensitivity to host defence metabolites can differ even among isolates of the same species. For instance, the *B. cinerea* DKUS-1 exhibits strong resistance to the toxic effects of camalexin, and as a result its virulence is unaffected by the *pad3-1* mutation (Kliebenstein et al. 2005).

Unlike the signalling phytohormone and signalling mechanisms discussed above, it is not expected that secondary metabolites produced in response to pathogen infection will be conserved between Arabidopsis and lettuce. For example, camalexin has only been detected in the Brassicaceae family. STLs are notable secondary metabolites which are abundant and diverse in Asteraceae species such as lettuce (Sessa et al. 2000). Several STLs have *in vitro* anti-fungal activity (Pickman 1984; Nawrot et al. 2021). Takasugi et al. 1985 identified an STL in lettuce, lettucenin A which was later shown to be produced in response to pathogen infection and had anti-microbial effects against *Bremia lactucae*, *B. cinerea* and *P. syringae* *in vitro* (Bennett et al. 1994). In strawberry, expression of terpene synthases (FaTPS) and terpene accumulation was upregulated after MeJA treatment. Overexpression of FaTPS1 increases *B. cinerea* resistance, over-accumulating sesquiterpene Germacrene D and monoterpenoid α -terpineol (Zhang et al. 2022).

1.5.7.3 Pathogenesis-related proteins

Pathogenesis-related (PR) proteins describe 17 gene families (named PR-1 to PR-17 respectively) which have been identified in several plant species, are upregulated upon pathogen infection, many of which show anti-microbial activity (Loon 1985; Van Loon et al. 1994; Sels et al. 2008). Some PR families have already been discussed. PR-1 mRNA is upregulated by NPR1, and acts as a “marker” of the SA-induced defence response, although the function of PR-1 remains elusive (Loon et al. 2006).

The PR-12 family encodes plant defensins (PDFs) which are marker genes of JA/ET gene expression in Arabidopsis (Penninckx et al. 1996; Manners et al. 1998; Brown et al. 2003). PDFs are ~5kDa cysteine-rich peptides containing three β -sheets and an α -helix strengthened by 3-5 disulphide bonds, conferring resistance to extreme temperature and pH (Lay and Anderson 2005). Numerous PDFs demonstrate anti-fungal activities both *in vitro* (Lacerda et al. 2014) and *in planta* promoting increased resistance to necrotrophic fungi such as

B. cinerea and *V. dahliae* (Gao et al. 2000; Khan et al. 2006; Aerts et al. 2007). PDFs form pores within the fungal cell membrane, facilitating ion leakage and fungal cell death (Thevissen et al. 1996).

The PR-15 family encode oxalate oxidases (OA-Ox), involved in the detoxification of oxalic acid, an important virulence factor in *S. sclerotiorum* (Chiriboga 1966). *B. cinerea* also produces oxalic acid, but it is not as important for virulence as in *S. sclerotiorum*. OA-Oxs have been identified in some grasses such as barley and sorghum, and when transgenically expressed in other species increases *S. sclerotiorum* resistance (Verma and Kaur 2021; Livingstone et al. 2005; Yang et al. 2019). Arabidopsis does not contain an OA-Ox, but AtAAE3 has oxalyl-CoA synthase activity and is able to detoxify oxalic acid, promoting *S. sclerotiorum* resistance (Foster et al. 2012).

Multiple PR families encode enzymes which degrade fungal cell wall polymers, such as β -1,3-glucanases (PR-2) and chitinases (PR-3, PR-4, PR-8, PR-11) (Loon et al. 2006). Neither of these polymers are abundant in the plant cell wall, but are major components of the fungal cell wall, hence both enzyme classes can be utilised to damage fungal cell wall integrity (Mohammadi and Karr 2002; Roberts and Selitrennikoff 1988). Transgenic strawberry expressing ch5B, a *Phaseolus vulgaris* (bean) chitinase show increased resistance to *B. cinerea* (Vellicce et al. 2006; Boller et al. 1983). Transgenic carrot plants expressing a wheat chitinase, a wheat β -1,3-glucanase and a rice cationic peroxidase (PR-9 family) show increased resistance to both *B. cinerea* and *S. sclerotiorum* (compared to wild-type plants), however carrot lines expressing just the β -1,3-glucanase only displayed increased resistance to *B. cinerea* (Wally et al. 2009). Yang et al. 2020 generated soybean plants which transgenically express the chitinase gene from *Coniothyrium minitans* (CmCH1), a mycoparasite that specifically infects *Sclerotinia spp*, resulting in increased *S. sclerotiorum* resistance. However, no comparisons between the activity of CmCH1 and plant chitinase activity were made.

The PR-6 family encodes a class of proteinase inhibitors (PI) first identified to accumulate in potato leaves after insect damage (Green and Ryan 1972), hence they are also known as potato inhibitor I. Hence PR-6 refers to a specific subclass of PI, however, other classes such as potato inhibitor II, Kunitz-type and Bowman-Birk PIs have also been demonstrated to show anti-fungal activity (Kunitz and Northrop 1936; Bowman 1944; Grosse-Holz and Hoorn 2016). PIs from *Isoglossa woodii* (buckweed) inhibit Subtilisin-like proteases from *B. cinerea in vitro* (Dunaevskii et al. 2005), and exogenous application of potato PIs inhibited *B. cinerea* growth in culture and repressed pathogenicity (Hermosa et al. 2006). SAP16, a trypsin-inhibitor from *Helianthus annuus* (sunflower) demonstrates potent inhibition of *S. sclerotiorum* spore germination (Giudici et al. 2000). Overexpression of PIs *in vivo* has been demonstrated to provide increased resistance to both insects and fungi. Overexpression of rice Bowman-Birk Inhibitor 2 (RBB2) promotes resistance to rice blast (*Pyricularia oryzae*) (Qu et al. 2003), and overexpression of unusual serine-protease inhibitor (UPI) promotes *B. cinerea* and cabbage looper (*Trichoplusia ni*) resistance (Laluk and Mengiste 2011).

Together, these examples summarise some of the anti-fungal strategies employed by plants to defend themselves against necrotrophic fungal pathogens.

1.6 Gene Regulatory Networks

Network theory is a branch of mathematics which describes the interactions (“edges”) between connected individuals (“nodes”). “Degrees” describe the connectivity of a given node within the network, defined as the number of edges between the given node and other nodes. Highly connected nodes are described as “Hubs” (Albert and Barabási 2002). Network theory has been utilised to understand social networks, and optimise transportation networks. However, the principles of network-theory can also be used to understand complex interactions between biological molecules. Biological networks have a specific architecture known as “scale-free topology”, meaning that they have nodes with very different scales (degrees), hence some nodes act as highly connected hubs, and some nodes have low connectivity (Barabási and Albert 1999; Jeong et al. 2000).

Biological networks can aid our ability to understand highly complex interactions that occur within a biological system, such as; metabolic interactions, protein-protein interactions (PPI) and transcriptional regulation (Jeong et al. 2000; Matija Dreze et al. 2011; Gerstein et al. 2012). A *Saccharomyces cerevisiae* PPI network revealed that highly connected hubs were more likely to cause a lethal phenotype in loss-of-function mutants (Jeong et al. 2001). This work provided an early demonstration that highly connected hubs in networks may also have high biological importance, with highly connected nodes more likely to be required for the survival of the organism. The application of network theory to biological networks has been reviewed in depth by Barabasi and Oltvai 2004.

High-throughput methods, such as microarrays and RNA-sequencing, have allowed detailed transcriptome-wide characterisation of the defence response (Windram et al. 2012; Hickman et al. 2017; Bjornson et al. 2021). Large datasets generated during these studies can be utilised to make predictions about the underlying networks of the transcriptional responses. In a relatively simplistic approach, edges are drawn between any two transcripts whose expression is highly correlated (often $R > 0.9$), hence termed co-expression networks (Langfelder and Horvath 2008). Co-expression networks are undirected and don't make any inferences about transcriptional regulation, but instead assign putative functions to uncharacterised genes based on the function of co-expressed genes. Zhang et al. 2017c constructed Arabidopsis co-expression networks after infection with 96 different *B. cinerea* isolates, identifying 5 core co-expression modules. Dong et al. 2015 constructed an integrated PTI-ETI network which was built on co-expression data as well as using available PPI and ChIP data to filter out false positives. Szymański et al. 2020 constructed a “co-abundance” network of mRNA transcripts and metabolites in 580 tomato lines after *B. cinerea* infection, candidate defence regulators were selected from mRNA/metabolite clusters that were abundant in *B. cinerea* resistant lines, such as pantothenic acid which displayed *in vitro* antifungal activity against *B. cinerea*.

Gene Regulatory Networks (GRNs) are a specific class of biological network, the edges of which represent transcriptional (or post-transcriptional) regulation events, nodes usually represent gene products (either RNA transcripts or proteins) (Thompson et al. 2015). A subset of GRN nodes are identified as possible regulators, these will be TFs in a transcriptional regulation network, but may include sRNAs in a GRN that incorporates post-transcriptional regulation. Unlike co-expression networks GRNs have directed edges originating only from regulator nodes. The use of a dense time series which captures the temporal dynamics of transcriptional reprogramming will aid the inference of regulatory edges (**time series-GRN-annrev**). GRNs may also differ depending on whether their edges represent direct or indirect regulation.

A GRN based on direct regulation may incorporate TF-DNA binding data such as DAP-Seq, ChIP-seq or chromatin accessibility data such as ATAC-seq (assay for transposase-accessible chromatin with sequencing), or the presence of the TF binding motifs within the promoters of putative targets. However, such models are challenging to construct on a transcriptome-wide scale due to the need for TF-DNA binding data in the specific biological conditions of interest. This level of information is only available in model species. Although, as discussed in Section 1.5.5.6, it is challenging to determine which TF-DNA binding events will result in differential regulation.

As part of the ENCODE (**Encyclopedia of DNA Elements**) project, a human direct-regulation GRN was constructed, integrating RNA-seq after siRNA gene silencing, ChIP-seq, TF-TF interaction, miRNA-seq and phospho-proteomics data (Gerstein et al. 2012). One of their key findings was a “combinatorial effect”, such that the DNA-binding sites of an individual TF could be substantially modified when present in different TF-TF pairs, demonstrating another level of complexity in attempting to identify direct TF targets.

Alternatively, a GRN can be constructed which does not attempt to discriminate between direct and indirect regulatory events. In this case, an edge does not represent a DNA-binding event, but instead demonstrates that a change in expression of the regulator/TF will likely result in an expression change of the target. While such a GRN lacks a mechanistic understanding of the underlying transcriptional regulation, it maintains the ability to identify biologically relevant hub genes and only requires expression data to construct. Geng et al. 2021 constructed an EXPLICIT (Expression Prediction via Log-linear Combination of Transcription Factors) model based on 24,500 publicly available Arabidopsis RNA-seq samples, which is able to predict expression of non-TF genes with high accuracy (real vs predict correlation > 0.98) using TF-only expression. EXPLICIT identified over $> 980,000$ TF-Target interactions whose coefficients were significantly different from zero ($\sim 2\%$ of all possible pairwise interactions).

EXPLICIT treats all RNA-seq samples as independent, however its training data will contain many samples that are part of a time series and are therefore not independent observations. time series expression is invaluable for GRN modelling, as it captures the chronology of dynamic gene expression changes, particularly in response to a stimulus (**time series-GRN-annrev**).

1.6.1 Methods for GRN inference

The task of GRN inference is to utilise high-throughput gene expression datasets and predict causal regulatory edges, i.e. identify the likely regulators of all nodes in the network. This will be done by identifying a group of targets that has a highly similar expression profile (either time series or single time-point) to a putative regulator. However, this is a computationally challenging task given the high dimensionality (often thousands of targets and hundreds of regulators) and regulator co-linearity (many TFs with very similar expression profiles) that exist within gene expression datasets.

Both Bayesian and random forest based algorithms are commonly used to infer gene regulatory networks (Perrin et al. 2003; Mercatelli et al. 2020). Bayesian inference is well suited for GRN construction, as prior information can easily be incorporated, and it deals well with messy data. Random forests (RFs) are an ensemble machine learning method in which n decision trees are trained on a random sub-section of the data using a random subsection of the available features (TF expression in our case), then results from each tree are averaged to obtain the result for the entire “forest” (Breiman 2001). This approach allows RFs to be more resistant to overfitting and enables them to ignore low importance features. When training RFs, “feature importance” is easily calculated, which is a measure of the relative error decrease when splitting a decision tree on a specific feature, across all trees in the forest. In an RF which is predicting expression of a target gene using TF expression, feature importances will represent the most important TFs in determining expression of the target. Additionally, the use of machine learning approaches, such as RFs, allows multiple gene expression datasets to be used in combination to infer a single GRN.

GENIE3 (GEne Network Inference with Ensemble of trees) is an RF-based GRN construction algorithm which utilises feature importance to rank regulatory interactions and performs well on steady-state data (Huynh-Thu et al. 2010). dynGENIE3 is a dynamic extension of GENIE3 which models the expression of a gene over time series as an ordinary differential equation (ODE) with a decay rate parameter (Huynh-Thu and Geurts 2018). dynGENIE3 significantly improved time series performance, however it was still outperformed on a synthetic dataset of 100 genes by Causal Structural Inference (CSI), a Bayesian method (Penfold and Wild 2011). CSI learns gaussian processes for all possible regulators of all genes, meaning it is very computationally intensive and has limitations on the number of genes included within a network. Windram et al. 2012 used CSI to generate a network for the response to *B. cinerea* infection Arabidopsis, identifying TGA3 as a defence

regulator. However, due to gene number restrictions of CSI, their network modelled 44 co-expression modules as opposed to individual genes.

OutPredict is another RF-based method which handles multiple time series and prior information (Cirrone et al. 2020). Two model types are supported by OutPredict; an ODE-log model similar to the ODE model in dynGENIE3 and a time-step model where expression of a TF at a given time-point is used to predict target expression at the subsequent time-point. OutPredict determines which model type is optimal for the inputted dataset by omitting the final time-point for each time series and evaluating performance on this unseen data point. Prior information can be easily incorporated into the OutPredict model, by increasing the probability that a specific TF will be included in the subset of features used by an individual decision tree (usually a random subset of features). Predicted TF-Target interaction confidence is reported using the RF feature importance. With these improvements, OutPredict has been demonstrated to outperform dynGENIE3 on several time series datasets (Cirrone et al. 2020), with the top 2% of predicted interactions on an *Arabidopsis* nitrogen-response time series being significantly enriched in experimentally validated interactions (Varala et al. 2018).

Algorithms such as OutPredict or dynGENIE3 could be utilised to infer GRNs from time series datasets detailing the transcriptomic response to pathogen infection or defence elicitors (such as Windram et al. 2012; Lewis et al. 2015; Hickman et al. 2017; Bjornson et al. 2021). Additional relevant steady-state mutant or overexpressor expression datasets could also be utilised to infer the GRN. Such a network could be used to identify “hub genes” that are key regulators of the defence response.

1.7 Sources of genetic disease resistance

Given that we have now developed a good understanding of how plants defend themselves against pathogens (Section 1.5), at least in the model plant *Arabidopsis*, we must now utilise this knowledge to develop disease-resistant cultivars. Classical breeding attempts rely on the identification of a desirable allele (e.g. R gene variant) that occurs naturally and introgressing it into elite cultivars via traditional crossing (Zhang et al. 2016a). This method allows alleles present in wild-relatives which may have been lost during domestication to be re-introduced. Methods such as Quantitative Trait Loci (QTL) mapping and Genome Wide Association Studies (GWAS) are used to identify markers associated with desirable traits to can be utilised for introgression in a process called marker assisted selection (MAS) (Collard and Mackill 2008).

These traditional breeding methods have been responsible for massive crop improvement, however, they rely on the presence of an allele in a population that is able to cross with a crop species and generate viable offspring. Many generations of backcrossing are often then required to remove undesirable alleles that are in linkage disequilibrium (LD) with an allele of interest. Several methods are available to introduce novel genetic variation which have varying levels of precision and public acceptability such as induced mutagenesis (Stein 1922), genetic modification (GM) (Herrera-Estrella et al. 1983) and genome editing (GE) (Jinek et al. 2012). The latter two methods allow for a reverse-genetics approach, utilising knowledge of gene function to target specific genetic changes such as the mutation or overexpression of a GRN hub. Whereas induced mutagenesis relies on chance events and subsequent screening for a desired phenotype, known as forward-genetics.

1.7.1 Detection of natural resistance: QTL and GWAS

QTL mapping and GWAS are two commonly used methods to identify genetic loci linked to a trait in plant breeding. Typically, QTL mapping is performed on a bi-parental mapping population of recombinant inbred lines (RILs). Two parent individuals which differ phenotypically for a trait of interest are selected as population founders and are crossed, the resulting F_1 and F_2 progeny will then be inter-crossed, generating F_3 progeny which contain different combinations of the parental alleles. F_3 progeny are then selfed by single-seed descent to generate a homozygous RIL population (around F_6 to F_8). Markers of genetic variation between the two parents such as single nucleotide polymorphisms (SNPs) or restriction fragment length polymorphisms (RFLPs) should segregate through the RIL population (Jiang 2013). Interval mapping can then be used to identify QTL, loci that are associated with quantitative phenotypic variation in the trait (Haley and Knott 1992). QTL mapping approaches and the *R/qtl* R package are discussed in detail by Broman and Sen 2009.

Although QTL mapping has been instrumental in identify major disease resistance loci against many pathogens including *B. cinerea* and *S. sclerotiorum* (Young 1996; Denby et al. 2004; Mamo et al. 2019), the

methodology has limitations. Generation of the RIL population is time-consuming and expensive, requiring up to eight generations of crossing and > 200 individuals. It may be difficult to detect minor effect QTLs due to insufficient statistical power, especially when number of genetic markers or RILs are low (Hu and Xu 2008). Also the precision of QTL detection is limited by recombination frequency, as recombination is required to break LD between two loci. As RILs have typically only gone through a few generations of crosses, QTLs exist in large haplotype blocks linked by LD making it challenging to identify the causative mutations underlying the phenotype.

GWAS is typically performed using a population of more distantly related individuals that are separated by many more generations of recombination, hence haplotype LD blocks are much smaller. As a result GWAS has much greater precision, with markers being tightly linked to the underlying causative mutation. However, the detection threshold of loci within GWAS is much lower than QTL mapping. Rare alleles (such as an R-gene present in a specific wild crop relative) would be present in approximately 50% of RILs in a bi-parental population but may be present in only 1 individual of a GWAS panel, which diminishes the statistical power to determine any phenotypic effects of the allele. Furthermore, some GWAS loci may be missed due to not having a marker present in the same haplotype block, hence a very high marker density is required for GWAS studies. The use of GWAS in plant breeding has been reviewed extensively (Korte and Farlow 2013; Tibbs Cortes et al. 2021; Demirjian et al. 2022).

1.7.2 Generating novel sources of genetic disease resistance

Despite the usefulness of marker-assisted selection of alleles identified through QTL mapping and GWAS, these approaches still have their limitations. Both require further generations of backcrossing into an elite cultivar which is very time-consuming. Also there is a finite amount of genetic diversity that is possible to introduce due to the reliance on mutations arising naturally in a closely-related species that's able to form viable offspring with the crop species of interest. Breeders have used mutagenesis approaches to produce random novel genetic variation since the 1920s, but GM and GE technologies enable a targeted approach to produce novel genetic variation.

1.7.2.1 Induced mutagenesis

Induced mutagenesis either by physical or chemical methods (such as gamma-irradiation or EMS respectively) has been heavily utilised in plant breeding, with over 2250 mutagenised cultivars having been released (Ahloowalia et al. 2004). These methods can induce 1000s of random mutations which have unknown consequences, plants can then be selected for a phenotype of interest. However, several generations of either backcrossing or introgression will be required to remove deleterious mutations that have arisen from the

mutagenesis.

“Diamant”, a gamma-irradiated barley cultivar released in 1965 had 12% yield increase and 15 cm reduced height compared to its parent line. Over 150 elite barley cultivars have since been released that are descendants of “Diamant” (Bouma and Ohnoutka 1991). “CM-72”, a radiation mutagenised chickpea cultivar which displayed increased resistance to *Ascochyta* blight was released in the early 1980s in Pakistan (Ahsanul, Sadiq, et al. 1983; Islam et al. 2017).

1.7.2.2 Genetic Modification (GM)

The ability to generate GM plants using *Agrobacterium tumefaciens* was revolutionary for our ability to study gene function and offered great potential for crop improvement. In the late 1970s it was demonstrated that *Agrobacterium* could stably incorporate a section of its Ti-plasmid DNA into a plant’s chromosome, with the incorporated DNA referred to as transfer DNA (T-DNA) (Chilton et al. 1977). The Ti-plasmid could then be engineered to facilitate the expression of “foreign genes” in transgenic plants (Herrera-Estrella et al. 1983). The Cauliflower Mosaic Virus (CaMV) 35S promotor (p35S) was then widely adopted for its ability to drive constitutive high-level expression in many plant tissues (Odell et al. 1985). *Bar*, a gene from *Streptomyces hygroscopicus* which is able to detoxify the BASTA herbicide has been incorporated in many engineered Ti-plasmids as a convenient selectable marker for transgenic plants (Thompson et al. 1987). Clough and Bent 1998 developed an Arabidopsis floral-dip transformation procedure, making it very simple to generate transgenic plants in the model species. Arabidopsis T-DNA mutant collections were released shortly afterwards, requiring the generation of > 200,000 transgenic lines which would have likely not been possible without floral-dip (Alonso et al. 2003a; McElver et al. 2001). The vast majority of all functional characterisation that was reviewed in Section 1.5 was carried out on T-DNA lines, either T-DNA mutants or p35S overexpression lines.

Countries which have allowed the cultivation of GM crops have reaped benefits of introducing novel traits that could never have been achieved through traditional breeding approaches. Using non-GM approaches, alleles which can be introduced into a crop species are limited to those present within a population that can be crossed with a crop species-of-interest (including naturally occurring alleles or radiation-induced alleles). However GM technology allows gene transfer between more distantly related species, such as the transgenic expression of oxalate oxidase enzymes (naturally occurring within grasses) within tomato and brassica to detoxify a *S. sclerotiorum* metabolite, oxalic acid (previously discussed in Section 1.4.2.1). Genes can also be introduced from non-plant species such as gram-positive bacterium *Bacillus thuringiensis* (Bt) which contains several genes encoding insecticidal toxins. Transgenic expression of Bt toxins in plants reduces insect damage and reduces the need for chemical insecticide sprays (Vaeck et al. 1987). Multi-site field trials in the US showed

that Bt-maize not sprayed with insecticide had less insect damage than non-GM varieties that had up to 8 insecticide sprays (Shelton et al. 2013). Over 100 million hectares of Bt crops were grown from 1996-2015 (Brookes and Barfoot 2017). While not every genetically modified (GM) crop innovation achieves significant success, “Golden rice” serves as a notable example. Created by incorporating two genes into the β -carotene (Vitamin A) synthesis pathway in rice, through the use of p35S and an endosperm-specific promoter (Ye et al. 2000b). Golden rice was intended to alleviate blindness caused by vitamin A deficiency. However, due to opposition to GM crops, it wasn’t approved until 2021 and has only been cultivated in limited pilot studies (Wu et al. 2021a; De Steur et al. 2022).

Many GM innovations have also been developed to enhance disease-resistance in crop species, however due to GM opposition very few of these have made it to the field. Rpi-Vnt1 is a wild potato (*Solanum venturii*) NLR that provides full resistance to potato blight (*Phytophthora infestans*) that has been approved for use in GM commercial potato varieties (Foster et al. 2009). NLRs (or other disease resistance alleles) from wild relatives can be introduced via *Agrobacterium* transformation instead of crossing if the target gene is known, as potentially deleterious alleles in LD with the trait of interest will not be introduced, therefore backcrossing generations are not required to remove them. Other disease resistance alleles can only be introduced via *Agrobacterium* transformation such as, alleles present in more distant species that cannot be crossed with the species of interest (Walz et al. 2008; Horvath et al. 2012), or modified NLRs that have been engineered to identify novel effectors (Kourelis et al. 2023).

1.7.2.3 Genome editing (GE)

Genome editing refers to highly-precise molecular tools which can be used to generate double-stranded breaks (DSBs) at specific genomic loci. Induced DSBs are often repaired via non-homologous end joining (NHEJ), an error-prone process which often introduces frame-shift mutation. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system is highly popular for genome editing, as the site of DSB cleavage can easily be reprogrammed using a single guide RNA (sgRNA) (Jinek et al. 2012). The potential applications of CRISPR to plant breeding has been reviewed by Scheben et al. 2017; Zaidi et al. 2020. TALENs (transcription activator-like effector nucleases) can also be used for GE, but use protein DNA binding domain, which are more challenging to re-program.

GE crops are generated using CRISPR-Cas9 to introduce mutations that could occur naturally, but in a targeted manner, and may be considered non-GM and as a result their use may not be restricted by GM legislation. The introduction of an assembled Cas9-sgRNA ribonucleoprotein (RNP) complex into protoplasts allows the generation of GE plants without the introduction of any foreign DNA. Although this methodology requires extensive tissue culture and often has low editing rate (Metje-Sprink et al. 2019). Recently, Yang

et al. 2023 developed an innovative protocol, where a wild-type scion (shoot) can be grafted onto a transgenic rootstock expressing mobile Cas9 mRNA and mobile gRNA transcripts, that can be transported to the scion. Seeds that are produced by the scion may contain CRISPR edits, but will be transgene-free.

Alternatively, *Agrobacterium*-mediated transformation could be used to generate a GM-plant that expresses Cas9 and a sgRNA from a T-DNA loci. However, the site of the T-DNA insertion will likely not be in linkage with the site of the edit. Therefore it is possible to backcross to a wild-type and isolate individuals which contain an edited allele, but have lost the T-DNA insert (i.e. no longer express Cas9 or the T-DNA). Such individuals would be considered non-GM and as such may be regulated independently from GM crops. The UK does not allow GM crops, but has recently passed the Precision Breeding Act (2023) in England which permits the development and marketing of GE crops (Coe and Ares 2023; Caccamo 2023). It is hoped that with effective science communication they will be accepted by the consumer (Kato-Nitta et al. 2019).

Using this methodology, a targeted mutation can be introduced directly into an elite cultivar, it is not necessary to search natural populations for a trait-linked allele (e.g. a QTL). Several GE crop varieties are now being grown for commercial use, in 2019 Calyxt released the first GE crop, Calyno, a TALEN-edited soybean variety with high oleic and low saturated fat oil content (Calyxt 2019). The next year, a CRISPR-edited high-GABA tomato with human health benefits such as reduced hypertension was released by Sanatech seeds in Japan (Ezura 2022; Waltz 2022).

As of yet, no gene edited crops with improved disease resistance have been released, but CRISPR-knockouts of plant susceptibility genes in several crop species have been generated which show enhanced disease resistance. Downy Mildew Resistant 6 (DMR6), encodes a Salicylic Acid 5-Hydroxylase which promotes susceptibility to several biotrophic pathogens (Van Damme et al. 2008; Zhang et al. 2017d). As a result, CRISPR knockout of DMR6 orthologues in crop species such as, banana and tomato, have been utilised to generate varieties resistant to biotrophic pathogens (Thomazella et al. 2021; Tripathi et al. 2021). Tomato pectin lyase and *Brassica napus* WRKY70 CRISPR knockout mutants have also been generated that display increased quantitative resistance to *B. cinerea* and *S. sclerotiorum* respectively (Silva et al. 2021; Sun et al. 2018).

A CRISPR-Cas9 induced DSB targeted to the coding regions of a gene will likely be repaired incorrectly by NHEJ causing a frameshift mutation, however, in some cases we may wish to activate the expression of a gene without introducing “foreign DNA” such as 35S promotor. One reason for this may be that many crop species have large polyploid genomes with highly duplicated gene families which display functional redundancy meaning that mutating a single gene may not display a phenotype (Uauy et al. 2017). Lin et al. 2021

induced in-frame 3 or 6bp deletions within miR396 recognition sites on OsGRF (rice growth regulating factor) transcripts, causing a 5-fold increase in expression, and increased grain size. Both activating and repressive cis-regulatory elements (CREs) within promoters can be targeted for deletion by CRISPR to fine-tune expression in specific conditions (Tang and Zhang 2023), however this requires an in-depth knowledge of CRE function.

Lu et al. 2021 induced 911Kb inversion in rice by generating two CRISPR DSBs, swapping the promotor regions of protoporphyrinogen IX oxidase (PPO), a gene involved in herbicide resistance and CP12 (a functionally redundant, but highly expressed Calvin cycle gene). Non-GMO rice plants generated using this method displayed upregulated PPO expression and herbicide resistance. This demonstrates that is possible to induce gain-of-function mutations with CRISPR.

Additionally, it is possible to mutate the catalytic domain of Cas9 so it can no longer produced DSBs (nuclease-dead CAS9, dCAS), but is still able to be targeted to a specific loci using sgRNAs. Transcriptional activators can then be recruited to the dCAS via gRNA scaffolds or peptide binding sites resulting in upregulation of a specific target, termed CRISPR activation (CRISPRa) (Selma et al. 2019; Gilbert et al. 2014). Multiplexed CRISPRa targeting multiple genes can induce phenotypes such as early flowering (Pan et al. 2021). However, under current regulation the use of CRISPRa would likely be treated as GM, as the dCAS T-DNA insert is required for the transcriptional activation.

As we increase our understanding of the plant defence response within crop species, through diverse methods of gene discovery including the inference of gene regulatory networks, CRISPR offers a tool for directly translating knowledge into the generation of improved crop varieties. Disease resistance can be enhanced by either inducing mutations which knockout "susceptibility genes" such as DMR6 or BnWRKY70 or attempting to fine-tune gene expression via the mutation of miRNA binding sites or promotor CREs.

1.8 Project Aims

The overarching aims of this research is to identify candidate genes to target which can be utilised to generate *L. sativa* cultivars with enhanced resistance to necrotrophic fungal pathogens *B. cinerea* and *S. sclerotiorum*. Numerous high-throughput transcriptomic datasets of pathogen infection in lettuce are analysed in order to identify these candidates such as high-density time series transcriptomics in response to infection, transcriptomic profiling of a lettuce diversity panel and bi-parental mapping population parents after infection. Network inference will be performed combining multiple transcriptomic datasets to predict key regulators of the lettuce defence response. Candidate genes will be then be validated for their role in plant defence using stable transgenic *Arabidopsis* or transiently infiltrated *N. benthamiana* leaves.

- Define *L. sativa* genes whose expression is associated with varying levels of susceptibility to necrotrophic pathogens in lettuce (**Chapter 2**)
- Identify genetic loci associated with *B. cinerea* and *S. sclerotiorum* resistance in a biparental wild lettuce mapping population, and predict underlying causative genes using transcriptomic approaches (**Chapter 2**)
- Construct a gene regulatory network integrating multiple lettuce-necrotroph transcriptomic datasets and predict high-influence hub transcription factors, regulating the response to infection (**Chapter 3**)
- Validate the *in planta* defence functions of some predicted lettuce defence regulators (**Chapter 3 and 4**)

Chapter 2

Identification of genetic loci in lettuce mediating quantitative resistance to fungal pathogens

2.1 Introduction

Lactuca sativa L. (lettuce) is an economically valuable leafy vegetable with production worth more than \$200 million in the UK (Department for Environment Food and Rural Affairs 2022) and \$2.4 billion in the USA (USDA-NASS, 2019). Lettuce is susceptible to a wide range of plant pathogens including the fungal necrotrophs *Botrytis cinerea* Pers. and *Sclerotinia sclerotiorum* (Lib.) de Bary, the causal agents of grey mould and lettuce drop, respectively. *B. cinerea* was ranked second for fungal pathogens of scientific and economic importance (Dean et al. 2012) while up to 50% of lettuce yields may be lost due to *S. sclerotiorum* (Young et al. 2004). Chemical control is routinely used but there is an urgent need to identify sources of host genetic resistance given the costs of preventative pesticide sprays, the prevalence of fungicide-resistant isolates of both pathogens in the field (Zhou et al. 2014; Rupp et al. 2016; Hou et al. 2018) and the increasing withdrawal of approved fungicides through legislation.

Pathogens with a biotrophic lifestyle parasitize and extract nutrients from living plant tissue, whereas necrotrophic pathogens rapidly kill their host, extracting nutrients from the dead tissue. A plant's response to infection varies depending on the pathogen lifestyle (Mengiste 2012). Complete disease resistance against specific isolates of biotrophic pathogens is often seen, conferred by a single dominant host gene. Many of these genes encode nucleotide binding leucine rich-repeat (NLR) proteins, which directly or indirectly detect the presence of pathogen effectors (virulence factors delivered into host cells to aid infection) (Lo Presti et

al. 2015). Similarly, resistance to host-specific necrotrophic pathogens, such as *Cochliobolus carbonum*, the causal agent of northern leaf spot in maize, is controlled by single gene traits conferring toxin sensitivity on susceptible host plants (Panaccione et al. 1992). In contrast, resistance to broad-host range necrotrophic pathogens (of which *B. cinerea* and *S. sclerotiorum* are prime examples) is usually a quantitative trait, with a continuum of phenotypes rather than two distinct classes of resistant and susceptible (Corwin and Kliebenstein 2017). This quantitative resistance is controlled by multiple genes with small to moderate effects (Roux et al. 2014).

Molecular analyses, mostly in the model plant *Arabidopsis*, have identified numerous components of the plant response to infection by *B. cinerea* (AbuQamar et al. 2017) and *S. sclerotiorum* (Wang et al. 2019b) incorporating pathogen detection, signal transduction and activation of host defences. For many of these individual components, mutants (knock-outs or overexpressors) have been used to assess their impact on disease outcome. However, these single-gene transgenic studies are much more difficult in non-model plants and do not help us understand the genetic variation in natural or managed populations, nor the relative contribution different genes/loci make to overall plant resistance. Genetic loci contributing to quantitative disease resistance (QDR) against *B. cinerea* have been mapped in a number of different plant species including *Arabidopsis* (Denby et al. 2004; Rowe and Kliebenstein 2008; Coolen et al. 2019), tomato (Finkers et al. 2007; Szymański et al. 2020), *Brassica rapa* (Zhang et al. 2016b) and *Gerbera hybrida* (Fu et al. 2017). Multiple studies investigating QDR against *S. sclerotiorum* in *B. napus*, sunflower and soybean using quantitative trait loci (QTL) mapping and genome-wide association studies (GWAS) (reviewed in Wang et al. 2019b) have identified many loci, each with a minor effect on QDR. However, what is lacking is knowledge of the molecular mechanisms underlying these loci. Recombination frequency within mapping populations and linkage disequilibrium in association panels typically limit resolution of the loci. Co-localisation of genetic loci for different traits (such as those mediating the accumulation of specific metabolites with QTL controlling QDR against *B. cinerea*) can be informative in predicting causal genes or mechanisms. For example, camalexin accumulation QTL co-localised with QTL influencing lesion size after *B. cinerea* infection in *Arabidopsis* (Rowe and Kliebenstein 2008) and QTL in *B. rapa* controlling the accumulation of glucosinolates were co-localised with *B. cinerea* resistance QTL (Zhang et al. 2016b). Szymański et al. 2020 combined metabolic QTL and expression QTL with QTL mediating tomato fruit resistance to *B. cinerea* to predict specific flavonoids important for host resistance.

In lettuce, QTL mapping has been used extensively to characterise dominant resistance phenotypes against the oomycete pathogen *Bremia lactucae*, which causes downy mildew. More than 30 downy mildew resistance genes have been identified (Parra et al. 2016; Parra et al. 2021). However, mapping of genetic determinants of QDR in lettuce against *B. cinerea* or *S. sclerotiorum* is in its infancy. Recently, two QTL were reported for field resistance to lettuce drop in the Reine des Glaces x Eruption mapping population (Mamo et al. 2019).

However, lettuce drop can be caused by both *S. sclerotiorum* and *Sclerotinia minor* and in this case the fields were inoculated with *S. minor*, which has a different infection strategy (infection via mycelia in the soil) than *S. sclerotiorum* (infection via germinating ascospores). To our knowledge no lettuce QTL have been reported for QDR against *B. cinerea* or *S. sclerotiorum*.

Here, we demonstrate genetic variation in resistance to *B. cinerea* and *S. sclerotiorum* in a lettuce diversity set (Walley et al. 2017) including *L. sativa* cultivars and wild relatives and exploit a bi-parental mapping population to identify QTL mediating resistance to both pathogens. Transcriptome profiling of a selection of the diversity set lines identified genes with expression correlated with disease resistance and highlighted post-transcriptional gene regulation (in particular, gene silencing) and pathogen recognition as determinants of resistance. Moreover, we integrated the diversity set and mapping population parent line transcriptome data to predict causal genes underlying the QTL.

2.2 Methods

2.2.1 Lettuce lines and plant growth

The Diversity Fixed Foundation Set (DFFS) comprises 96 lettuce accessions selected from the lettuce collection at the UK Vegetable Genebank, Wellesbourne, UK and the international *Lactuca* collection at the Centre for Genetic Resources, Netherlands. The set includes 17 wild species accessions as well as a range of cultivated varieties (Walley et al. 2017). For detached leaf inoculation assays, two lettuce seeds for each line were sown into 90 mm x 90 mm x 100 mm plastic pots filled with well-packed Levington's M2 growing media (Harper Adams University) or into 56 mm x 56 mm x 50 mm plug plant cells filled with well-packed Levington's F2S growing media (Universities of Warwick and York). The seeds were covered with a thin layer of vermiculite and watered frequently to ensure the growing media remained damp. Following germination, seedlings were thinned to one per pot. Plants were grown in a glasshouse with supplemental lighting provided for 16 h and heating set to 18°C. Experiments were carried out at Harper Adams University (52°46'46.02"N, 2°25'37.68"W), University of Warwick (52°12'37.31"N, 1°36'0.42"W) and University of York (53°56'44.16"N, -1°03'28.44"W) between 2015 and 2017. (Table 2.1).

2.2.2 Detached Leaf Infection Assays

The *S. sclerotiorum* isolate L6 was used in all experiments (Taylor et al. 2018a). Sclerotia and apothecia were produced and ascospores captured onto filter paper as described by Clarkson et al. 2014. Spore suspensions were prepared by agitating a section of filter paper in 5 mL distilled water until the water appeared cloudy.

Inoculum	Year	Populations	Location
<i>Botrytis cinerea</i>	2015	Full diversity set and mapping population parents	Harper Adams
	2016	Armenian <i>L. serriola</i> × <i>L. sativa</i> PI251246 mapping population	York
	2018	Selected accessions from diversity set matched to transcriptome profiling	York
<i>Sclerotinia sclerotiorum</i>	2015	Full diversity set and mapping population parents	Warwick
	2016	Armenian <i>L. serriola</i> × <i>L. sativa</i> PI251246 mapping population	Harper Adams
	2018	Selected accessions from diversity set matched to transcriptome profiling	York

Table 2.1: Location of detached leaf inoculation experiments

The suspension was filtered through two layers of miracloth and diluted to 5×10^5 spores per mL following counts using a haemocytometer. *B. cinerea* inoculum (pepper isolate) (Denby et al. 2004) was prepared by inoculating sterile tinned apricot halves. The inoculated apricot halves were sealed in Petri dishes and left in the dark at 25°C for 14 days to facilitate sporulation. Spore suspensions of *B. cinerea* were prepared by washing off conidiospores in 3 mL distilled water and filtering through two layers of miracloth. The suspension was again diluted to 5×10^5 spores per mL.

The third leaf from four-week-old lettuce plants (BBCH Stage 13 or 14 depending on the accession) was removed and placed on 0.8% (w/v) agar in sealable propagator trays. Leaves that were damaged were discarded. Each leaf was inoculated with a 5µL drop of either *S. sclerotiorum* or *B. cinerea* spore suspension on either side of the mid-vein. The trays of leaves were covered to maintain humidity and placed in a controlled environment cabinet at 22 °C, 80% humidity and a cycle of 12 h light: 12 h dark. Overhead photographs were taken of each tray between 48 and 72 h post-inoculation (hpi) including a scale bar to enable measurement of lesion area using ImageJ2 v1.51 (Rueden et al. 2017). Overlapping lesions, lesions that had spread to the leaf edge or lesions that had failed to initiate were not measured. For assessment of the complete diversity set one leaf (two inoculation sites) of each lettuce line was included in a single experiment, and experiments were repeated for ten consecutive weeks. To compensate for differences between experimental replicates and missing lesions, a Restricted Maximum Likelihood (REML) analysis was used to identify sources of variation between square root lesion size. Means were predicted using the least-squares method.

For the assessment of lesion size coupled to gene expression, sixteen lines were used in experiments with both pathogens, with a further 10 lines inoculated with a single pathogen (five with *B. cinerea*; five with *S. sclerotiorum*). These lines were chosen to capture a range of susceptibility to each pathogen whilst maximising the overlap between accessions used for each pathogen. Only *L. sativa* lines were selected to help ensure a

similar level of read mapping to the reference genome across the lines. Detached leaf infection assays were carried out using three replicate leaves of each accession for each pathogen (apart from eight varieties with two replicates for *S. sclerotiorum* infection and four varieties with two replicates for *B. cinerea* infection) giving 114 leaves in total. Lesion size was measured at 42 hpi for *S. sclerotiorum* and 46 hpi for *B. cinerea* with leaves harvested at 43 hpi (*S. sclerotiorum*) and 48 hpi (*B. cinerea*) for transcriptome profiling.

For assessment of lesion size in the mapping population in each replicate 120 lines (i.e. approximately half the population) were grown to four weeks old before leaf 3 was detached and placed on agar prior to droplet inoculation. This was repeated in a randomised design to a total of eight experiments. Mean lesion size per line was estimated using a least squares method.

2.2.3 Polytunnel Assay

Whole-head lettuce disease assays for both *B. cinerea* and *S. sclerotiorum* were performed in duplicate at the University of Warwick and Harper Adams University. Eighteen plants per accession (per pathogen inoculation) were grown to 4 weeks old in the glasshouse (as above) before being transplanted to 24 cm diameter pots in Levington's F2S soil. Plants were placed in a blocked randomisation design within a sealed polytunnel. Spores were collected as above in 500 mL sterile distilled water and diluted to 1×10^5 spores/mL before being sprayed directly onto each plant using a hand-sprayer. Plants were sprayed with inoculum until saturation and inoculum run off, and irrigated via spray irrigation from 1.5 m tall irrigators every 2 h from 6 am until 2 am the next day, for 10 min at each interval. Plants were assessed twice weekly, with the following scale: 0—no symptoms, 1—visible lesions on lower leaves, 2—visible lesions on majority of leaves, 3—severe disease symptoms over entire plant, 4—total plant collapse. Plants were monitored to 6 weeks post-transplanting for disease symptoms. Area under the disease progression curve (AUDPC) was calculated using the trapezoid rule with the Agricolae R package (de Mendiburu 2021). ANOVA was used to determine significant differences in variation followed by a Tukey HSD test to determine significant differences between varieties ($p < 0.05$).

2.2.4 Quantitative Trait Loci (QTL) Analysis

The RIL population of 234 F6 lines generated from crossing the Armenian *L. serriola* 999 and *L. sativa* PI251246, including genotyping of the population and generation of a genetic map using 2677 markers, was previously described (Han et al. 2021). QTL analysis was performed in the R/qtl package (Broman et al. 2003). Recombination fraction was estimated using the `est.rf` function. The least-squares predicted mean of square-root lesion size (mm^2) was used as the phenotyping score for each RIL. `calc.genoprob` was used (with an error probability of 0.001 and a step-limit of 2 cM), which utilises hidden Markov models to estimate true underlying genotype between markers. A single QTL scan was performed, using `scanone` as a

preliminary measure using Haley-Knott regression (Haley and Knott 1992), for which the significance threshold was calculated by a permutation test of 1,000 imputations with an alpha value of 0.05. A search for epistatic interactions between loci was conducted using *scantwo* with significance calculated based on a permutation test (750 imputations). QTL above the permutation threshold for either algorithm were then fitted to the multi-QTL model selection pipeline using *makeqtl* and *fitqtl*. Percentage variance explained statistics were calculated by *fitqtl*. Once fitted, *addqtl* was used to search for additional QTL missed by the preliminary scan. Peak QTL positioning was further adjusted using *refineqtl*. Finally, a forward/backwards selection model was applied with *stepwiseqtl* to give the final QTL, with model penalties calculated at an alpha level of 0.05 (Manichaikul et al. 2009). Haley-Knott regression (Haley and Knott 1992) was used for the model selection stages.

Epistatic interactions between the QTL loci were passed to the *stepwiseqtl* algorithm, but none passed the significance threshold. To identify confidence regions surrounding each QTL, *lodint* was used, a size of 1.5 Logarithm of the odds score (LOD) around each QTL peak was selected and expanded to the next marker. Flanking markers of the 1.5 LOD confidence interval were mapped back to the *L. sativa* cv. Salinas v8 genome (Reyes-Chin-Wo et al. 2017) to identify genes located within the QTL. Another R package, *LinkageMapView* was used to visualise the genetic map (Ouellette et al. 2018).

2.2.5 Gene expression profiling

Leaves were infected with *S. sclerotiorum* (Taylor et al. 2018b) or *B. cinerea* as per the detached leaf assays outlined above, and samples were harvested using a size 6 cork borer centred on the lesion. All expression profiling experiments used *B. cinerea* pepper isolate (Windram et al. 2012). For *S. sclerotiorum* the L6 isolate was used for the diversity set and mapping population parent expression analysis, with the P7 isolate used for response to infection expression (mock vs. inoculated). Infected tissue was snap frozen in liquid nitrogen before RNA extraction using Trizol (Thermo Fisher Scientific) with a lithium chloride purification. Sequencing libraries from mRNA were prepared using the Illumina TruSeq RNA V2 kit and sequenced using a HiSeq 2500 generating 100 bp paired-end reads or HiSeq 4000 with 75 bp paired-end reads. Read quality was checked with FastQC (Andrews et al. 2010). Sequencing reads were aligned to a combined lettuce–pathogen transcriptome using Kallisto (Bray et al. 2016). The reference transcriptomes were obtained from Derbyshire et al. 2017; Van Kan et al. 2017; and Reyes-Chin-Wo et al. 2017. Counts were summarised at the gene level and differential expression analysis was performed using the Limma-Voom pipeline (Law et al. 2014) with a threshold of ≥ 1.2 log₂ fold change and an adjusted $p < 0.05$ (Benjamini and Hochberg 1995). Principal component analysis was performed on gene counts using the *prcomp* function in R and Euclidean distance between samples was used for hierarchical clustering.

2.2.6 Lesion size-gene expression correlation

Diversity set reads aligned to lettuce genes were normalised using the trimmed mean of M-values from edgeR (Robinson et al. 2010). Genes with low expression (under 50 counts) and transcripts with low variance (≤ 1.2 logFC between samples) were removed. Spearman correlations were calculated for the relationship between trimmed mean of M expression values and square-root lesion size for each gene (23,111 in *S. sclerotiorum* and 23,164 in *B. cinerea*). Correlation p-values were calculated using cor.test in R and corrected using the Benjamini–Hochberg method (Benjamini and Hochberg 1995).

2.2.7 Gene Ontology (GO)-enrichment analysis

GO enrichment analysis was performed for genes whose expression significantly correlated with increased pathogen resistance or susceptibility using the org.At.tair.db and clusterprofiler R packages (Wu et al. 2021b). Arabidopsis orthologs are taken from (Reyes-Chin-Wo et al. 2017).

2.3 Results

2.3.1 Lettuce accessions vary in lesion development after inoculation with *S. sclerotiorum* or *B. cinerea*

A detached leaf assay was previously developed for infection of Arabidopsis by *B. cinerea* and shown to be effective in quantitative assessment of lesion development and correlated with fungal growth within the leaf (Windram et al. 2012). We had also used a similar assay to evaluate susceptibility to *S. sclerotiorum* in multiple Brassica lines (Taylor et al. 2018b). We adapted these assays to measure lesion development in lettuce after inoculation with the two closely related necrotrophic fungal pathogens (*B. cinerea* and *S. sclerotiorum*) to assess susceptibility to disease. Using these quantitative assays, we measured lesion size in 97 lettuce accessions (96 lines comprising a Diversity Fixed Foundation set (Walley et al. 2017) plus the cultivar Lolla Rossa) after inoculation with *B. cinerea* or *S. sclerotiorum* spores. Crucially, although these assays use detached leaves, they use spores (ascospores of *S. sclerotiorum* and conidiospores of *B. cinerea*) to mimic the type of infection occurring naturally. This contrasts with the commonly used *S. sclerotiorum* inoculation method of mycelial plugs (Joshi et al. 2016; Barbacci et al. 2020; Chittem et al. 2020). With occasional lack of plant growth and/or lack of infection from the droplet, the average number of lesions (and leaves) per lettuce line inoculated with *B. cinerea* was 16 (eight leaves) and for *S. sclerotiorum* 17 (seven leaves). REML least-squares predicted mean lesion size was calculated for each lettuce accession to account for variation between replicate experiments.

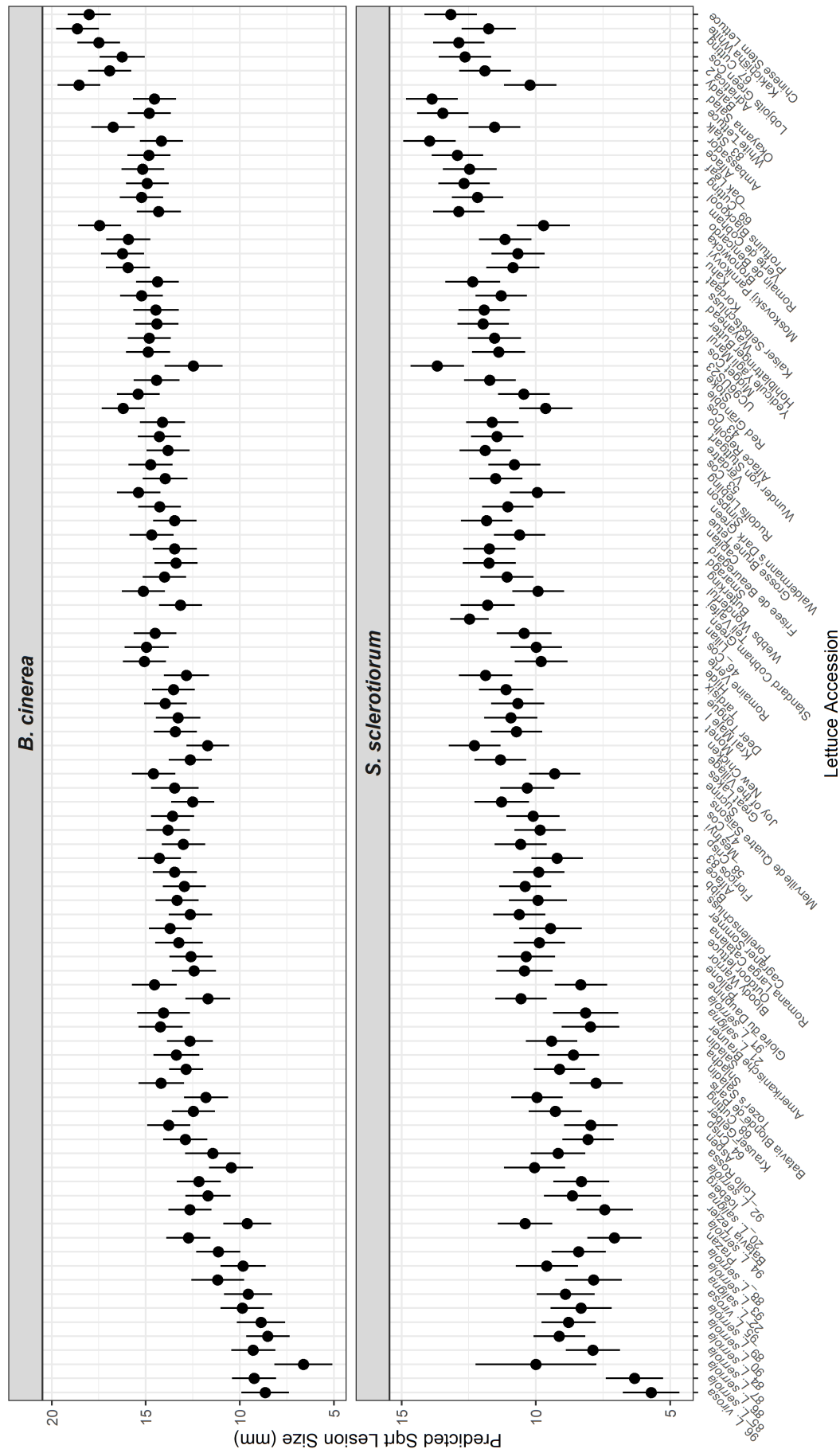


Figure 2.1: Variation in lesion size after inoculation with *Botrytis cinerea* or *Sclerotinia sclerotiorum* in a set of lettuce accessions. Least-squares REML predicted mean square root of lesion size 64 h after *Botrytis cinerea* (top) or *Sclerotinia sclerotiorum* (bottom) inoculation of detached lettuce leaves is shown in ascending order of mean lesion size across the two pathogens. Error bars are REML standard error. Lesions measured per accession per pathogen ranges from two (#86 *L. serriola*—*S. sclerotiorum*) to 213 (Tozer Salad—*B. cinerea*) with a median $n = 16$.

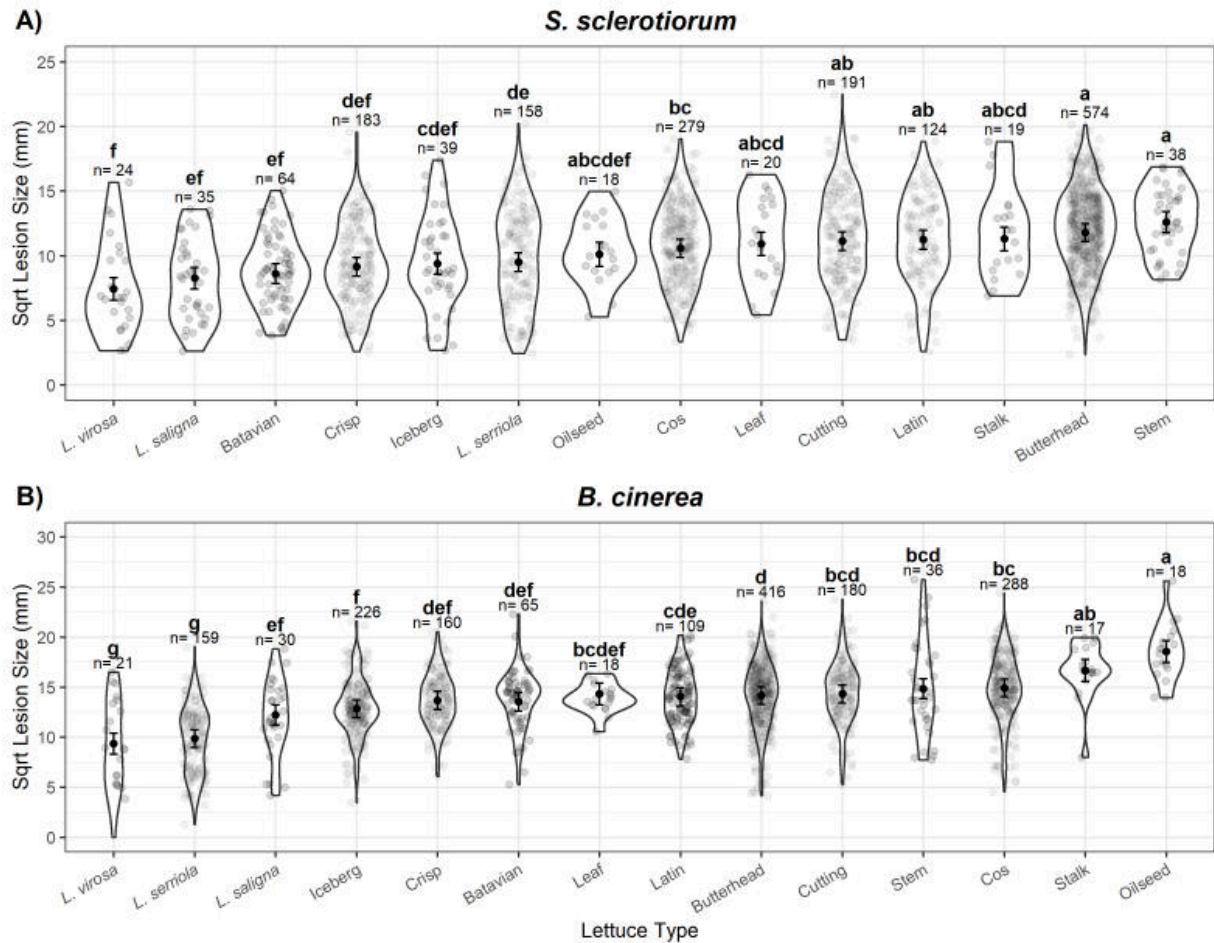


Figure 2.2: Variation in lesion size after inoculation with *B. cinerea* or *S. sclerotiorum* between lettuce types. Square root lesion size in response to *S. sclerotiorum* (top) or *B. cinerea* (bottom) on detached lettuce leaves of the Lettuce Diversity Fixed Foundation Set. Grey points show individual measured lesions, violins show the distribution. Black points show REML predicted (accounting for random variation between experimental replicates). Error bars indicate REML predicted standard error. Letters shown represent Tukey HSD significance groupings ($p < 0.05$). n = the number of lesions measured from each type in response to each pathogen.

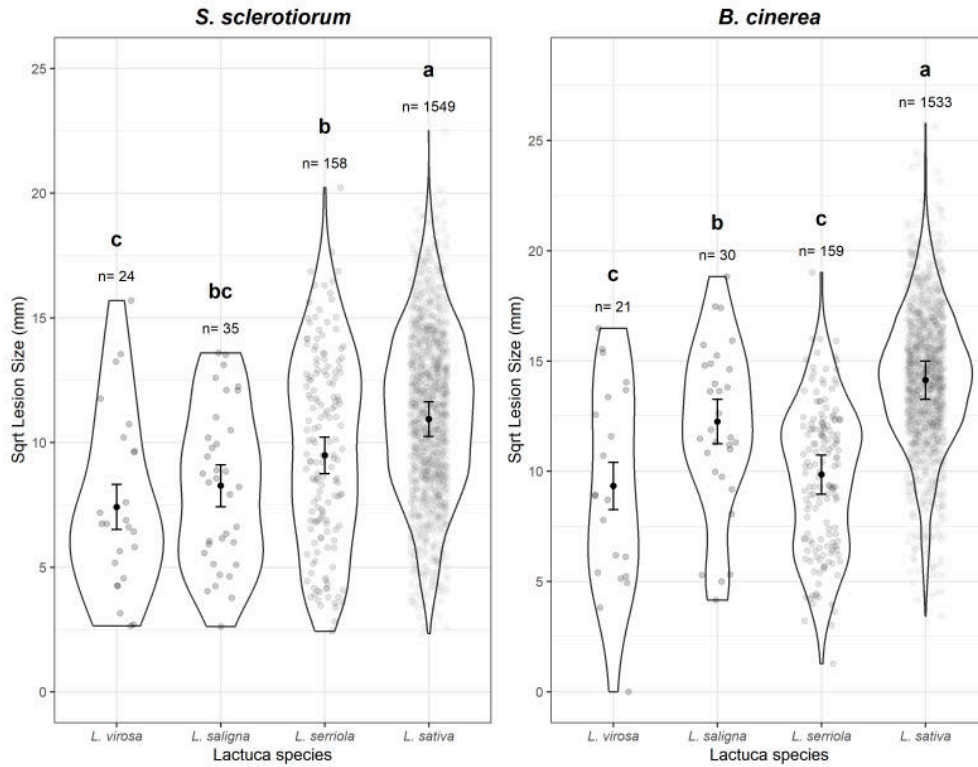


Figure 2.3: Variation in lesion size after inoculation with *Botrytis cinerea* or *Sclerotinia sclerotiorum* in different lettuce species. Square root lesion size 64 h after *Sclerotinia sclerotiorum* (left) or *Botrytis cinerea* (right) inoculation of detached lettuce leaves is shown. Grey circles represent individual measured lesions, with areas within the lines showing the distribution of data points. Black circles are the REML predicted mean lesion size per species (correcting for random variation between experimental replicates) with error bars showing REML predicted standard error. Letters represent Tukey post-hoc significance groupings ($p < 0.05$) performed on the REML model. n is the number of lesions measured from each species.

The lettuce diversity set clearly contains genetic variation for susceptibility to *B. cinerea* and *S. sclerotiorum*, as judged by lesion development on detached leaves (Figure 2.1). Lettuce lines exhibited significant variation in lesion size ($p < 0.001$ for both pathogens), which likely reflects the ability of the pathogens to grow, and hence the effectiveness of the plant defence response to combat infection. Lesion size varied by lettuce type; for example, Iceberg lettuces were significantly more resistant to *B. cinerea* and *S. sclerotiorum* than Butterhead and Cutting types (Figure 2.2). Different lettuce types have large architectural differences (Walley et al. 2017) which alters the ability of pathogens to infect the plant in the field; for example, a more open structure reduces moist environments for spore collection and germination. A benefit of the detached leaf assay is that it identifies architecture-independent sources of resistance that could be deployed across multiple lettuce types. Wild relatives of lettuce (*L. virosa*, *L. saligna*, and *L. serriola*) were significantly more resistant to both *S. sclerotiorum* and *B. cinerea* than the cultivated *L. sativa* (Tukey HSD $p < 0.05$) (Figure 2.3), suggesting that alleles conferring quantitative resistance against these fungal pathogens may have been lost during the domestication of lettuce.

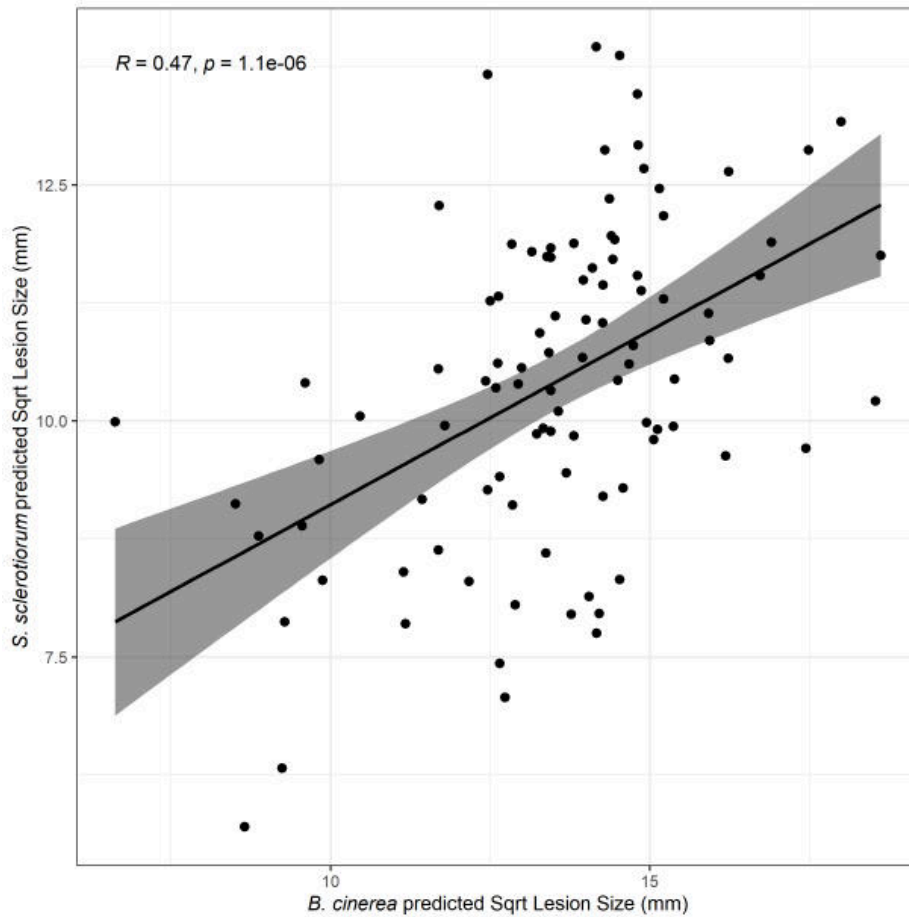


Figure 2.4: Correlation between lesion size 64 hours post inoculation with *S. sclerotiorum* and *B. cinerea*. Least-squares predicted mean square root lesion size of *B. cinerea* (x-axis) vs. *S. sclerotiorum* (y-axis) on detached lettuce leaves, where n ranges from two to 20 for each accession/pathogen combination. Linear regression line is shown in black, with 95% confidence intervals shaded in grey.

B. cinerea and *S. sclerotiorum* are closely related necrotrophic fungal pathogens sharing many genes and virulence strategies Amselem et al. 2011, although *B. cinerea* contains a higher number and diversity of genes involved in secondary metabolism (e.g. the production of plant toxins). Consistent with their similarity, we found a correlation ($r = 0.47$, $p = 1.1E-6$) across the diversity set between lesion size after inoculation with each of the two pathogens (Figure 2.4), raising the prospect of identifying lettuce alleles conferring quantitative resistance against both pathogens.

We performed a whole-head lettuce inoculation experiment to determine whether such an assay could be used in a quantitative manner and if the detached leaf assay data were relevant to whole plants. Four-week-old plants of seven lettuce accessions were sprayed with spore suspensions of *S. sclerotiorum* or *B. cinerea* and humidity was kept high through regular mist irrigation. A disease score was captured for each plant from 14 to 49 days post inoculation, and the AUDPC was calculated to quantify disease symptoms over time. Plots of disease score progression for each accession are shown in Figure 2.5.

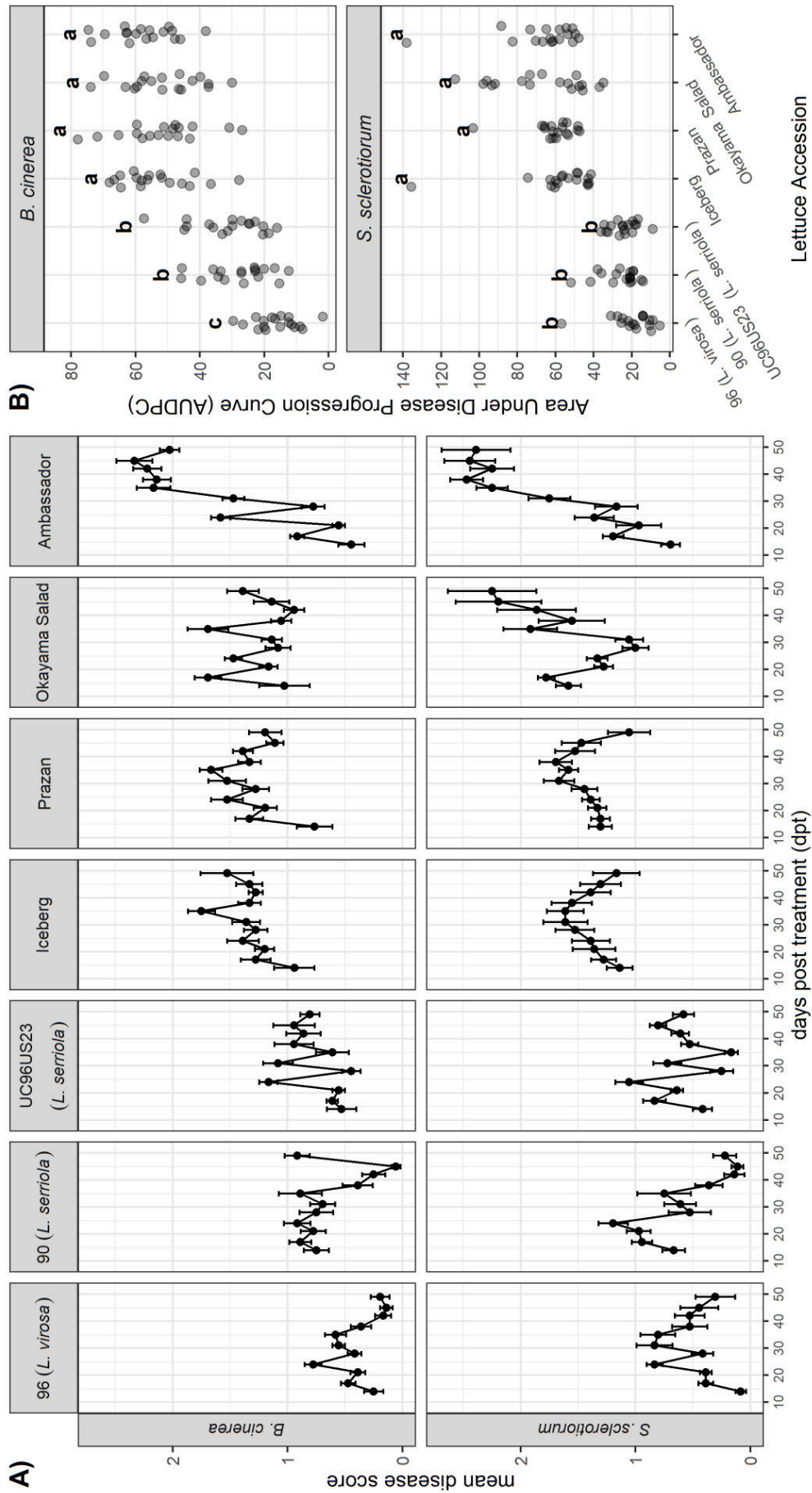


Figure 2.5: Whole Plant Disease incidence scoring on seven lettuce accessions. All accessions are *L. sativa* unless otherwise indicated. **(A)** Mean disease symptom scoring out of 4 for each lettuce accession from 14 to 49 days post treatment in response to *B. cinerea* and *S. sclerotiorum*. Error bars are standard error, $n=18$. **(B)** Area under the disease progression curve (AUDPC) calculated to summarise disease progression over time for each individual plant, $n=18$. Letters represent Tukey HSD statistical significance groupings ($p < 0.05$).

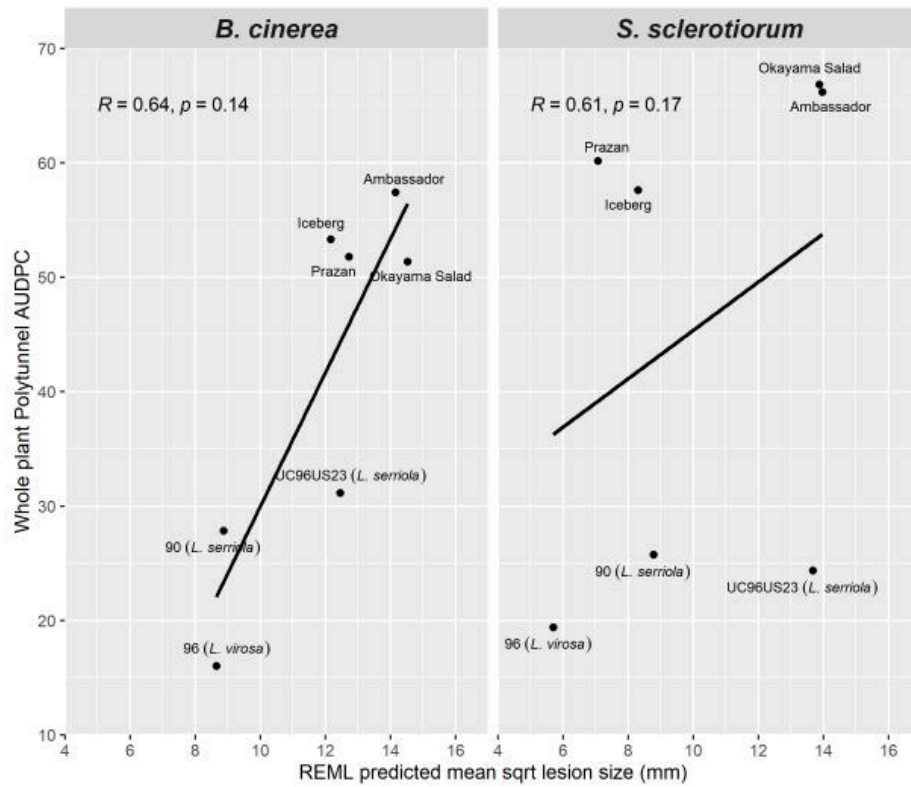


Figure 2.6: Correlation of REML predicted detached leaf assay square root lesion size (mm) with AUDPC in whole plant inoculations of *B. cinerea* (left) and *S. sclerotiorum* (right) for seven lettuce accessions. Pearson's correlation coefficient (R) values and p -values are shown. All accessions are *L. sativa* unless otherwise indicated.

The relationship between AUDPC from the whole-head assay and lesion size in the detached leaf assay for the seven selected lettuce accessions is shown in Figure 2.6. There is a positive trend between the two measurements: accessions with a higher whole plant disease score (AUDPC) also had a higher detached leaf assay lesion size in response to *B. cinerea* ($r = 0.64, p = 0.14$) and *S. sclerotiorum* ($r = 0.61, p = 0.17$). However, neither correlation showed statistical significance. Notably, the accession with the smallest lesion size in the detached leaf assay (*L. virosa*, line 96) and the accessions with the largest lesion size (Okayama Salad, Ambassador) showed a clear difference in the whole plant assay suggesting that the detached leaf assay phenotypes do have relevance to whole head disease progression. However, the whole plant assay appears unable to distinguish varieties with intermediate levels of resistance. Hence, we proceeded with the detached leaf assay as it provides quantitative measurements over a wider range of values and is carried out under more controlled conditions.

2.3.2 Diversity set transcriptome profiling identifies gene expression correlated to disease progression

We selected accessions from the lettuce diversity set that exhibited a wide range of lesion size after infection with both pathogens whilst focusing on *L. sativa* varieties to ease transcriptome analysis. The Armenian *L. serriola* line was also included as it is a parent of a key mapping population. Despite a lower number of replicates than in the full diversity set experiment, capturing the lesion size from the exact leaves used for transcriptome analysis enables us to directly link gene expression and lesion size in the same leaf.

A disc of tissue around each developing lesion was sampled for RNAseq transcriptome profiling with three biological replicates for each accession/pathogen combination. Reads were mapped to a combined *S. sclerotiorum*- or *B. cinerea*-lettuce (*L. sativa* var. Salinas) transcriptome (Derbyshire et al. 2017; Van Kan et al. 2017; Reyes-Chin-Wo et al. 2017). Approximately 25,000 lettuce genes were present in at least one sample. As lesion size reflects pathogen growth in planta, the percentage of reads mapping to the fungal transcriptome in each sample significantly correlated with measured lesion size (*S. sclerotiorum*: $r = 0.69$, $p = 6.3E-9$; *B. cinerea*: $r = 0.4$, $p = 0.0018$) (Figure 2.7). Correlation is not absolute most likely due to the fact that lesion size is a two-dimensional measurement, whereas pathogen growth will take place in three dimensions; accordingly, correlation is weaker in *B. cinerea*-inoculated leaves with larger lesion sizes. Hierarchical clustering of the normalised expression data demonstrates high similarity between the biological replicates of each accession, with *B. cinerea* and *S. sclerotiorum* inoculated samples of an accession often also clustering together (Figure 2.8). There is no clear grouping of the expression data by lettuce type.

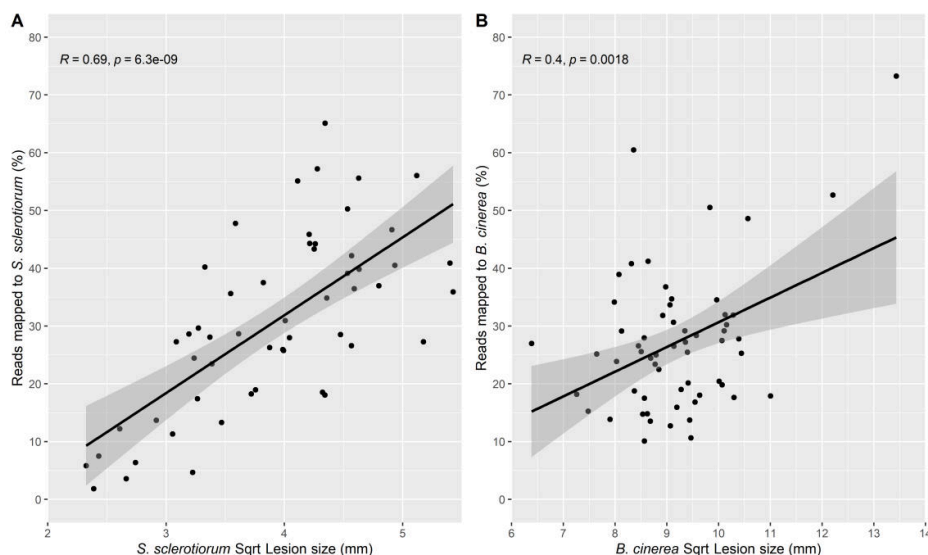


Figure 2.7: Pearson's correlation of RNAseq reads in each sample that map to fungal transcripts versus lesion size in (A) *S. sclerotiorum* and (B) *B. cinerea* inoculated samples.

Spearman correlations were calculated between lesion size and lettuce gene expression after both *B. cinerea* and *S. sclerotiorum* infection for each gene detected in our transcriptome profiling. After false discovery rate correction, 1605 and 9936 lettuce genes exhibited expression levels significantly correlated with *B. cinerea* and *S. sclerotiorum* lesion size, respectively ($p < 0.05$). The difference in the number of genes showing correlation between expression and lesion size for each pathogen infection is likely due to the timing of sampling. Disease symptoms appear much faster following *B. cinerea* inoculation compared to *S. sclerotiorum*; hence, these samples are at a later stage of infection and the profiling has perhaps missed the critical dynamic period of transcriptome reprogramming.

These genes with expression correlated to lesion size are likely to include many genes where the difference in expression between accessions is simply due to the dynamics of infection progression, rather than differences in gene expression being a potential driver of varying lesion size. For example, a gene upregulated during pathogen infection would likely have higher expression in a more susceptible accession simply because the infection has progressed faster and more tissue is responding to the pathogen. In contrast, genes downregulated during pathogen infection could have higher expression in a more resistant accession (compared to a susceptible accession) simply because infection has progressed more slowly and less plant tissue has responded. We therefore removed these categories of genes (upregulated genes correlated with susceptibility and downregulated genes correlated with resistance).

To determine whether genes were up or downregulated during pathogen infection of lettuce, we used an RNAseq dataset comparing lettuce gene expression in leaves after *B. cinerea* or *S. sclerotiorum* inoculation to mock inoculation. Three biological replicates were harvested from leaves of the lettuce variety Saladin at 24 hpi with *B. cinerea* (and mock) and 42 hpi with *S. sclerotiorum* (and mock). A total of 8130 (4165 up/3965 down) and 5466 (3329 up/2137 down) genes were significantly differentially expressed in response to *B. cinerea* and *S. sclerotiorum*, respectively (absolute log₂ fold change ≥ 1.2 and adjusted $p < 0.05$). Integrating this data with the diversity set RNAseq data and removing upregulated genes correlated with susceptibility and downregulated genes correlated with resistance resulted in 305 and 3724 lettuce genes correlated with resistance to *B. cinerea* and *S. sclerotiorum*, respectively, as well as 326 and 1580 correlated with susceptibility across the different accessions. Of these, 174 genes correlated with resistance to both pathogens and 211 with susceptibility to both pathogens.

Figure 2.9a illustrates the expression profiles for the 50 lettuce genes with the highest correlation with resistance against *S. sclerotiorum*, and Figure 2.9b shows the 50 genes with the highest correlation with susceptibility to *S. sclerotiorum*. The filtered lists of genes with expression significantly correlated with resistance or susceptibility contain several genes whose Arabidopsis orthologs have a known role in defence against *B. cinerea* and *S. sclerotiorum*, providing an initial validation of the data and indicating the ability of

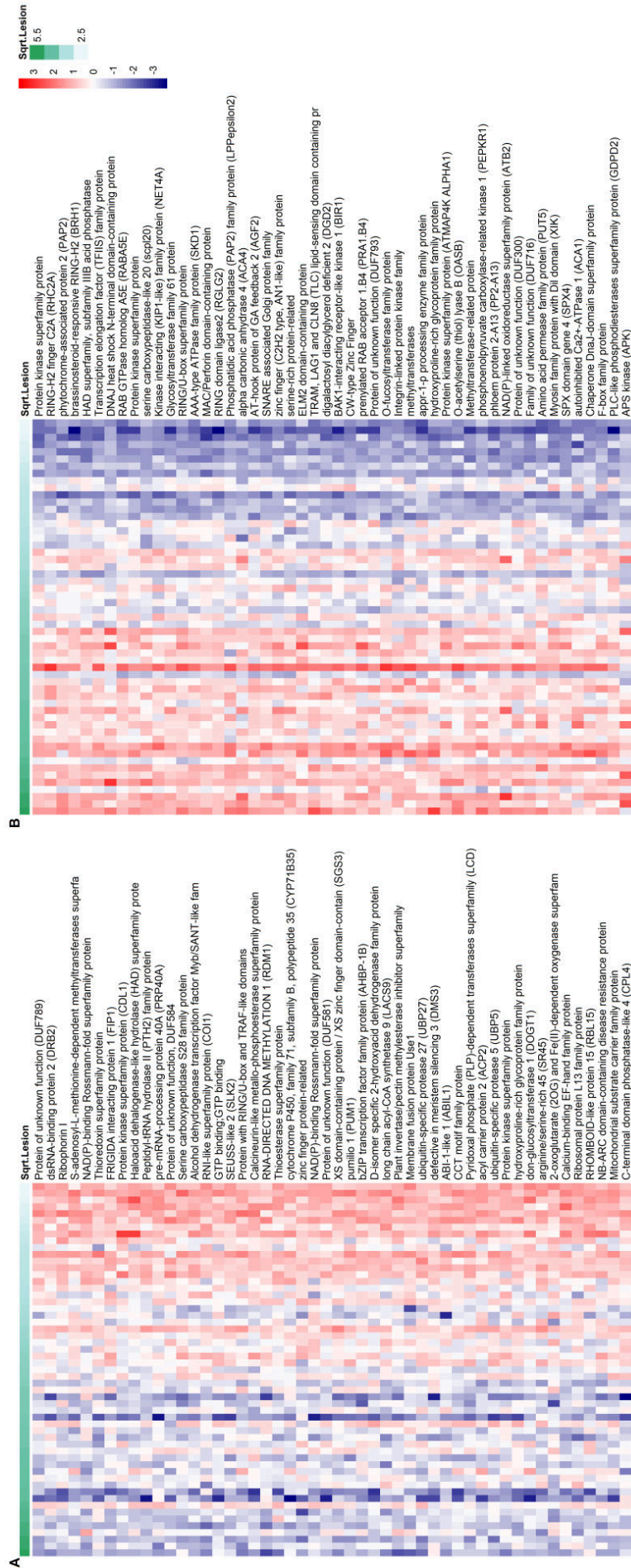


Figure 2.9: Heatmap of the top 50 lettuce genes identified with expression that correlates with *S. sclerotiorum* **A**) resistance and **B**) susceptibility across the diversity set accessions. The samples in the heatmap are ordered, with most susceptible (largest *S. sclerotiorum* lesion size) on the left and most resistant (smallest *S. sclerotiorum* lesion size) on the right. Log2 expression is indicated by the red/blue scale

our approach to identify genes acting both positively and negatively on host immunity against these pathogens. For example, expression of two lettuce orthologs (Lsat_1_v5_gn_2_122000 and Lsat_1_v5_gn_9_61461) of coronatine insensitive 1 (COI1), the jasmonic acid receptor that is required for defence against *B. cinerea* (Feys et al. 1994; Rowe et al. 2010), are significantly inversely correlated with *S. sclerotiorum* lesion size ($r_s = -0.67$, $p = 3.8E-6$; $r_s = -0.61$, $p = 2.9E-5$). Two orthologs of TOPLESS (TPL) (Lsat_1_v5_gn_1_31280 and Lsat_1_v5_gn_5_63700) are significantly correlated with *S. sclerotiorum* resistance ($r_s = -0.59$, $p = 8.1E-5$; $r_s = -0.57$, $p = 1.01E-4$, respectively). Arabidopsis triple mutants of TPL and the highly similar TOPLESS-related proteins (TPRs) 1 and 4, *tpl/tpr1/tpr4*, show increased susceptibility to *B. cinerea* (Harvey et al. 2020). In addition, an ortholog of MAP kinase substrate 1 (MKS1), Lsat_1_v5_gn_1_8801, has expression correlated with *S. sclerotiorum* susceptibility ($r_s = 0.65$, $p = 7.3E-6$) and in Arabidopsis, MSK1 is known to directly bind the key defence regulator WRKY33 with overexpression of MKS1 resulting in *B. cinerea* susceptibility (Petersen et al. 2010). These examples demonstrate our ability to identify defence genes from this dataset and increase our confidence in identifying novel lettuce defence components.

2.3.3 GO-term analysis reveals enrichment of lettuce RNA binding proteins amongst genes with expression correlated with *S. sclerotiorum* resistance

We examined the biological processes represented by genes correlated with the defence response across the diverse lettuce accessions using GO-term enrichment. Lettuce genes have poor GO annotation; therefore, we performed GO-term enrichment analysis using the Arabidopsis orthologs of the lettuce genes correlated with resistance and susceptibility to *S. sclerotiorum* (2985 and 1254 genes respectively) (Figure 2.10). Strikingly, amongst the GO-terms enriched in genes correlated with *S. sclerotiorum* resistance were multiple terms associated with post-transcriptional RNA processing and regulation including gene silencing (GO:0016458), RNA interference (GO:0016246), dsRNA processing (GO:0031050), RNA modification (GO:0009451), exosome RNase complex (GO:0000178), and RNA splicing (GO:0008380). Genes correlated with increased susceptibility to *S. sclerotiorum* were enriched for vesicle transport and cell growth related GO-terms.

As shown above, *S. sclerotiorum* resistance correlated genes show a remarkable enrichment for GO-terms involved in RNA production, processing and RNA-mediated regulation. Post-transcriptional gene regulation via small RNAs is known to be a critical component of the host immune response and to contribute to reciprocal host–pathogen manipulation during infection by different types of plant pathogens (Huang et al. 2019). Seventy-two lettuce genes significantly correlated with resistance to *S. sclerotiorum* ($p < 0.05$) were identified as orthologs of Arabidopsis genes involved in gene silencing (GO:0016458). These 72 genes include several core components of the RNAi-mediated gene silencing pathway (Borges and Martienssen 2015) such as Dicer-like (DCL)2, DCL3, DCL4, Argonaute 1 (AGO1) and RNA-dependent RNA polymerase 2 (RDR2). In Arabidopsis, the gene silencing mutants *dcl4-2*, *ago9-1*, *rdr1-1*, *rdr6-11* and *rdr6-15* have been shown to increase susceptibility to *S. sclerotiorum* (Cao et al. 2016a) while *dcl1* increased susceptibility to *B. cinerea* (Weiberg et al. 2013). Our data suggest a similar role for gene silencing in the defence response of lettuce against these broad host range pathogens.

Pentatricopeptide Repeat (PPRs) proteins are a family of RNA-binding proteins expanded in plants and involved in base editing and processing of organellar RNAs (Barkan and Small 2014), and their transcripts are a major source of secondary small interfering RNAs (siRNAs) in plants (Howell et al. 2007). The lettuce genome contains 513 putative PPRs (Reyes-Chin-Wo et al. 2017), 184 of which show correlation of expression with lesion size in our data. Expression of 178 lettuce PPRs is correlated with increased resistance to *S. sclerotiorum* (and six with increased susceptibility) suggesting a potential key role for PPRs in the lettuce immune response. Figure 2.11 shows the expression profile of the top 20 resistance correlated PPRs.

2.3.4 Multiple pathogen recognition genes have expression correlated with *S. sclerotiorum* lesion size across diverse lettuce accessions

Pathogen recognition by the host plant is mediated by both cell-surface (receptor-like kinases, RLKs and receptor-like proteins, RLPs) and intracellular (nucleotide binding site leucine-rich repeat proteins, NLRs) proteins. Our analysis suggests both groups of receptors impact lettuce resistance to *S. sclerotiorum*. Twenty-six RLKs and 14 RLPs had expression correlated with *S. sclerotiorum* lesion size; 12 RLKs and three RLPs with resistance and 14 RLKs and 11 RLPs with susceptibility (Figure 2.12). Notably RLKs whose Arabidopsis orthologs have well-established and interacting roles in necrotrophic pathogen recognition were identified in this analysis. For example, mutants of BAK1 (BRI1-associated receptor kinase 1) show increased susceptibility to *B. cinerea* (Kemmerling et al. 2007) and expression of LsBAK1 (Lsat_1_v5_gn_9_117621) correlates with increased *S. sclerotiorum* resistance ($r_s = -0.38$, $p = 0.01$). BAK1 directly interacts with the flagellin-sensitive receptor FLS2 (following flg22 perception) initiating downstream defence responses (Sun et al. 2013) and a lettuce ortholog of FLS2, Lsat_1_v5_gn_7_32940, also had expression correlated with *S. sclerotiorum* resistance ($r_s = -0.47$, $p = 0.002$). In contrast, BIR1 (BAK1-interacting receptor-like kinase 1), directly interacts with BAK1 (Ma et al. 2017) and negatively regulates defence (Gao et al. 2009). Consistent with this function, the expression of Lsat_1_v5_gn_0_1380, an ortholog of BIR1, was found to correlate with lettuce susceptibility to *S. sclerotiorum* ($r_s = 0.69$, $p = 1.6E-6$). Our detection of this group of interacting known immune regulators provides confidence in our approach and suggests other RLKs identified could have genuine impacts on *S. sclerotiorum* and/or *B. cinerea* resistance. Similarly, there is a precedent for the involvement of RLPs in plant response to necrotrophic fungal infection with Arabidopsis RLP23 required for proper defence against *B. cinerea* (Ono et al. 2020).

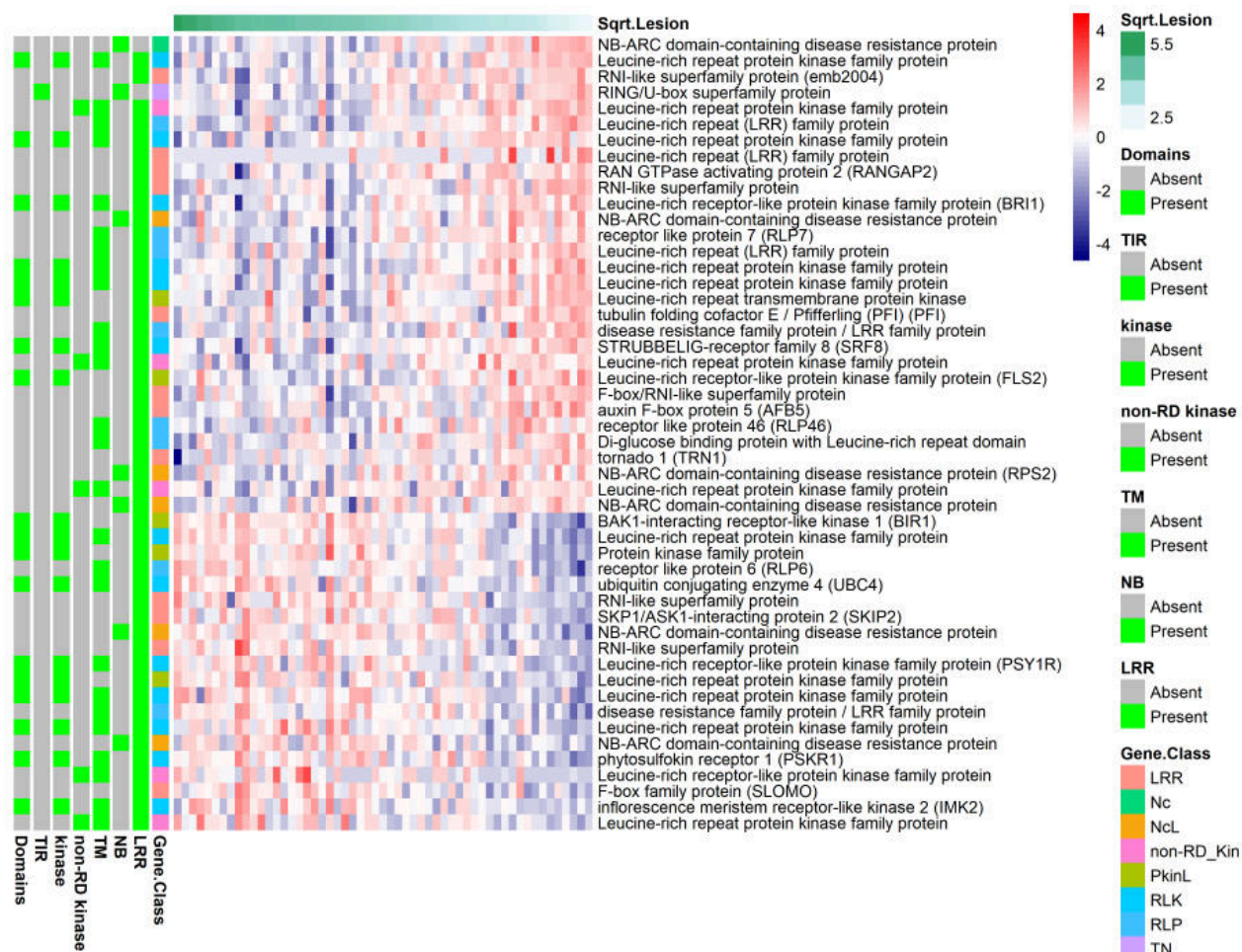


Figure 2.12: Expression of the top 50 lettuce genes whose expression is correlated with *S. sclerotiorum* lesion size, and which were classified as non-NLR pathogen recognition receptors (Christopoulou et al. 2015) classification indicated in the column gene.class). This classification was based on the presence of any combination of the following domains; leucine-rich repeats (LRR), nucleotide-binding (NB), NB Coiled-coil type (Nc), transmembrane (TM), kinase, non-arginine-aspartate kinase (non-RD kinase) and TOLL/interleukin-1 receptor (TIR). Additional gene nomenclature includes NcL: NC plus L domains; PkinL: kinase plus L; RLK: receptor-like kinase; RLP receptor-like protein. The individual lettuce samples are ordered left to right on the basis of lesion size after inoculation with *S. sclerotiorum*, with the most susceptible (largest lesion size) on the left and most resistant (smallest lesion size) on the right. Log2 expression is indicated by the red/blue scale

In total, 236 genes in the lettuce genome encode intracellular NLRs, with 47 encoding coiled-coil NLRs (CNLs) and 187 encoding Toll/Interleukin-1 type NLRs (TNLs) (annottaions from Christopoulou et al. 2015). Of these NLRs, 20 showed significant ($p < 0.05$) correlation of expression with lesion size after *S. sclerotiorum* infection with expression of 12 correlating with increased resistance and eight with increased susceptibility (Figure 2.11). As for the majority of the genes with expression correlated with lesion size, all 20 do not change in expression in response to pathogen infection (at least from our single time point dataset), suggesting that it is basal expression levels of these NLR genes that is impacting quantitative disease resistance to *S. sclerotiorum* in lettuce.

2.3.5 Parents of existing lettuce mapping populations show quantitative variation in susceptibility to necrotrophic fungal pathogens

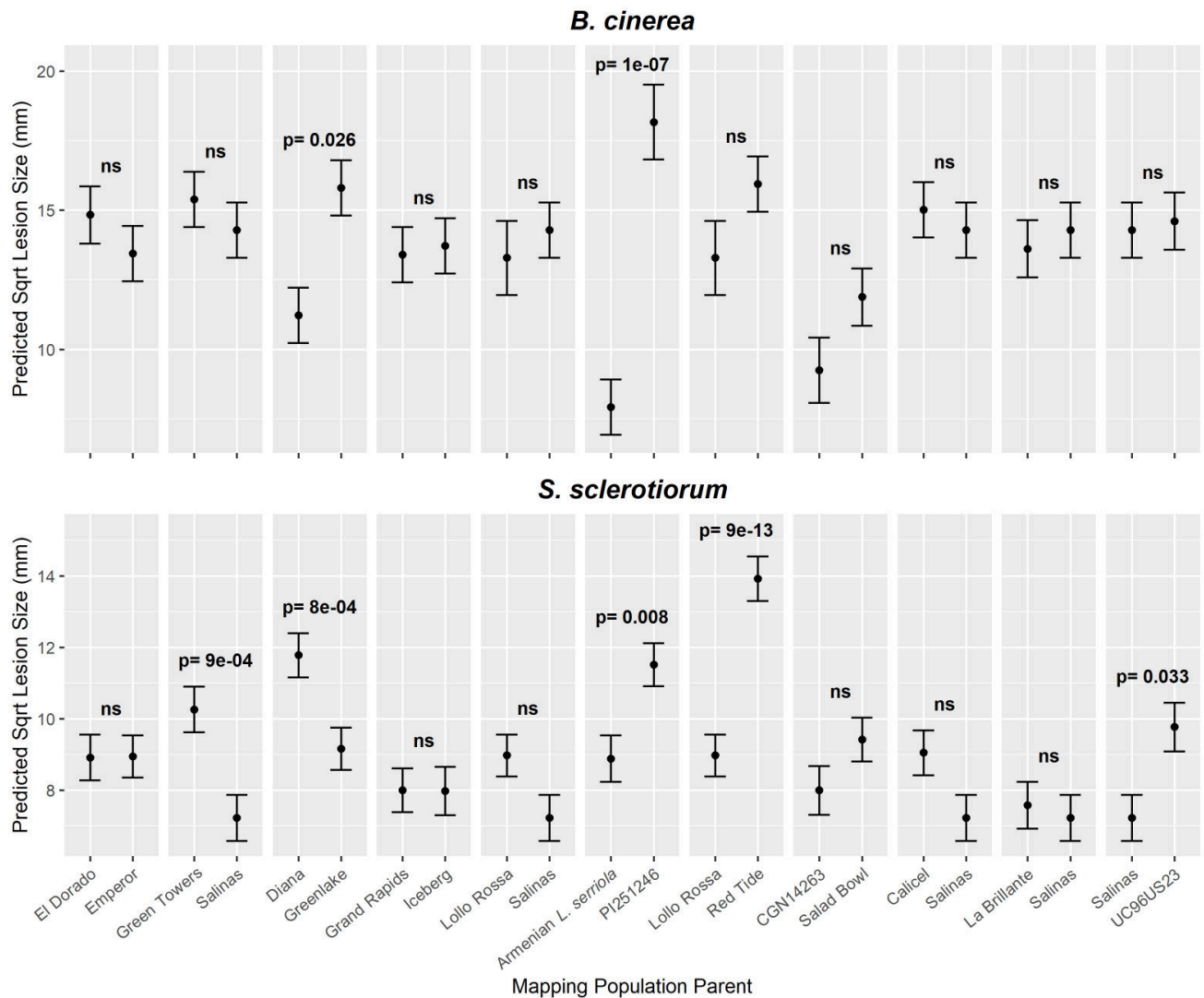


Figure 2.13: Parent lines of lettuce mapping populations differ in lesion size after inoculation with *B. cinerea* or *S. sclerotiorum*. REML predicted square root mean lesion size of *B. cinerea* (top) or *S. sclerotiorum* (bottom) on detached lettuce leaves of mapping population parents available from UC Davis. Lines are shown grouped as parents of mapping populations. Multiple cases of the same line represent one set of data that is repeated to allow comparison within a different parental pair. Error bars are REML predicted standard error, where n is between 15 and 29. Tukey HSD p-values are shown where there is a significant difference ($p < 0.05$), otherwise “ns” indicates not significant.

We screened the parents of existing lettuce mapping populations to test whether these populations would be suitable for dissecting the mechanistic basis of quantitative variation in resistance to *B. cinerea* and *S. sclerotiorum*. Seventeen lettuce accessions, the parents of 11 different mapping populations, were phenotyped using the detached leaf inoculation assay with both *B. cinerea* and *S. sclerotiorum*. Of the 11 parental combinations, two exhibited significantly different lesion size after *B. cinerea* inoculation, and five exhibited significantly different lesion size after *S. sclerotiorum* inoculation ($p < 0.05$) (Figure 2.13). The parents of two mapping populations exhibited significantly different lesion sizes in response to both pathogens. Notably,

in the Greenlake x Diana cross, *S. sclerotiorum* lesions on lettuce variety Greenlake were significantly larger than lesions on variety Diana, while *B. cinerea* lesions were significantly smaller, indicating that Greenlake and Diana exhibit contrasting susceptibility to the two pathogens. The second set of parental lines, *L. sativa* PI251246 and an Armenian *L. serriola* (Mamo et al. 2021), showed consistent responses to *B. cinerea* and *S. sclerotiorum* with lesions caused by both pathogens larger on PI251246 than on leaves of the Armenian *L. serriola* line. This mapping population was investigated further to identify genomic regions mediating this difference in lesion development.

2.3.6 Five genomic loci mediating lettuce resistance to fungal pathogens in a detached leaf assay

A total of 234 F₆ recombinant inbred lines (RILs) resulting from the cross between the Armenian *L. serriola* and PI251246 (*L. sativa*) were phenotyped in a replicated incomplete experimental design using both *B. cinerea* and *S. sclerotiorum* detached leaf assays. QTL mapping identified five loci impacting lesion size following inoculation with *B. cinerea* or *S. sclerotiorum* (Figure 2.14, Figure 2.15). Three QTL impacted the size of *S. sclerotiorum* lesions and two impacted the size of *B. cinerea* lesions. Despite correlation between resistance to these two pathogens across the lettuce diversity set ($r = 0.47$, $p = 1.1 \times 10^{-6}$) and the parental lines demonstrating significant differences in response to both pathogens ($p < 0.05$) (Figure 2.13), QTL mediating quantitative resistance to the two pathogens did not co-locate. This suggests five QTL exist between these two parent lines independently contributing to disease resistance. No evidence for epistatic interactions between the QTL loci was detected.

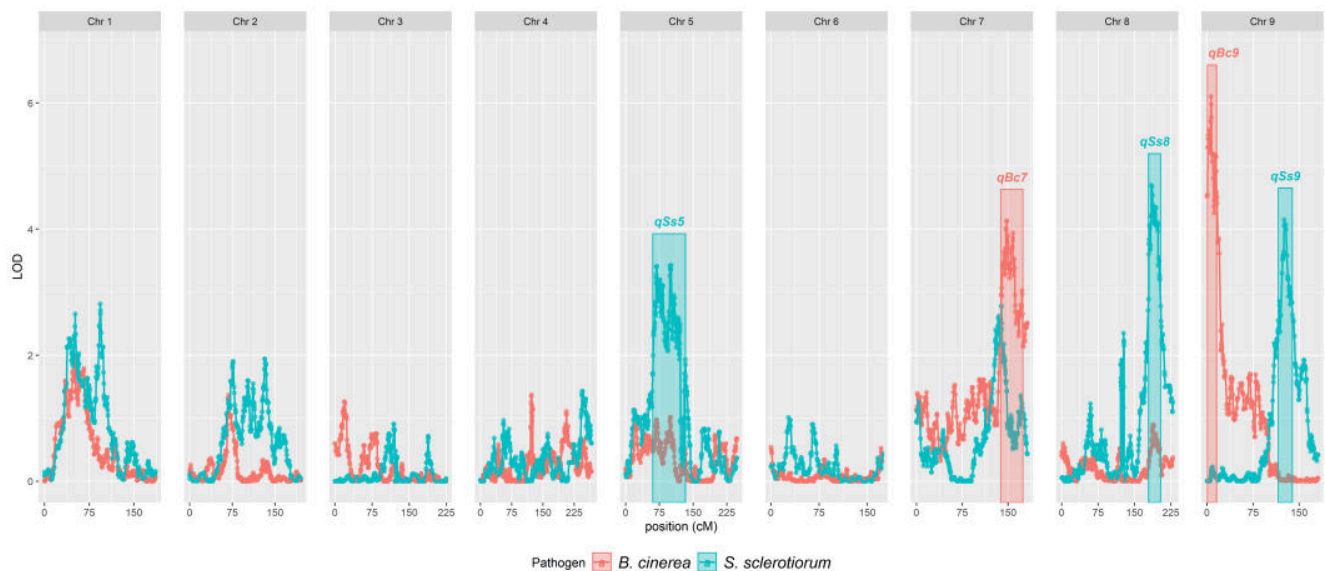


Figure 2.14: Quantitative trait loci associated with reduced lesion size of *B. cinerea* or *S. sclerotiorum*. LOD scores from 'stepwiseqtl' multi-QTL selection models using the Haley-Knott algorithm, genotyping-by-sequencing markers and predicted mean lesion size from the detached leaf assay data for each pathogen are shown. Data relating to *B. cinerea* inoculation are shown in red, whereas those from *S. sclerotiorum* inoculation are shown in blue. Five significant QTL (*qSs5*, *qSs8*, *qSs9*, *qBc7* and *qBc9*) were maintained in the final model after backwards elimination of insignificant loci. Boxes represent the 1.5 LOD confidence intervals around the peak LOD of each QTL. The nine lettuce chromosomes are shown along the x-axis.

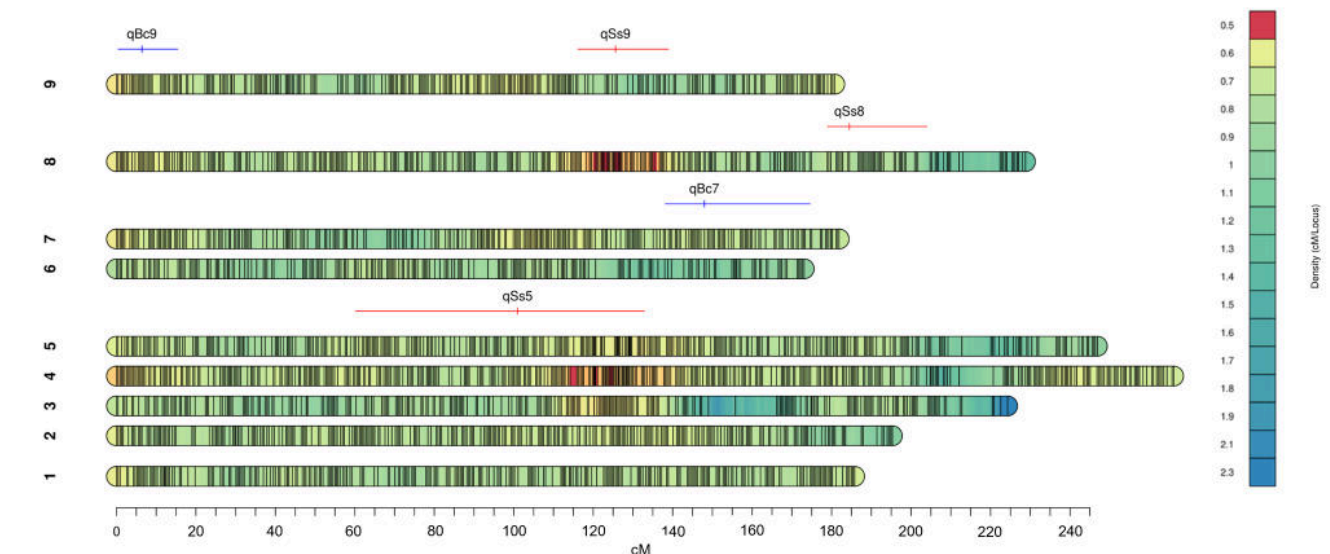


Figure 2.15: Location of resistance QTL on the Armenian *L. serriola* × PI251246 marker density map. Horizontal bars show the 1.5 LOD confidence interval of the QTL loci and the vertical bar shows the location of the peak QTL marker

Each QTL explains between 7 and 11% of the lesion size variation (Table 2). For four of the five QTL, the alleles conferring reduced lesion size were derived from the Armenian *L. serriola* parent, which showed increased resistance to *B. cinerea* and *S. sclerotiorum* compared to the other parental line, PI251246. However, at *qSs9* (*S. sclerotiorum* QTL on Chromosome 9), the resistance allele originates from the susceptible parent,

PI251246. This suggests the presence of alleles with both positive and negative effects on disease resistance, which can be separated by recombination. RILs that contain the resistance alleles at both *B. cinerea* QTL or all three *S. sclerotiorum* QTL have significantly reduced lesion size compared to RILs with susceptibility alleles at the same loci ($p < 0.05$) (Fig 2.16).

QTL	Pathogen	Chr	Pos (cM)	pLOD	Variance exp (%)	Genetic size (cM)	Physical size (Mb)	#Genes	#Genes Expressed
qBc7	<i>B. cinerea</i>	7	148	4.1	7.29	36.5	34.9	639	313
qBc9	<i>B. cinerea</i>	9	6.4	6.1	10.75	15	26.7	776	415
qSs5	<i>S. sclerotiorum</i>	5	101	3.4	7.02	72.7	118.4	1353	565
qSs8	<i>S. sclerotiorum</i>	8	184.5	4.7	8.14	25.1	40.1	292	142
qSs9	<i>S. sclerotiorum</i>	9	125.6	4.1	8.54	22.8	18.8	204	83

Table 2.2: Summary statistics for resistance quantitative trait loci (QTL) identified in the Armenian *L. serriola* × PI251246 mapping population

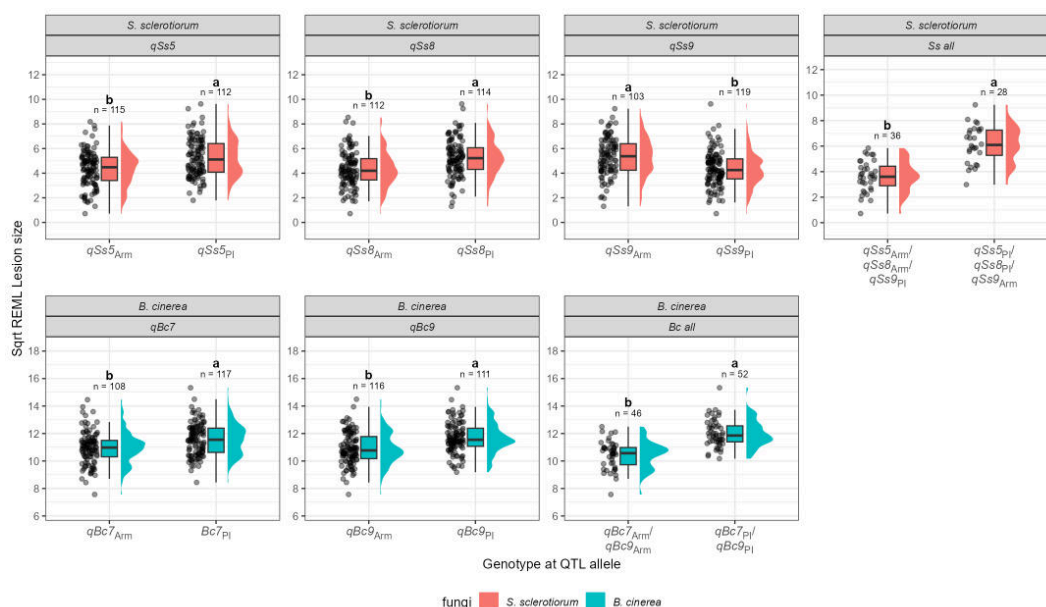


Figure 2.16: Sqrt Restricted Maximum Likelihood (REML) lesion size of Armenian *L. serriola* (Arm) × PI251246 (PI) recombinant inbred lines (RILs) grouped by their genotype at identified QTL markers. *B. cinerea* lesion sizes are shown in red, *S. sclerotiorum* are shown in blue. Letters show statistical significance groupings (Tukey HSD $p < 0.05$) and the number of RILs tested is indicated for each genotype.

To define boundary markers for each QTL, confidence intervals of 1.5 LOD surrounding each QTL peak were calculated using 'lodint', which were expanded to the next marker. QTL boundary markers were mapped onto the *L. sativa* cv Salinas v8 genome (Reyes-Chin-Wo et al. 2017). Predicted genes positioned between the QTL boundary markers in the Salinas cultivar could then be identified (Table 2.2). A large variation in QTL size (and gene number) was observed, with qSs5 the largest at 72.7 Mb and containing 1353 genes. The smallest QTL (by gene number) is qSs9, at 22.8 Mb with the region containing 204 genes.

2.3.7 Identification of candidate causal genes in the QTL through transcriptome profiling

We attempted to predict causal genes within the identified QTL regions using transcriptome and genome data. Both parent lines, the Armenian *L. serriola* and PI251246, were included in the lettuce diversity set whose transcriptomes were profiled 48 hpi and 43 hpi with *B. cinerea* and *S. sclerotiorum*, respectively. We carried out a second inoculation of these parental lines and generated an additional four biological replicates of transcriptome profiles (48 hpi after *B. cinerea* and 64 hpi after *S. sclerotiorum* inoculation). Principal component analysis of the 28 samples (three/four replicates x two experiments x two pathogens x two lettuce lines) demonstrates clear differences between the parental lines and similarity of the replicates within and across experiments (Fig 2.17). Prior to any differential expression analysis, numbers of potential QTL candidate genes could be reduced by 46–59%, by removing genes which failed to pass the low-expression filter, i.e. they had no detectable expression after either *B. cinerea* or *S. sclerotiorum* infection (Table 2.2).

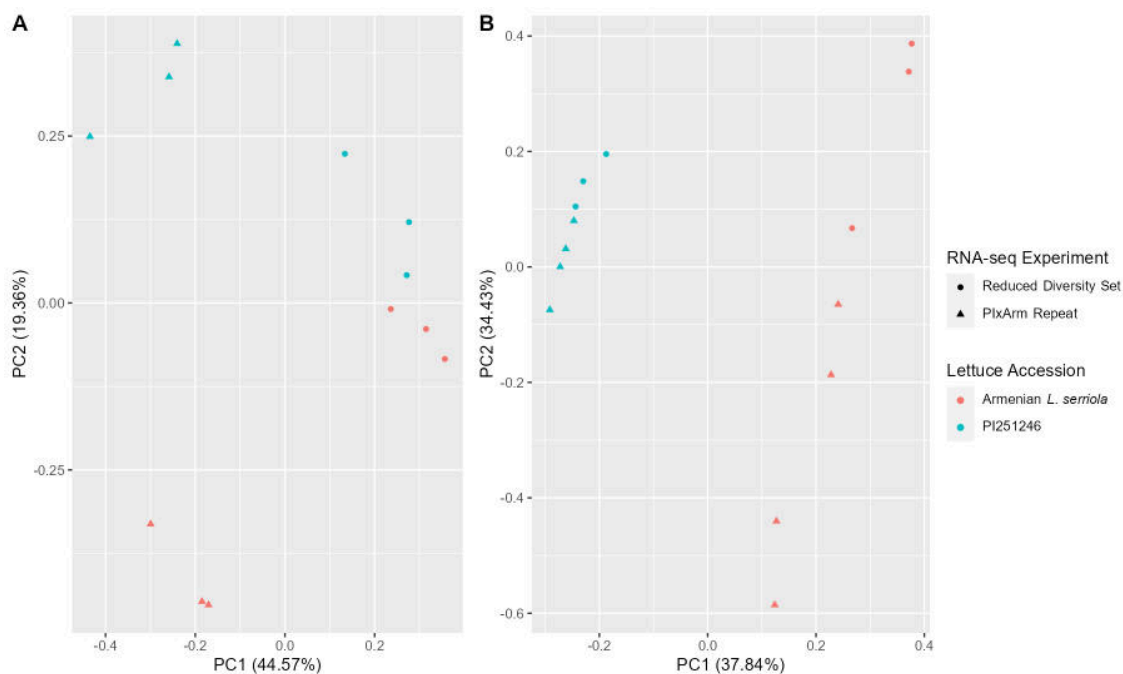


Figure 2.17: Principal Component Analysis (PCA) of PI251246 (blue) and Armenian *L. serriola* (pink) RNAseq data after pathogen infection with **(A)** *B. cinerea* and **(B)** *S. sclerotiorum*. The PCA plot shows two independent experiments: Diversity Set RNAseq (circles) and the mapping population parent repeat (triangles). In *S. sclerotiorum* infected samples, there is a clear separation across PC1 between the parental lines that is consistent across experiments. In *B. cinerea* infected samples, the largest separation across PC1 appears to reflect different experiments, but each parent line clearly separates within experiment across PC2.

Differential expression analysis was carried out on each experiment/pathogen combination separately with threshold of absolute log2 fold change ≥ 1.2 and adjusted $p < 0.05$ used to determine significantly differentially expressed genes (DEGs) between the parental lines. PI251246, the more susceptible parent, was used as the “control” in the differential expression analysis, hence upregulated genes have higher expression in the Armenian *L. serriola* line (and vice versa for downregulated genes). After *S. sclerotiorum* inoculation, there were 1,198 DEGs (425 up/773 down) across both sets of data with 96 (24 up/72 down) of these DEGs located within

identified resistance QTL. One hundred forty-five DEGs (58 up/87 down) were common to both *B. cinerea* inoculation datasets with 10 (5 up/5 down) of these located within QTL. Ninety genes (36 up/54 down) were differentially expressed (in the same direction) between the parent lines in both *B. cinerea* and *S. sclerotiorum* inoculated samples, of which seven (three up/four down) are in QTL.

For the lettuce genes located within the QTL regions, we integrated the parental line transcriptome data above with information on whether expression of the gene in the lettuce diversity set was correlated with lesion size in response to pathogen infection to predict candidate causal genes underlying the identified resistance QTL (Fig 2.18). This analysis highlighted a number of genes with expression patterns consistent with a potential role in mediating fungal pathogen resistance within this mapping population; for example, Lsat_1_v5_gn_5_91640 (LsPDR12) is an ortholog of the Arabidopsis gene Pleiotropic Drug Resistance 12 (AtPDR12), known to mediate camalexin secretion in response to *B. cinerea* infection (He et al. 2019). LsPDR12 is significantly correlated with *S. sclerotiorum* resistance in the diversity set ($r_s = -0.37$, $p = 0.01$), is upregulated in Armenian *L. serriola* compared to PI251246 in both experiments and is located within the QTL *qSs5* (for which the resistance allele comes from the Armenian *L. serriola* parent).

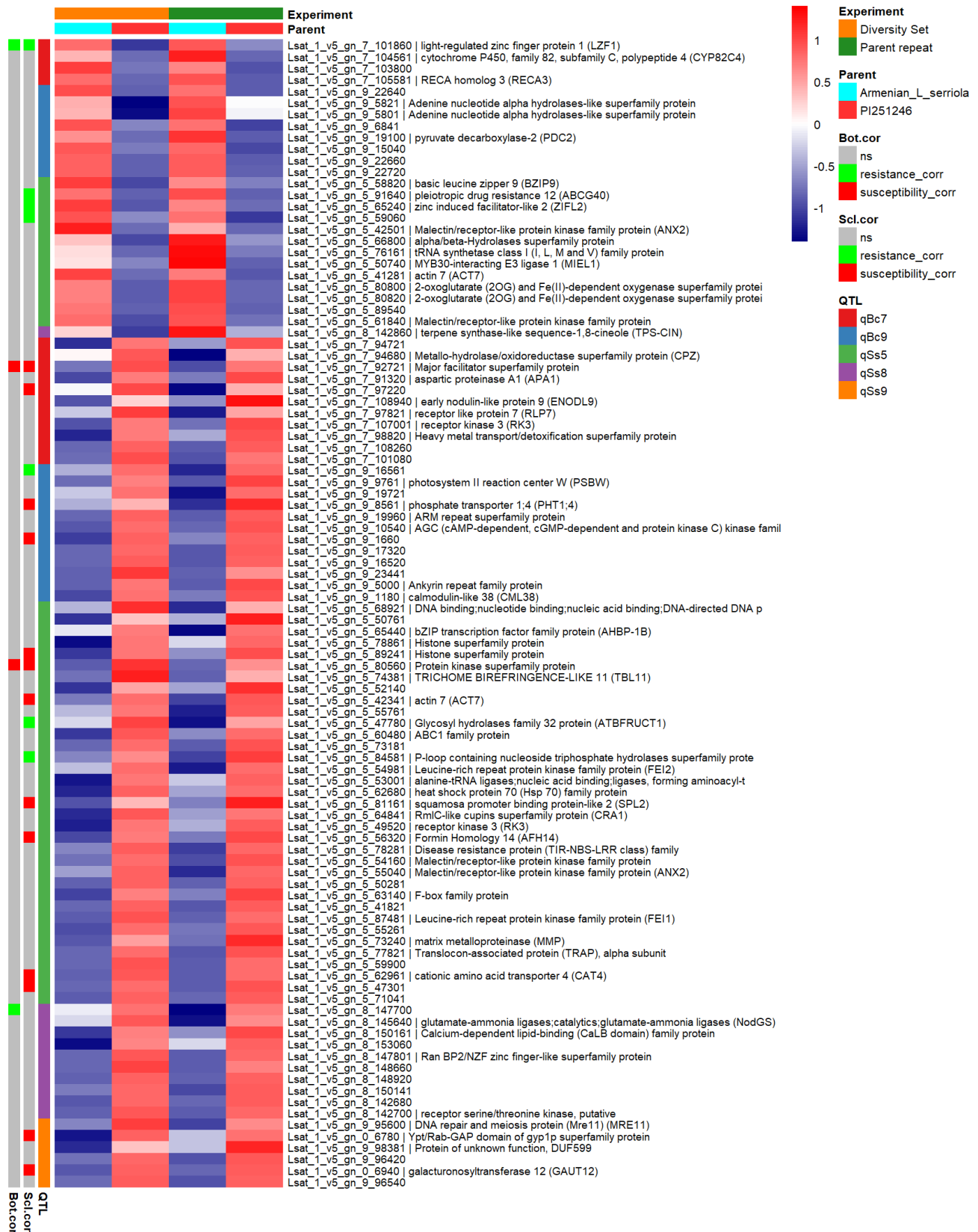


Figure 2.18: Integration of gene expression data with QTL to predict potential causal genes. The expression of genes differentially expressed between the mapping population parents (Armenian *L. serriola*, PI251246) after *S. sclerotiorum* infection in two datasets (as part of the diversity set and a specific repeat of the two lines) and located within a QTL are shown. For all QTL, except for qSs9, the resistance allele originates in the Armenian *L. serriola* line. The two columns on the left indicate genes whose expression is significantly correlated with pathogen resistance or susceptibility in the lettuce diversity set ($p < 0.05$) (ns=no significant correlation). Log₂ expression is indicated by the red/blue scale

Another potential candidate, *Lsat_1_v5_gn_9_1180*, encodes an ortholog of calmodulin-like 38 (CML38), is located within the *qBc9* QTL and is upregulated in PI251246 compared to the Armenian *L. serriola*, i.e. higher expression in the susceptible parent. However, consistent with this, Arabidopsis CML38 promotes degradation of suppressor of gene silencing 3 (SGS3) (Field et al. 2021), a lettuce ortholog of which is correlated with resistance against *S. sclerotiorum* ($r_s = -0.65$, $p = 6.4E-6$).

2.4 Discussion

We demonstrated that genetic variation for resistance to the fungal pathogens *S. sclerotiorum* and *B. cinerea* exists within a lettuce diversity set comprised of both *L. sativa* varieties and wildtype relatives (Fig 2.1). Quantitative resistance within this diversity set was assessed using a detached leaf assay. Although this does not necessarily correlate with field resistance, it has the advantage of reliable and consistent inoculation, and of measuring immunity in a manner that is not dependent on the overall plant architecture. In this way, we anticipate identifying traits that could be exploited in a range of lettuce morphotypes. Crucially, we used inocula of spore suspensions for both pathogens, which mimics the natural infection route, whereas most publications investigating variation in resistance against *S. sclerotiorum* use mycelial plugs (e.g. Chittem et al. 2020) due to the difficulties, and time taken, to produce ascospores.

We identified parents of existing lettuce mapping populations that differed in susceptibility to the two fungal pathogens (Figure 2.13) and five QTL that impacted quantitative resistance differences between *L. sativa* PI251246 and an Armenian *L. serriola* (Fig 2.14). For four of these QTL the resistance allele originated in the Armenian *L. serriola* line. Although PI251246 was the more susceptible parent in this work, this accession was previously shown to have lower *S. sclerotiorum* disease incidence, but similar disease severity to a standard lettuce Butterhead variety, Rachel, in an inoculated glasshouse trial (Whipps et al. 2002). This suggests that PI251246 is able to escape *S. sclerotiorum* (due to architecture and/or rapid bolting) but lacks tissue resistance once infection becomes established. This is consistent with our results from detached leaf assays and a field trial where observed resistance of PI251246 to *Sclerotinia minor* was attributed to rapid bolting characteristics (Hayes et al. 2010). These architectural and rapid bolting attributes would not be beneficial in cultivated lettuce. In contrast, the Armenian *L. serriola* had significantly higher resistance than PI251246 to both *S. sclerotiorum* and *B. cinerea* in the detached leaf assay, suggesting this accession may have beneficial traits which could be exploited in lettuce varieties with varying architectures. *L. serriola* is a wild lettuce believed to be the progenitor of domesticated *L. sativa* (Uwimana et al. 2012). Wild relatives of crop plants are often sources of disease resistance and in previous work with this mapping population, four QTL conferring quantitative resistance to *Verticillium dahliae* race 2 have been identified (Sandoya et al. 2021) with all four beneficial alleles from the Armenian *L. serriola* parent. Three of these *V. dahliae* QTL are on

different chromosomes to the QTL identified here, and the fourth (on chromosome 8) is located in the middle of the chromosome far from the *qSs8* locus impacting resistance to *S. sclerotiorum*.

Although QTL have been identified for field resistance to *S. minor* (Mamo et al. 2019), to our knowledge these are the first lettuce QTL reported for resistance against *S. sclerotiorum* and *B. cinerea*. Of the two *S. minor* resistance QTL identified in repeat field experiments one slightly overlaps with *qSs5* (with the other located on chromosome 1). Multiple *S. sclerotiorum* resistance QTL mapping studies exist in sunflower and *Brassica napus* (for example, Behla et al. 2017), as well as a small number of GWAS in soybean and *B. napus*, all excellently reviewed in Wang et al. 2019b. As in our study, where the QTL explained between 5 and 10% of the variation, published QTL have minor effects on disease resistance (less than 10%). A similar polygenic basis for resistance has been seen against *B. cinerea* with QTL studies in *Arabidopsis*, *Solanum* species and *Brassica rapa* and GWAS in *Arabidopsis* (Corwin and Kliebenstein 2017).

Although the *S. sclerotiorum* isolate used here was initially obtained from field grown lettuce, our analysis was restricted to single isolates of both pathogens. Previous studies using *B. cinerea* have indicated that there is a high level of isolate-specificity to quantitative resistance loci (Zhang et al. 2016b) and data on disease outcome using 98 *B. cinerea* isolates and 90 genotypes of eight plant hosts (including lettuce) demonstrated a much greater impact on lesion size from the *B. cinerea* isolate (40–71%) than the host genotype (3–8%) (Caseys et al. 2021). Similarly, in *B. napus* both pathotype-specific and pathotype-independent resistance against *S. sclerotiorum* has been identified (Neik et al. 2017). Hence, determining whether the QTL identified here can mediate resistance to a broad range of pathogen isolates will be critical to their value in crop improvement.

Despite multiple QTL/GWAS analyses, relatively little is known about the molecular mechanisms driving resistance, and the small effect of each QTL increases the complexity of the fine-mapping process. Hence, we used transcriptome data to predict potential causal genes. We go beyond just simply comparing differential expression of genes located within QTL in two contrasting parental lines (e.g. Qasim et al. 2020). The power of our approach is identifying genes whose expression is correlated with resistance or susceptibility (as judged by lesion size) across 21 different lettuce accessions (Fig 2.9). The use of multiple accessions (rather than the commonly seen comparison of one resistant and one susceptible line, for example Zhao et al. 2009) gives us better ability to identify expression differences genuinely contributing to disease resistance. Our analysis identified genes where an increase in expression is correlated with smaller lesion size (resistance) or larger lesion size (susceptibility). Although this correlation analysis (unlike expression QTL analysis) does not pinpoint the genetic region responsible for the variation in expression, it does enable us to identify genes that impact infection outcome (positively or negatively) but are not necessarily differentially expressed during infection and not necessarily directly involved in the defence response (for example, genes influencing cell wall composition

or metabolite content of leaves).

One key group of genes that we identified as highly correlated with disease resistance were those involved in RNA-mediated post transcriptional regulation, in particular gene silencing. In well-studied plants, such as *Arabidopsis* and tomato, gene silencing (via microRNAs, miRNAs, or small interfering RNAs, siRNAs) is known to play a crucial role in the host immune response—both in regulating expression of plant genes, as well as silencing of genes in the pathogen (Qiao et al. 2021b). Our data suggest a similar role for gene silencing in lettuce, and the prominence of orthologs of genes known to increase susceptibility in *Arabidopsis* to both *S. sclerotiorum* and *B. cinerea* provides confidence in this approach to highlight key mechanisms of quantitative resistance. One such potential mechanism is PPR-driven silencing of pathogen virulence genes. Increased expression of many lettuce PPR genes was correlated with decreased lesion size after pathogen inoculation (Fig 2.11). Intriguingly PPR transcripts are a major source of secondary siRNAs in plants, whose generation is triggered by both direct miRNA binding to specific PPR transcripts and via miRNA-mediated generation of trans-acting siRNAs (tasiRNAs) (Howell et al. 2007). Furthermore, siRNAs derived from PPR transcripts accumulate after *Phytophthora capsici* infection, can potentially target known pathogen virulence genes, and an effector from the pathogen can suppress accumulation of these siRNAs to promote infection (Hou et al. 2019). *Arabidopsis* RNA-dependent RNA polymerase 6 (RDR6) is required for generation of siRNAs from endogenous transcripts, and *rdr6* mutants show enhanced susceptibility to *B. cinerea* (Cai et al. 2018). Furthermore, mutation of the Pentatricopeptide repeat protein for germination on NaCl (PGN) in *Arabidopsis* led to increased susceptibility to *B. cinerea* (Laluk et al. 2011). Clearly, further research is needed to determine the importance and mechanism of PPR siRNA production in lettuce, especially given that in a comparative study across nine plant species, several new and potentially species-specific miRNAs were shown to drive production of these siRNAs (Xia et al. 2013).

Small RNAs are also thought to play a key role in regulating expression of NLR genes, helping regulate their expression (and hence inadvertent triggering of the defence response) in the absence of infection. NLRs are mostly known as an integral part of effector-triggered immunity (ETI), whereby pathogen effectors are directly or indirectly (as guards or decoys of effector targets) recognised by NLRs (Cui et al. 2015b). As such, they have typically been associated with qualitative (all or nothing) disease resistance. Due to the lack of complete resistance phenotypes against broad host range necrotrophic fungal pathogens and very limited number of NLR genes shown to impact resistance, it was thought that NLR proteins and ETI are not important in defence against these pathogens (Mengiste 2012). However, in our data expression of multiple lettuce NLRs was shown to be correlated with resistance suggesting that, in addition to their well-known role in lettuce resistance against biotrophic pathogens (Simko et al. 2013; Parra et al. 2016; Parra et al. 2021), NLR genes in lettuce may play a role in quantitative resistance against *B. cinerea* and *S. sclerotiorum*. NLRs show huge diversity both within a single genome and in populations, and a multitude of incomplete NLRs (lacking one or

more of the canonical domains but thought to still be able to function as adapters or helpers for other NLRs) are also found in all plants (Baggs et al. 2017). Several lettuce incomplete NLRs had expression correlated with *S. sclerotiorum* lesion size (Fig 2.12) and there is a precedent for incomplete NLRs impacting resistance to broad host range necrotrophic pathogens, with mutations in the Arabidopsis gene RLM3 (containing TIR and nucleotide binding domains) causing increased susceptibility to these pathogens, including *B. cinerea* (Staal et al. 2008). We also noted in our analysis that there were several NLRs whose increased expression was correlated to increased susceptibility to *S. sclerotiorum* (Fig 2.11) suggesting that NLRs may have both a positive and negative effect on resistance to this pathogen. Indeed, a Toll interleukin-1 receptor (TIR) type NLR in Arabidopsis, LAZ5, has been shown to increase susceptibility to *S. sclerotiorum* infection, with *laz5-1* mutants showing increased resistance (Barbacci et al. 2020).

Although the lettuce diversity set we used here is small compared to a collection that has been recently genome sequenced (Wei et al. 2021), our analysis demonstrated useful genetic variation for quantitative disease resistance, indicated crosses that could be useful in mapping this trait and identified multiple potential mechanisms for experimental testing. It is likely that different lettuce lines harbour different quantitative resistance mechanisms, and our gene expression correlation analysis has identified strong candidates for experimental testing that are not obviously segregating in the Armenian *L. serriola* × PI251246 population. However, combining transcriptome data from the parents and diversity set with QTL analysis has also identified a small number of potential causal genes for the resistance QTL in this population, with the strongest candidate being the lettuce ortholog of Arabidopsis Pleiotropic Drug Resistance 12 (AtPDR12) within the QTL *qSs5* (Fig 2.18). Obviously, the molecular mechanism underlying the resistance QTL may not necessarily be regulatory variation of a gene within the QTL itself (and hence identifiable in our analysis approach) but could be driven by sequence variation driving changes in post-transcriptional gene regulation or protein function.

In summary, we have identified multiple potential architecture-independent resistance mechanisms that may be successful for enhancing disease resistance in lettuce. Future work will aim to validate candidate genes, for example via fine-mapping of QTL and/or the generation of lettuce lines with gain or loss of function mutations/transgenes. The resistance traits could be incorporated into cultivated varieties (via marker-assisted selection) with genome editing of validated candidate genes offering an exciting route to exploit the genetic variation from these lettuce accessions without losing the beneficial traits stacked up in elite breeding lines.

Chapter 3

Identification of *Lactuca sativa* transcription factors impacting resistance to *Botrytis cinerea* through predictive network inference

3.1 Introduction

Botrytis cinerea is a devastating plant pathogen able to infect over 200 plant species including numerous important crop species, costing over \$10 billion per year in control attempts and crop losses. *B. cinerea* can infect pre or post-harvest, and isolates have been identified which show resistance to fungicides (Rupp et al. 2016). *B. cinerea* is a generalist necrotrophic pathogen, secreting a vast arsenal of phytotoxins and cell wall degrading enzymes to induce cell death in its host (Williamson et al. 2007). *Sclerotinia sclerotiorum* is another necrotrophic fungal pathogen which is closely related to *B. cinerea* and employs similar infection strategies (Amselem et al. 2011). *Lactuca sativa* (lettuce) is a nutritionally and economically important crop species with a global value of \$US2.4 billion. Lettuce is highly susceptible to a number of plant pathogens including the fungal pathogens, *B. cinerea* and *S. sclerotiorum*.

Arabidopsis thaliana-*B. cinerea* is one of the most extensively studied pathosystems in plant pathology, providing a high-level understanding of the complex plant-pathogen interactions. Upon *B. cinerea* infection, microbe-associated molecular patterns (MAMPs) or damaged associated molecular patterns (DAMPs) are recognised by pathogen recognition receptors (PRRs), which trigger downstream signalling to activate a defence response. For example, fungal cell wall component chitin acts as a MAMP, which is recognised

by CERK1 (Chitin Elicitor Receptor Kinase 1) (Miya et al. 2007). Chitin-activated CERK1 phosphorylates PBL27, which in turn initiates a MAP kinase cascade via MAPKKK5 (Shinya et al. 2014). This recognition pathway is required for defence against multiple necrotrophic fungi including *B. cinerea* and *Alternaria brassicicola* (Yamada et al. 2016; Liu et al. 2018). Such signalling cascades trigger large-scale transcriptional reprogramming changes to combat pathogen infection. In response to *B. cinerea*, Arabidopsis undergoes massive transcriptional reprogramming, differentially expressing over 9000 genes (Windram et al. 2012). Pathogen-induced transcriptional reprogramming is significantly impacted by plant hormone signalling networks, which themselves exhibit multiple levels of crosstalk. Two key defence hormones, jasmonic acid (JA) and salicylic acid (SA), induce differential expression of thousands of genes in Arabidopsis (Hickman et al. 2017; Hickman et al. 2019; Zander et al. 2020).

Generally, JA and ethylene (ET) promote defence against necrotrophic pathogens, and SA against biotrophic pathogens. However, exogenous application of SA increases *B. cinerea* resistance (Ferrari et al. 2003) indicating that effective defence requires complex interaction between hormone networks to fine-tune gene expression in response to different pathogens based on their lifestyle. Transcription factors (TFs) are central to integrating multiple hormone signals and fine-tuning the defence in specific scenarios (Nomoto et al. 2021; Aerts et al. 2021; Caarls et al. 2017; Ndamukong et al. 2007; Zhang et al. 2014b; Tsuda and Somssich 2015). There are several major TF families which coordinate these defence responses such as ERFs (Ethylene Responsive Factors), WRKYs, MYBs, NACs (NAM-ATAF1-CUC2 family) and bHLHs (basic helix-loop-helix) (Tsuda and Somssich 2015). Many TFs within these families, such as ERF1 (Berrocal-Lobo et al. 2002), ORA59 (ERF) (Pré et al. 2008), WRKY33 (Birkenbihl et al. 2012; Liu et al. 2015), WRKY70/WRKY54 (Li et al. 2017), BOS1 (MYB) (Mengiste et al. 2003), MYC2 (bHLH) (Lorenzo et al. 2004) and NAC019/NAC055 (Bu et al. 2008) have been identified as key regulators of the defence response to *B. cinerea*, either promoting resistance or susceptibility to the fungus. What is not clear is how these individual TFs operate within gene regulatory networks (GRNs) to shape the defence response against a particular pathogen, or whether orthologues of these TFs (and/or different TFs) are important in lettuce defence against *B. cinerea*.

With recent advances in high-throughput sequencing and the availability of whole-transcriptome expression data, it is possible to take a systems biology approach to identify regulators and understand the complex GRNs in which these regulators interact. Inference of GRNs can be performed to predict key regulators, or “hub genes”, which regulate other genes within a network. In causal network inference, nodes (genes) are linked by directional edges and expression of the upstream gene is predicted to impact expression of the downstream gene. This contrasts with co-expression networks where nodes are linked by an edge if they share a similar expression profile across the input data sets, with no prediction of upstream regulation. For causal network inference, high-resolution temporal transcriptome data is critical. We previously constructed a GRN based on an Arabidopsis time series gene expression data set (*B. cinerea*- and mock-inoculated samples, 24

time points) which identified TGA3 as a network ‘hub’ positively regulating *B. cinerea* resistance (Windram et al. 2012). However, incorporating multiple complementary datasets into network inference is likely to increase the accuracy of the resulting network model. Here, we present a high-resolution *B. cinerea* infection (and mock) time-series from a lettuce cultivar (*L. sativa* cv. Saladin). We construct a GRN integrating our *B. cinerea*-infection time-series data with three additional transcriptomic datasets from lettuce infected with *B. cinerea* and *S. sclerotiorum* (Pink et al. 2022; Ransom et al. 2023). Together, these data provide an unprecedented level of detail about the transcriptional defence response for a crop species, identify a core set of genes that respond to *B. cinerea* and *S. sclerotiorum*, and generates a high-confidence GRN model underlying the lettuce defence response. This GRN accurately predicts LsBOS1 and LsNAC53 as key regulators of defence against *B. cinerea*, as well as validated downstream target genes of LsNAC53.

3.2 Methods

3.2.1 Pathogen inoculation time series experiment

Lettuce cv. Saladin was grown for 4 weeks in Levington’s M2 soil in the greenhouse at approx. 18°C with day length supplemented to 16 hours. The third leaf from each plant was removed and placed on 0.8% agar in 35 x 23 cm propagator trays. Leaves were inoculated with four 10 μ L droplets of 5×10^5 mL *B. cinerea* ‘pepper’ isolate (Windram et al. 2012) spore suspension in 50% (w/v) potato dextrose broth (PDB), 1% (w/v) guar, or mock inoculated with four 10 μ L droplets of 50% PDB, 1% guar. *B. cinerea* spore suspensions were prepared as in (Pink et al. 2022). Inoculations were carried out halfway through the 16-hour light period. Lidded trays were placed in a controlled environment chamber under 16-hour light: 8-hour dark, 22°C at 95% humidity. A 1 cm cork borer was used to harvest a leaf disc surrounding each inoculation droplet, with the four discs from one leaf pooled and flash-frozen in liquid nitrogen. Three leaves (each from separate plants) were harvested for mock and *B. cinerea*-inoculations at each of the 14 time points: 3-hour intervals from 9 to 48 hours post inoculation (84 samples in total).

3.2.2 Gene expression profiling

RNA was extracted using Trizol (Thermo Fisher Scientific) with a lithium chloride purification step. Sequencing libraries were prepared using the Illumina TruSeq RNA V2 kit and sequenced on a HiSeq 3000 generating 75 bp paired-end reads at the Wellcome Trust Human Genetics Centre. Read quality was checked with FastQC (Andrews et al. 2010). Raw reads were trimmed with trimmomatic (Bolger et al. 2014) and aligned to a combined *L. sativa* cv. Saladin – *B. cinerea* transcriptome using STAR (Dobin et al. 2013), achieving a median alignment of 90%. Transcript abundances were calculated with RSEM (Li and Dewey 2011). We applied a low expression filter, keeping genes > 1 count per million in at least 3 samples. Principal component

analysis was performed using 'prcomp' R function.

3.2.3 Differential expression analysis

Pairwise differential expression analysis between mock and *B. cinerea* inoculated samples at each time point was performed using the Limma-voom pipeline (Ritchie et al. 2015; Law et al. 2014). P-values for each time point were combined using the Simes method (Simes 1986; Sarkar and Chang 1997) obtaining a single combined p-value per gene for the time series, these were subsequently adjusted using Bonferroni-Hochberg (BH) (Benjamini and Hochberg 1995) to account for multiple testing. Genes with a final adjusted p-value < 0.01 were considered differentially expressed. The Time of First Differential Expression (TOFDE) was determined by examining the pairwise time point comparisons. Genes with BH adjusted p-values < 0.01 were considered differentially expressed at a specific time point. TOFDE was determined for each gene that exhibited differential expression across the entire time-series, by the earliest time point at which they were identified as differentially expressed.

3.2.3.1 Re-analysis of publicly available datasets

Publicly available lettuce-defence transcriptomic datasets were downloaded from short-read archives; Fletcher et al. 2019 (www.ncbi.nlm.nih.gov/sra/?term=PRJNA523226) and Verwaaijen et al. 2019 (ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-4762). Reads were trimmed with fastp (Chen et al. 2018) and mapped to combined pathogen- *L. sativa* V8 transcriptomes (Reyes-Chin-Wo et al. 2017). GCA_004359215.2 (ncbi.nlm.nih.gov/datasets/genome/GCA_004359215.2/) was used as the reference transcriptome *Bremia lactucae* data and GCF_016906535.1 (ncbi.nlm.nih.gov/datasets/genome/GCF_016906535.1/) was used for *Rhizoctonia solani*. Biological samples were filtered to have at least 2 million reads mapping to the lettuce transcriptome.

3.2.4 Wigwams Modules

The Wigwams algorithm (Polanski et al. 2014) (github.com/cyversewarwick/wigwams) was used to identify co-expressed modules within the set of DEGs common to both *B. cinerea* and *S. sclerotiorum* infection. The following parameters were used: SizeThresholds = 30, Merging_Overlap = 0.82, Merging_CorrelationFilter = 0.89, Mining_CorrelationNet = 0.5, Merging_MeanCorrelation = 0.93.

3.2.5 Functional Enrichment

Gene-ontology GO term enrichment analysis was performed using `enrichGO` function within the `clusterProfiler` R package (Wu et al. 2021b), performed with a significance threshold of $p \leq 0.05$; p-values were corrected for multiple testing using Bonferroni-Hochberg. Arabidopsis GO term annotation was used and enrichment performed using the closest Arabidopsis orthologue (Reyes-Chin-Wo et al. 2017) of lettuce genes in the test set, against a background of all Arabidopsis genes with an identified lettuce orthologue expressed in the *B. cinerea* time-series.

Protein domain enrichment was performed using previously published InterProScan annotations (Reyes-Chin-Wo et al. 2017). A hypergeometric test was performed with the `phyper` R function using all genes expressed in the lettuce *B. cinerea* time series as the background. P-values were corrected for multiple testing using Bonferroni-Hochberg.

3.2.6 Transcription factor binding motif enrichment

Lettuce promoter sequences 1000 bp upstream from the predicted transcription start site (TSS) were extracted from the *L. sativa* cv. Saladin V8 genome (Reyes-Chin-Wo et al. 2017). The enrichment of Arabidopsis DAP-seq transcription factor binding sites (O'Malley et al. 2016b) was tested within these lettuce promoter sequences using SEA (Simple Enrichment Analysis) within MEME-suite (Bailey et al. 2009; Bailey and Grant 2021). Shuffled input sequences (1000 bp lettuce promoters) were used as background.

3.2.7 Gene Regulatory Network

A gene regulatory network was constructed with OutPredict (Cirrone et al. 2020) with genes differentially expressed in both *B. cinerea* and *S. sclerotiorum* time series in the same direction used as input. 251 of these DEGs, identified as TFs using the PlantTFDB predictor (Jin et al. 2016), were identified as potential regulators. The GRN was trained on expression of these genes in two pathogen infection time series (*B. cinerea* – this study, *S. sclerotiorum* – Ransom et al. 2023 and two previously published lettuce diversity panel data sets (Pink et al. 2022). All expression data was scaled to ensure comparability between experiments. The OutPredict random-forest model was trained with 300 estimators and a test-train split ratio of 0.15. The model was trained on both the time series and single time point data, with the leave-out test set from the time-series. OutPredict calculates an Importance score for the influence of each of the 251 TFs on every non-self-target gene. The top 1% highest confidence edges were included in the final network, comprising 3,382 nodes (including 226 TFs) and 10,947 edges. Pairwise Jaccard-index (TF1-TF2 target intersection/TF1-TF2 target union) was calculated to quantify predicted target overlap of TFs.

3.2.8 Transgenic Arabidopsis lines

The *nac53-1* (SALK_009578C) Arabidopsis mutant line was obtained from NASC. Lettuce BOS1 (Lsat_1_v5_gn_6_70301) and NAC53 (Lsat_1_v5_gn_2_103381) sequences were amplified from *L. sativa* cv. Saladin cDNA generated from mRNA extraction of *B. cinerea* infected leaf material. Primers with attB1 and attB2 extensions were used to amplify the full-length coding sequence of LsBOS1 and a truncated version of LsNAC53 which lacked the C terminal transmembrane domain. Sequences were cloned and verified in the pDONR-Zeo vector, before cloning into the destination vector, pB2GW7 (Karimi et al. 2002) containing a 35S promoter. Stable Arabidopsis transformants were generated using the floral-dip method (Clough and Bent 1998). Multiple independent homozygous transformed lines were selected, and expression of the transgene determined via qPCR of T₃ homozygous plants. T₃ lines were subsequently used for pathogen infection assays and analysis of downstream target genes.

3.2.9 Arabidopsis-*B. cinerea* susceptibility assay

Arabidopsis-*B. cinerea* infection assays were performed as previously described (Windram et al. 2012). In summary, Arabidopsis plants were grown in P24 trays on Levington's F2+Sand soil in controlled environment growth chambers (16 hour day length, 22°C day and night, 60% relative humidity) for 4 weeks. A single leaf was detached from a plant and placed in propagator trays containing a layer of 0.8% agar. Detached leaves were inoculated with 10 µL of *B. cinerea* spore suspension (pepper isolate) at a concentration of 1×10^5 spores/mL, diluted in 50% filter-sterilised grape juice. Trays are sealed and placed back in the growth chamber at a relative humidity of 90%. Photographs are taken of the developing lesions at 72 hpi and lesion size is measured using ImageJ software. Statistical differences between genotypes was determined using Tukey HSD test $p < 0.05$ (Tukey 1949).

3.2.10 qPCR expression analysis

Arabidopsis seedlings were grown on 1/2 strength Murashige and Skoog (MS) media agar plates for 10 days under a 16 hour photoperiod. RNA extractions were performed from whole seedlings using Qiagen RNeasy Plant columns, with an on-column DNase digestion step. cDNA synthesis was performed using SuperScript III (Invitrogen), qPCRs with SYBR green. All qPCRs are performed with three technical replicates of three biological replicates (separate seedling pools). PUX1 (At3g27310) or PP2AA3 (At1g13320) was used as an internal control to normalise expression. Delta Ct ($2^{-\Delta Ct}$) was used to analyse transgene expression level relative to an endogenous gene and delta-delta Ct ($2^{-\Delta\Delta Ct}$) to calculate relative expression of an endogenous Arabidopsis gene.

3.2.11 Data Analysis and Visualisation

Statistical analysis, data analysis and data visualisation were performed using R, unless stated otherwise. The “Tidyverse” collection of R packages was used for all data analysis and data manipulation (Wickham et al. 2019). Heatmap figures were visualised with ComplexHeatmap R package (Gu et al. 2016). Phylogenetic trees were generated using MEGA X (Kumar et al. 2018) and visualised with treeio (Wang et al. 2020a) and ggtree R packages (Yu et al. 2017).

3.3 Results

3.3.1 Shared transcriptional reprogramming in lettuce during *Botrytis cinerea* and *Sclerotinia sclerotiorum* infection

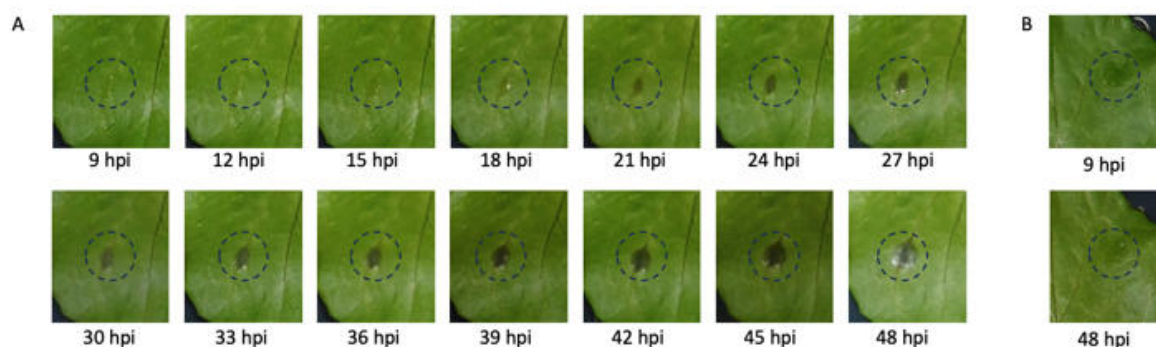


Figure 3.1: Time series of *Botrytis cinerea* infection on lettuce leaves. **A)** 10 μ L droplets of a suspension of *B. cinerea* spores (5×10^4 spores mL) were placed on detached leaves from 4 week old lettuce cv. Saladin plants. Images show the same leaf every 3 hours from 9 hours post inoculation (hpi) to 48 hpi. **B)** A mock inoculated leaf at 9 hpi and 48 hpi. The dashed line indicates the size of the 1 cm diameter disc used for sampling.

We profiled the transcriptome of lettuce leaves (cv. Saladin) following inoculation with spores of *Botrytis cinerea* pepper isolate (Windram et al. 2012). The third leaf from 4-week old plants was inoculated with four droplets of 5×10^5 mL spore suspension or mock control. One cm diameter leaf discs around each inoculation site were harvested every 3 hours between 9 and 48 hours post-inoculation (hpi) with the four discs from one leaf pooled as one sample Figure 3.1. Three leaves were sampled at each of the 14 time points, for both inoculated and mock, as biological replicates. Total RNA was extracted from each sample and mRNA profiled using Illumina short read sequencing and reads mapped to a combined lettuce-*B. cinerea* transcriptome. As expected, the proportion of reads mapping to the *B. cinerea* genome increased over time (Figure 3.5a) although even at the later time points the vast majority of reads were mapping to lettuce transcripts. After quantification of reads, principal component analysis of lettuce gene expression highlighted the similarity between biological replicates and showed clear differences in lettuce gene expression as infection

progressed (Figure 3.2). As expected, we observe diel oscillation in expression of some genes across the time series demonstrating the need for a mock-inoculated time series as opposed to a single time point control (Fig 3.3). Differential expression analysis was performed using limma-voom (Ritchie et al. 2015) revealing 6713 differentially expressed genes (DEGs) over the time series, 3524 up- and 3189 down-regulated (Fig 3.4). Hence, in the hours leading up to and during initial visible lesion development, there is large-scale transcriptional reprogramming in lettuce leaves in response to *B. cinerea* inoculation.



Figure 3.2: Principal component analysis of the lettuce gene expression data (TPM) demonstrates variability between samples is reflected by time point after inoculation. Mock inoculated samples (red), early infection time points (9 18 hpi, green), mid infection time points (21 33 hpi, blue) and late infection time points (36 48 hpi, purple) are indicated. The proportion of variance explained by the first and second principal components is 32% and 8.5% respectively. Ellipses representing 90% confidence intervals around each group's data points.

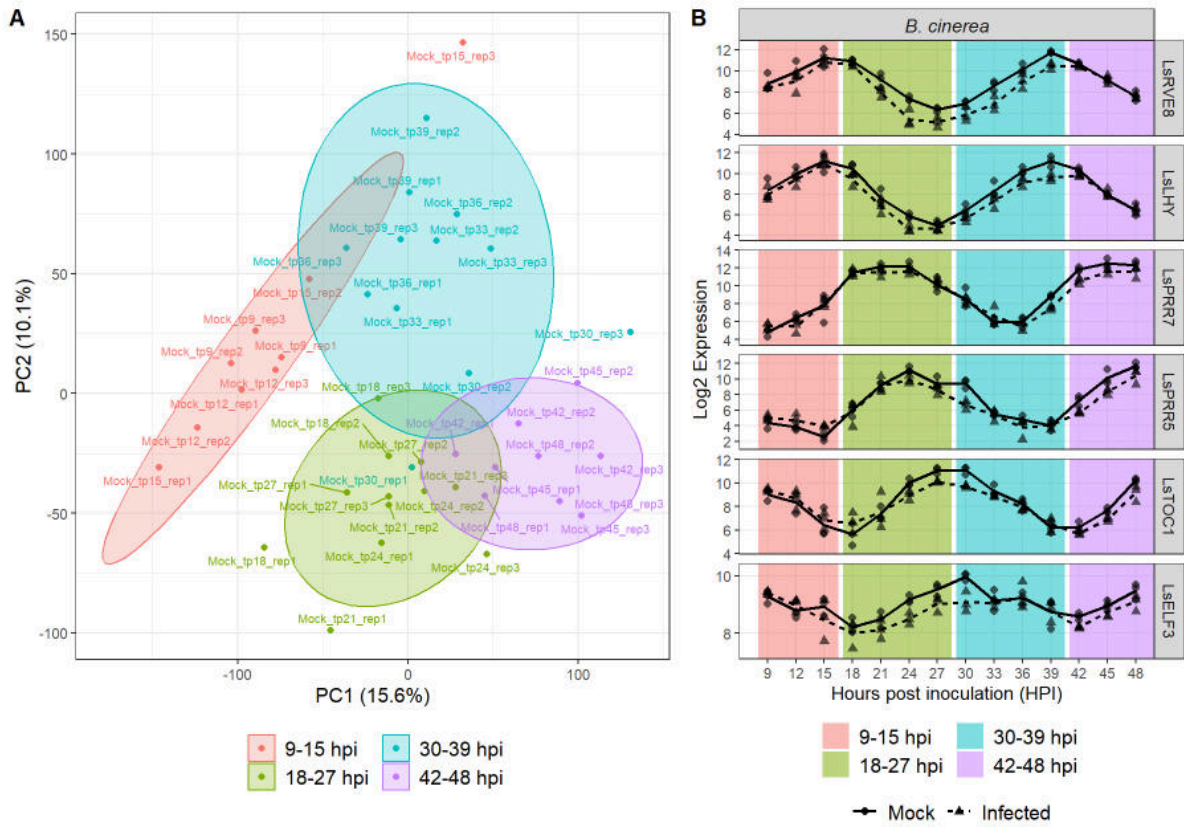


Figure 3.3: Circadian Oscillation observed in Lettuce time-series. **(A)** Principal Component Analysis of mock inoculated gene expression, PC1 is plotted on the x axis, PC2 is plotted on the y axis accounting for 15.6% and 10.1 % of variation respectively. Samples between 9 and 15 hours post inoculation (hpi) are coloured in red, 18 to 27 hpi samples are green, 30 to 39 hpi in blue and 42 to 48 hpi. **(B)** Expression of circadian regulators in lettuce time-series, mock expression shown by circles and solid lines *B. cinerea* infected expression shown by triangles and dotted lines. *LsRVE8* (*Lsat_1.v5_gn.2.109801*), *LsLHY* (*Lsat_1.v5_gn.3.140720*), *LsPRR5* (*Lsat_1.v5_gn.8.4341*), *LsPRR7* (*Lsat_1.v5_gn.2.115441*), *LsTOC1* (*Lsat_1.v5_gn.1.14320*) and *LsELF3* (*Lsat_1.v5_gn.6.7561*).

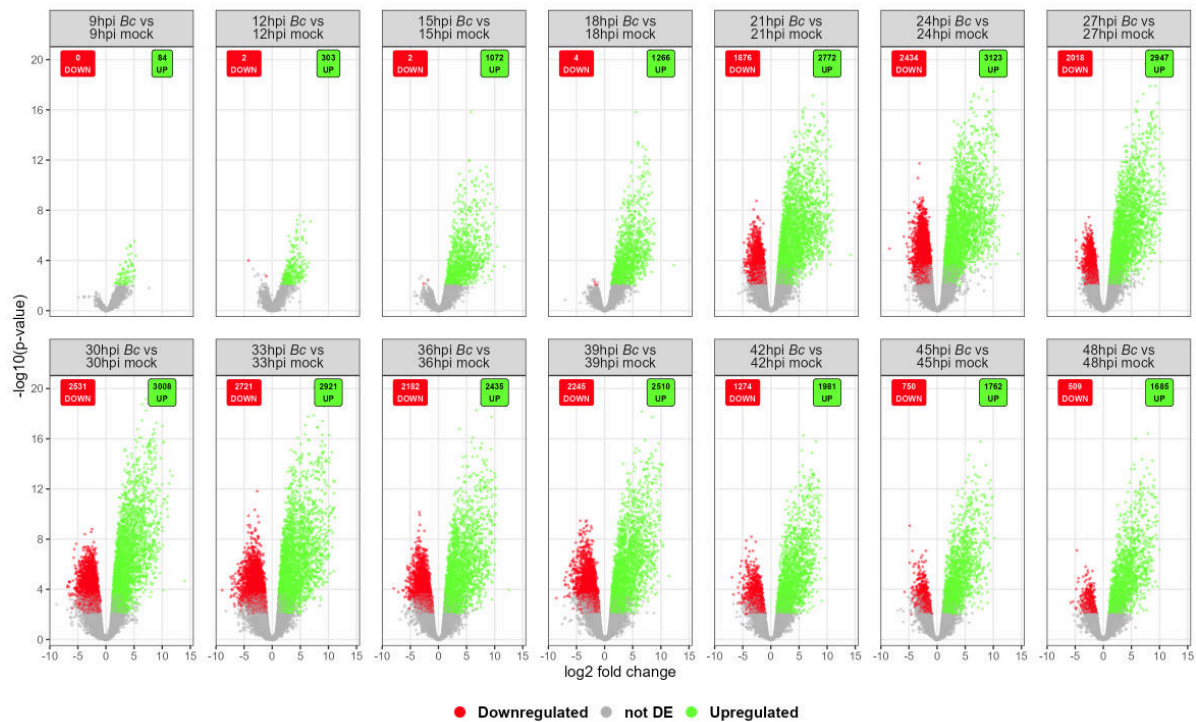


Figure 3.4: Volcano plots of *B. cinerea* vs mock inoculated at each individual time point highlighting the scale of differential gene expression. Log₂ fold change is displayed on the x axis, and -log₁₀ transformation of Bonferroni-Hochberg adjusted p-values (for the single time point) are shown on the y axis. Significantly upregulated genes (*B. cinerea* inoculated versus mock inoculated samples) are highlighted in green, and significantly downregulated genes in red.

Previously, we conducted an independent RNAseq time-series, capturing the transcriptome response in lettuce to inoculation with *S. sclerotiorum*, a fungal pathogen closely related to *B. cinerea* (Ransom et al. 2023). This analysis identified a similar number of DEGs compared to mock-inoculated controls (6446; 4346 up-regulated, 2100 down-regulated). There is a striking similarity between the transcriptional reprogramming occurring in lettuce in response to *B. cinerea* and *S. sclerotiorum*, with 4390 DEGs common to both. Furthermore, the changes in gene expression are overwhelmingly in the same direction (3040 DEGs upregulated and 1322 DEGs downregulated in response to both *B. cinerea* and *S. sclerotiorum*, with only 28 genes showing an opposite change in expression). This reveals a core set of 4362 DEGs which have the same direction of differential expression in response to both pathogens (Figure 3.5B).

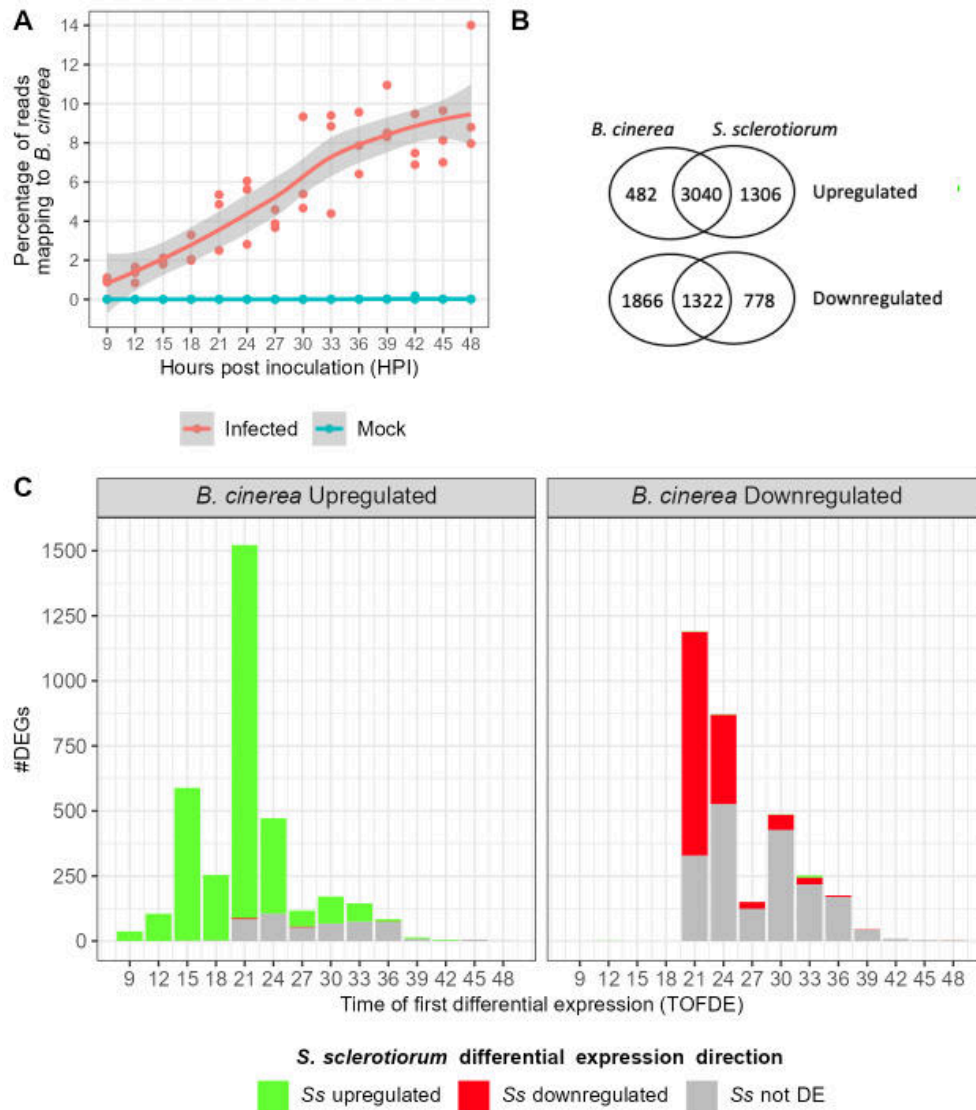


Figure 3.5: *B. cinerea* infection leads to large-scale transcriptional reprogramming in lettuce. **(A)** The percentage of RNAseq reads mapping to the *B. cinerea* transcriptome compared to the total number of mapped reads in each times series sample. Individual data points are shown along with a smoothed regression line and 95% confidence interval, in grey. As expected, there are no or extremely low numbers of reads mapping to *B. cinerea* in the mock-inoculated samples. The proportion of reads in each sample mapping to the *B. cinerea* transcriptome increased over time after inoculation, an indication of pathogen growth during the infection. Red indicates infected samples and blue, mock. **(B)** Up- and down-regulated lettuce genes following inoculation with *B. cinerea* (this study) and *S. sclerotiorum* (Ransom et al. 2023). 4362 genes are differentially expressed after inoculation with both pathogens with the same direction of expression change. **(C)** Timing of First Differential expression (TOFDE) of lettuce DEGs during *B. cinerea* infection, separated into upregulated (left panel) or downregulated (right) genes. The number of DEGs with a TOFDE at each time point is indicated. Colouring indicates whether the DEGs are upregulated, downregulated or not differentially expressed (not DEG) in response to *S. sclerotiorum* infection (Ransom et al. 2023).

To determine critical periods for transcriptional reprogramming in response to these generalist necrotrophic pathogens, we identified the time of first differential expression (TOFDE) for all lettuce DEGs during *B. cinerea* infection. The early phase of transcriptional reprogramming (9-18 hpi) is dominated by upregulation of DEGs (Figure 3.5C) with 99.6% (982/986) of these early DEGs are also upregulated in response to *S. sclerotiorum*, indicating an early conserved defence response. The vast proportion of DEGs after *B. cinerea* inoculation

are first differentially expressed at 21 and 24 hpi (60%: 4055/6713). The late phase of transcriptional reprogramming (27-48 hpi) consists of more down-regulated DEGs than upregulated and has the least overlap with genes differentially expressed during *S. sclerotiorum* infection (Figure 3.5C). At least part of the reduced overlap for DEGs with later TOFDE is likely due to the slower progress of infection by *S. sclerotiorum* compared to *B. cinerea*, with the lettuce-*B. cinerea* downregulated genes typically having later TOFDE and the lettuce-*S. sclerotiorum* time series capturing less of the later response.

3.3.2 Conserved and species-specific defence responses in lettuce and Arabidopsis

The Arabidopsis-*B. cinerea* pathosystem has been extensively characterised over the last 20 years and is now well-understood. Although lettuce (Asterid) and Arabidopsis (Rosid) are distant species (diverging approx. 125 million years ago, Zeng et al. 2017), we expect there to be similarities in defence strategies against both *B. cinerea* and *S. sclerotiorum*, given the broad host range of these pathogens. Time-series transcriptome profiling of the Arabidopsis defence response against *B. cinerea* was previously carried out using microarrays capturing gene expression from 2 to 48 hpi Windram et al. 2012. There is overlap between the DEGs in lettuce and Arabidopsis after *B. cinerea* inoculation (Fig 3.6) with approx. 30% and 43% respectively of up- and down-regulated lettuce DEGs with their Arabidopsis orthologue differentially expressed in the same direction. However, a significant proportion of DEGs in lettuce are orthologous to Arabidopsis DEGs with an opposite direction of expression change, or which do not change in expression during *B. cinerea* infection of Arabidopsis. This suggests that there are both conserved and species-specific aspects to the defence response.

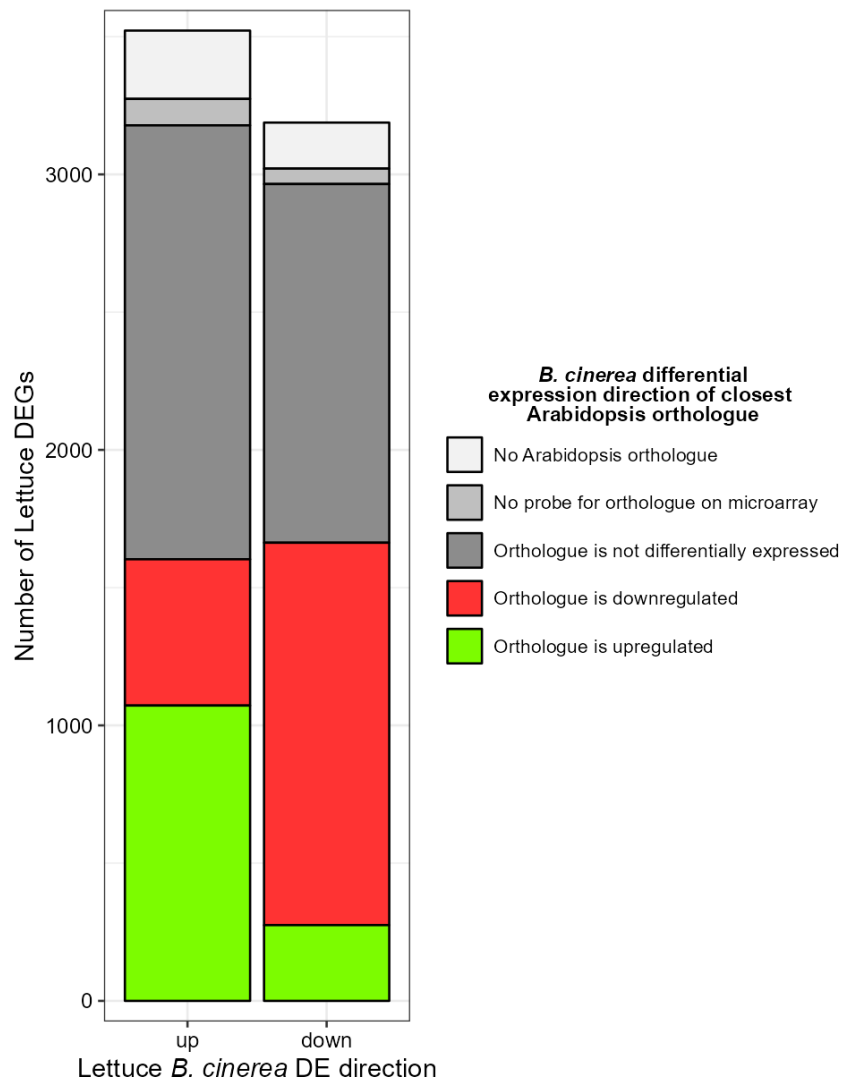


Figure 3.6: Species specificity of transcriptional reprogramming during *B. cinerea* infection. Bar chart showing the number of lettuce genes up and downregulated after inoculation with *B. cinerea*. Bars are coloured by the direction of differential expression following *B. cinerea* inoculation of the single closest Arabidopsis orthologue for each lettuce DEG (Windram et al. 2012). Orthologues were as described in Reyes-Chin-Wo et al. 2017.

Many lettuce orthologues of well-characterised Arabidopsis regulators with a known role in defence against *B. cinerea* were identified as DEGs in both time-series data sets (Fig 3.7), including genes involved in JA and ET signalling (*LsERF1*, *LsMYC2*, *LsWRKY33*), JA and ET biosynthesis (Allene oxide synthase, *LsAOS*; lipoxygenase 1, *LsLOX1*), SA signalling (*LsWRKY54*, *LsWRKY70*, Enhanced disease susceptibility 1, *LsEDS1*) and SA biosynthesis (*LsICS2*). *WRKY54*, a known SA regulator, has two lettuce orthologues differentially expressed, one of which is upregulated, the other downregulated. The expression profiles of these genes also illustrate the slower progression of infection by *S. sclerotiorum* with changes in gene expression delayed compared to the *B. cinerea* time series.

A striking species difference is expression of plant defensins (PDFs), some of which are key marker genes of the JA-activated defence pathway in Arabidopsis Brown et al. 2003; Manners et al. 1998. *AtPDF1.2* shows

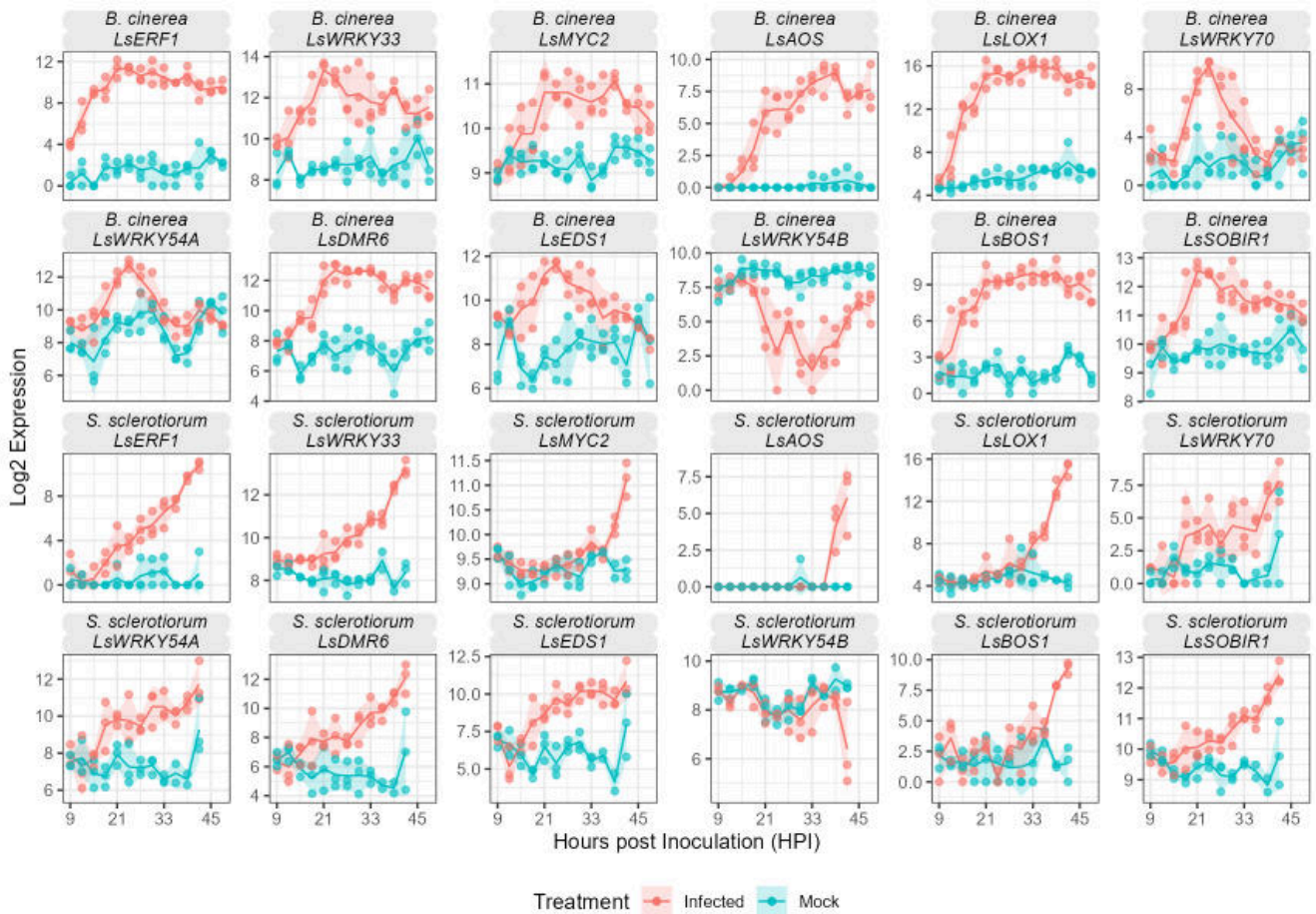


Figure 3.7: Lettuce orthologues of known Arabidopsis defence regulators are differentially expressed during both *B. cinerea* and *S. sclerotiorum* infection. Individual data points (log2 expression) are shown along with the mean and 95% confidence interval. Red indicates pathogen inoculated samples, blue are mock inoculated. Lsat_1_v5_gn_3.121961 (LsERF1), Lsat_1_v5_gn_8.103681 (LsWRKY33), Lsat_1_v5_gn_6.65141 (LsMYC2), Lsat_1_v5_gn_3.26481 (LsAOS), Lsat_1_v5_gn_9.123300 (LsLOX1), Lsat_1_v5_gn_9.38680 (LsWRKY70), Lsat_1_v5_gn_2.126880 (LsWRKY54A), Lsat_1_v5_gn_0.17841 (LsDMR6), Lsat_1_v5_gn_5.18140 (LsEDS1), Lsat_1_v5_gn_2.126920 (LsWRKY54B), Lsat_1_v5_gn_6.70301 (LsBOS1) and Lsat_1_v5_gn_1.28600 (LsSOBIR1)

dramatic upregulation in response to *B. cinerea* and *Alternaria brassicicola* infection, which is abolished in both *coi1-1* mutants and *ORA59* RNAi lines Pré et al. 2008. *AtPDF1.1* and *AtPDF1.3* also show upregulation in response to *B. cinerea* Ingle et al. 2015. We identified 13 putative PDFs in lettuce (*LsPDFs*), which contain the gamma-thionin domain (Pfam PF00304) and were shorter than 150 amino acids in length. Phylogenetic analysis of these with Arabidopsis defensins (*AtPDFs*), and characterised plant defensins with anti-fungal activity from other species Lacerda et al. 2014 (Fig 3.8a) indicated the similarity of the putative lettuce defensins to these proteins, particularly to AtPDF families 1 and 2. However, only 8 *LsPDF* genes had detectable expression in leaves and none were upregulated after *B. cinerea* infection (Fig 3.8b). Five predicted *LsPDFs* have very low levels of expression in our samples, two show constitutively high levels of expression, and *Lsat_1_v5_gn_5_70941* is downregulated after *B. cinerea* infection. It is possible that there is sufficient anti-fungal activity from the defensin genes with high levels of expression or that the pathogen may be preventing the upregulation of *LsPDFs*, or potentially driving reduction of *Lsat_1_v5_gn_5_70941* mRNA through effector molecules introduced into the plant.

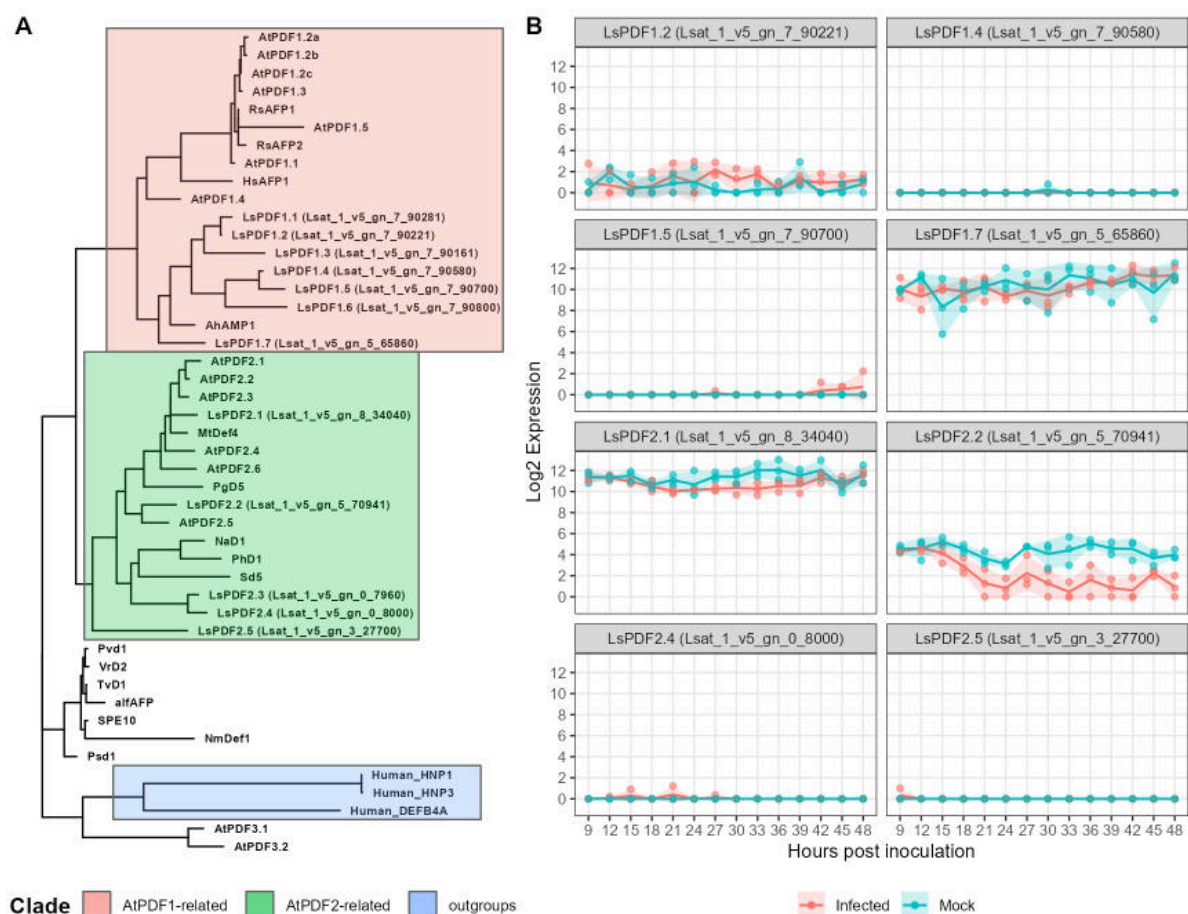


Figure 3.8: Phylogeny and expression profile during *B. cinerea* infection of lettuce defensins. **A)** A 750 bootstrap maximum likelihood phylogenetic tree of 13 putative lettuce defensins, Arabidopsis Plant Defensins (AtPDFs) and characterised anti fungal defensins from other species (Lacerda et al. 2014). **B)** *B. cinerea* and mock inoculated time series expression profiles of putative LsPDFs. Only 8 of the 13 LsPDFs were detected in at least 1 sample, only 4 of which were consistently detected across the time series. Only 1 was differentially expressed, *Lsat_1_v5_gn_5_70941*, which was downregulated.

3.3.3 Co-expression modules highlight specific biological functions and processes during infection

We used the Wigwams (Polanski et al. 2014) algorithm to identify non-redundant modules of lettuce genes which are co-expressed following *B. cinerea* and *S. sclerotiorum* infection. Wigwams does not force every gene into a module, unlike typical clustering algorithms, and evaluates each putative module for statistical significance in an attempt to identify co-expression due to true co-regulation, rather than simply because of the frequency of a particular expression profile. 3129 (72%) of the common 4362 DEGs were grouped into 20 co-expressed modules, with a median size of 90 genes (Fig 3.9). Two of the modules are very large (module 1 and 3), containing 942 and 527 genes respectively.

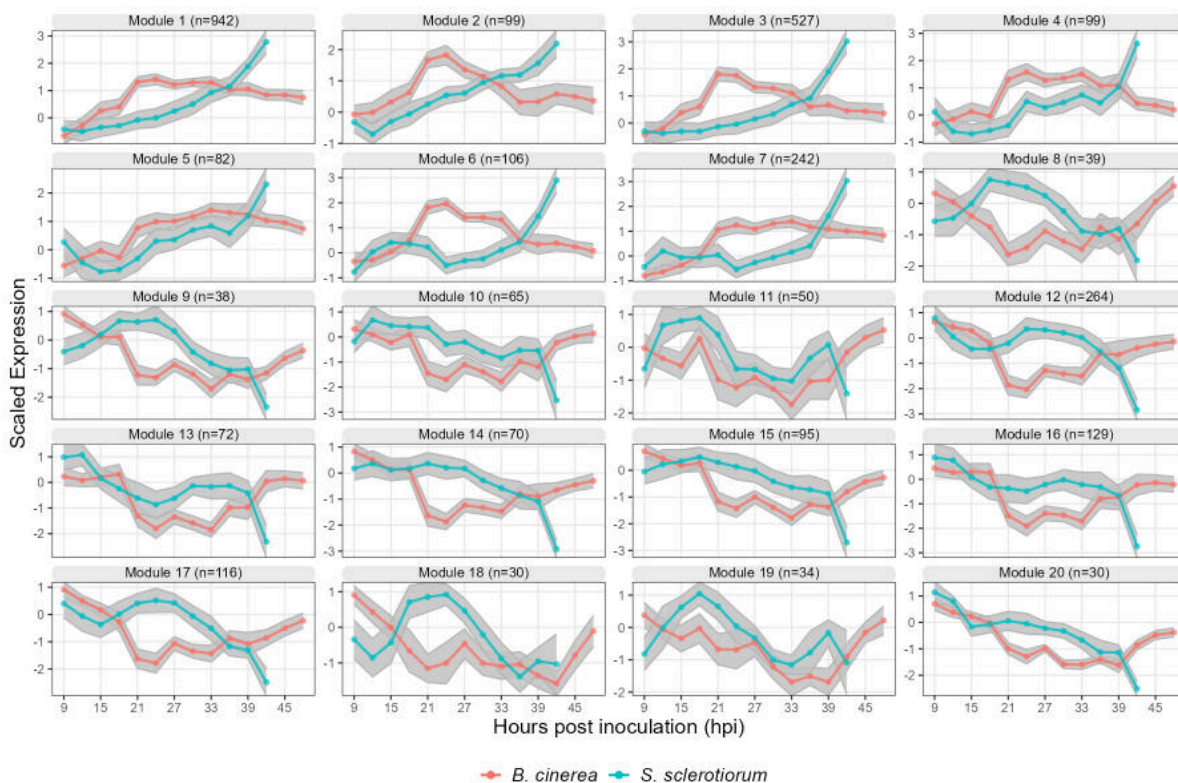


Figure 3.9: Modules of lettuce DEGs co-expressed following both *B. cinerea* (red) and *S. sclerotiorum* (blue) infection. 3129 of the 4362 lettuce genes were differentially expressed in both the lettuce-*B. cinerea* and lettuce-*S. sclerotiorum* time-series are included in a module, with the mean scaled log₂ expression profile (solid line) and 95% confidence interval (grey area) of the genes in each module shown. N represents the number of genes within a module. Modules 1 – 7 contain upregulated DEGs, and modules 8 to 20 contain downregulated DEGs (compared to mock-inoculated control in each time series).

It is clear from the expression profiles of these modules, particularly modules 1 to 7, that the lettuce response to *B. cinerea* infection is faster than that to *S. sclerotiorum*, with a significant transcriptional shift by 21 hpi for *B. cinerea* and only happening by the end of the time series (42 hpi) for *S. sclerotiorum*. In the *B. cinerea* time series, several modules show transient upregulation of mRNA levels (e.g. modules 2 and 6) while others (e.g. modules 1 and 7) have increased expression levels that last throughout the time series. However, expression

of all the downregulated modules during *B. cinerea* infection starts to recover either immediately after 21 hpi (e.g. modules 12 and 14) or from 39 hpi (e.g. modules 9, 15 and 19). Many of these modules show expression profiles still decreasing in the *S. sclerotiorum* time series.

We tested whether these modules were enriched for specific biological functions using gene ontology (GO), this enrichment was performed using annotations of the Arabidopsis orthologues of lettuce genes within each module. Arabidopsis orthologues of all the lettuce genes detected in the mock or *B. cinerea*-inoculated time series were used as the background set. 13 modules were significantly enriched for genes with a particular GO term. Additionally, we performed protein domain enrichment, using Pfam and Panther annotations, again comparing against a background set of all genes with detectable expression in either time-series.

General defence-related GO terms were enriched across multiple modules, with “response to fungus”, “secondary metabolic processes” and “response to bacterium” being significantly over-represented in 3 modules each (Figure 3.10). This was expected, as general responses like these are unlikely to be limited to a single group of genes. However, we also identified GO terms enriched in a single module, suggesting that these modules contain distinct biologically relevant groups of genes. Module-specific functions include response to ethylene (module 1), cell death (module 3), unfolded protein binding (module 6), response to UV (module 10), pigment biosynthetic process (module 13) and chloroplast relocation (module 16).

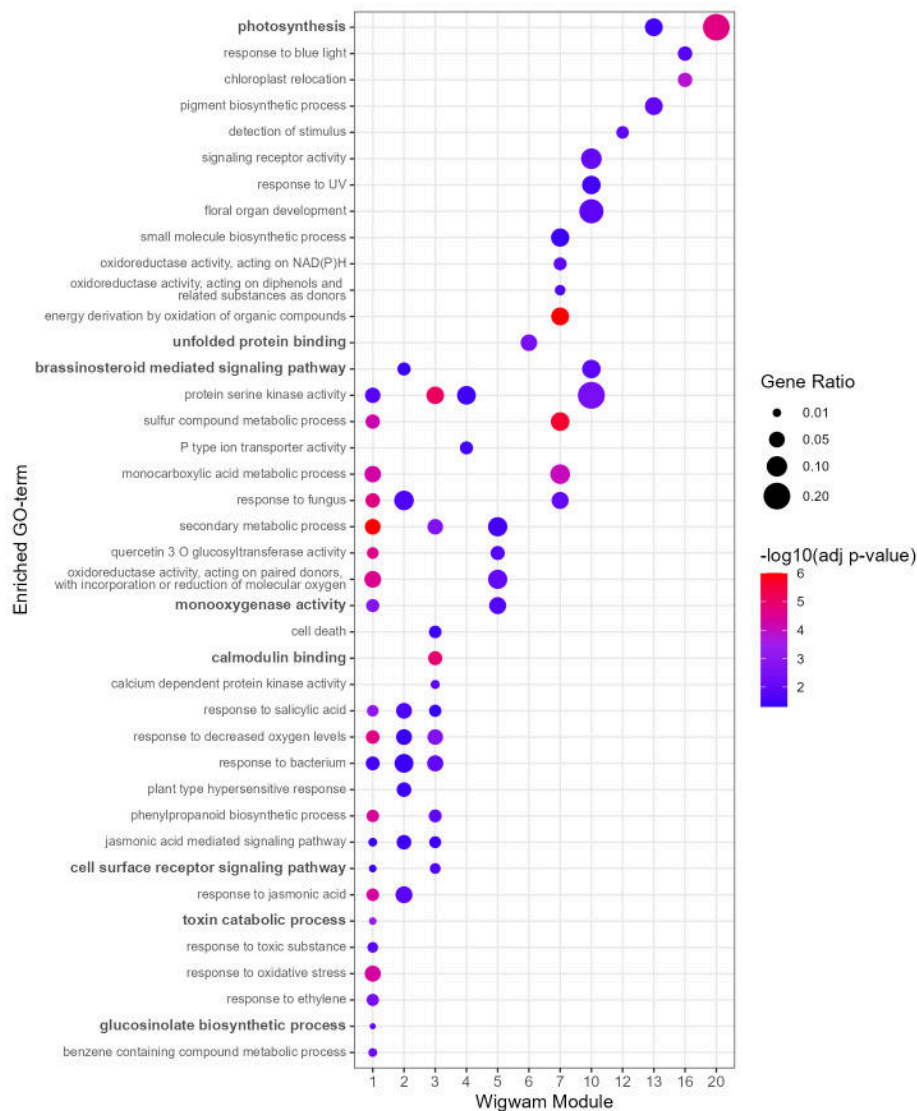


Figure 3.10: Modules of co-expressed lettuce genes are enriched for genes involved in different biological processes. Selected gene ontology (GO) terms significantly enriched in modules of lettuce genes co-expressed in response to *B. cinerea* and *S. sclerotiorum* are indicated, with colour indicating the statistical significance of the enrichment, and the size of the circle indicating the scale of enrichment for that term. Enrichment analysis was carried out using annotations of the Arabidopsis orthologue of each lettuce gene (where available) against a background of Arabidopsis orthologues of all lettuce genes detected in the mock- or *B. cinerea*-inoculated time series.

Module 1 is a large group of 942 genes, accounting for 21.6% of all common *B. cinerea* / *S. sclerotiorum* DEGs and 30.0% of all DEGs assigned to a module, demonstrating that despite the integration of two high-density time series datasets, large proportions of the transcriptome appear to change within a very short time-frame. Enrichment for biological functions associated with JA and ET signalling (known regulators of defence against *B. cinerea* in Arabidopsis) was evident. The GO terms “response to jasmonic acid”, “jasmonic acid biosynthetic process”, “response to ethylene” and “ethylene-activated signalling pathway” as well as AP2/ERF protein domains all show their highest levels of overrepresentation in module 1 (Fig 3.10). Lettuce orthologues of many key Arabidopsis JA/ET biosynthetic and/or response genes are identified in this module: 17 ERF domain TFs (including ERF1, three ERF13 orthologues and three ERF-1 orthologues),

WRKY33, MYC2, LOX2 and EFE (ethylene forming enzyme). Downstream genes responding to JA/ET signalling have not yet been characterised in lettuce, however the large group of genes that are co-expressed with known JA/ET regulators and biosynthetic enzymes in module 1 may represent such genes in lettuce. The biosynthesis of key Arabidopsis phytoalexins such as camalexin and glucosinolates is JA/ET regulated (Hickman et al. 2017; Zhou et al. 2022) and promotes resistance to *B. cinerea* (Ferrari et al. 2003; Denby et al. 2004; Kliebenstein et al. 2005) and *S. sclerotiorum* (Stotz et al. 2011; Zhang et al. 2015c). Module 1 shows enrichment of cytochrome P450 protein domains as well as “glucosinolate biosynthetic process”, “isoprenoid metabolic process” and “secondary metabolic process” GO terms. Module 1 also contains three orthologues of PDR12, a transporter responsible for secreting camalexin in Arabidopsis (He et al. 2019). While camalexin is a phytoalexin specific to Arabidopsis, these transporters may be secreting other anti-fungal compounds in lettuce.

Lettuce is known to synthesise a diverse range of sesquiterpene lactones (STLs), including sulfate, oxalate and amino acid conjugates (Yang et al., 2022) with one compound, lettucenin A, shown to have anti-fungal activity against *B. cinerea in vitro* (Bennett et al. 1994; Sessa et al. 2000). Germacrene A synthase (Bennett et al. 2002; Kwon et al. 2022), germacrene A oxidase (Nguyen et al. 2010) and costunolide synthase (Ikezawa et al. 2011) catalyse the production of costunolide, a key precursor of STLs. Genes encoding all three of these enzymes are in module 1 along with 37 additional cytochrome P450 encoding genes. Downstream of costunolide, there is significant diversity in STL structures, with the biosynthetic pathways unknown. Hence these uncharacterised P450s, co-expressed with known STL biosynthetic genes, are good candidates for roles in the synthesis of STLs in lettuce. The presence of STL biosynthetic genes in module 1, may further suggest STL biosynthesis in lettuce is JA/ET regulated.

In addition to the production of anti-microbial compounds, defence against *B. cinerea* is likely to require detoxification of pathogen toxins, including botrydial and botcinic acid (Zhang et al. 2019). The GO terms “Toxin catabolic process” along with Glutathione S-transferase (GST) and Aflatoxin B1 aldehyde reductase (AFAR) protein domains are overrepresented in module 1 genes. Aflatoxin B1 a mycotoxin produced by the saprophytic fungus *Aspergillus flavus* during infection of maize and peanut, is detoxified by AFAR enzymes (Judah et al. 1993; Klich 2007). Although *B. cinerea* and *S. sclerotiorum* do not produce aflatoxin, the lettuce AFAR-like enzymes may have roles in detoxification of other pathogen-derived metabolites, with their presence in module 1 suggesting potential JA/ET regulation.

Module 3 (527 genes) is enriched for genes involved in pathogen perception, annotated with GO term “cell surface receptor signalling pathway”, as well as being the only module enriched for “cell death”. Orthologues of chitin-binding pathogen recognition receptors (PRRs) are present in module 3 such as lettuce orthologues of chitin-elicited receptor kinase 1 (CERK1) and lysin motif (LysM) receptor kinase 4 (LYK4), both of which

have been shown to play a role in resistance to *B. cinerea* in Arabidopsis (Liu et al. 2018; Cao et al. 2014). A lettuce orthologue of SOBIR1 is also present in this module, along with orthologues of a receptor-like protein (RLP) and SERK4/BKK1 (BAK1-LIKE 1). SOBIR1 is a PRR known to promote *B. cinerea* and *S. sclerotiorum* resistance via recognition of elicitor peptides in coreceptor complexes with BAK1 and RLPs (Zhang et al. 2013; Albert et al. 2015; Albert et al. 2019; Ono et al. 2020), with SERK4/BKK1 (BAK1-LIKE 1) having functional redundancy with BAK1 (He et al. 2007; Schoonbeek et al. 2022). The presence of lettuce orthologues for these known pathogen recognition complexes in a single module suggests co-regulation of these genes in response to initial pathogen perception. Interestingly, a lettuce orthologue of BIR1 is also present in module 3. BIR1 negatively regulates the SOBIR1-BAK1 interaction (Liu et al. 2016; Ma et al. 2017) indicating coordinated regulation of mechanisms to dampen plant defence responses, potentially balancing effective defence with the physiological impact on the host plant. In the same co-expression module, we see overrepresentation of EF-hand protein domain and the presence of 5 genes encoding calcium-dependent protein kinases (CPKs), suggesting an important role for calcium signalling. Arabidopsis CPK mutants, *cpk1-1* and *cpk5/6/11*, show hyper-susceptibility to *B. cinerea*, with *cpk5/6/11* also showing a reduced response to oligogalacturonide DAMPs (Coca and San Segundo 2010; Gravino et al. 2015).

Module 6 is enriched for the GO term “unfolded protein response” (UPR), a response triggered by the accumulation of misfolded proteins in the endoplasmic reticulum, inducing chaperone expression to maintain correct protein folding (Bao and Howell 2017). UPR has been shown to promote resistance to *Alternaria alternata*, a necrotrophic fungus, in *Nicotiana attenuata* (Xu et al. 2019). Lettuce orthologues of key chaperone proteins including ERdj3B, CNX1 and HSP89.1 (Liu et al. 2017) are present in module 6.

Amongst the downregulated clusters, multiple photosynthesis and growth-related GO terms are significantly enriched, indicating a switch from growth to defence during necrotrophic pathogen infection. This has been seen in many plant defence responses to pathogens including during *B. cinerea* infection of Arabidopsis (Windram et al. 2012) and includes genes involved in chlorophyll synthesis (module 13), chloroplast localization (module 16) and photosynthesis reactions (module 20). Module 10 was significantly enriched for the GO term “brassinosteroid (BR) mediated signalling pathway”, containing orthologues of the BR receptor, BR insensitive 1 (BRI1) and a downstream signalling kinase, BR signalling kinase 2 (BSK2) (Tang et al. 2008). BRI1 is also a co-receptor of BAK1, and BR signalling inhibits BAK1-mediated immune signalling (Albrecht et al. 2012; Belkhadir et al. 2012), however, several BSKs have now been shown to interact with PRRs and promote *B. cinerea* resistance (Majhi et al. 2019; Majhi et al. 2021).

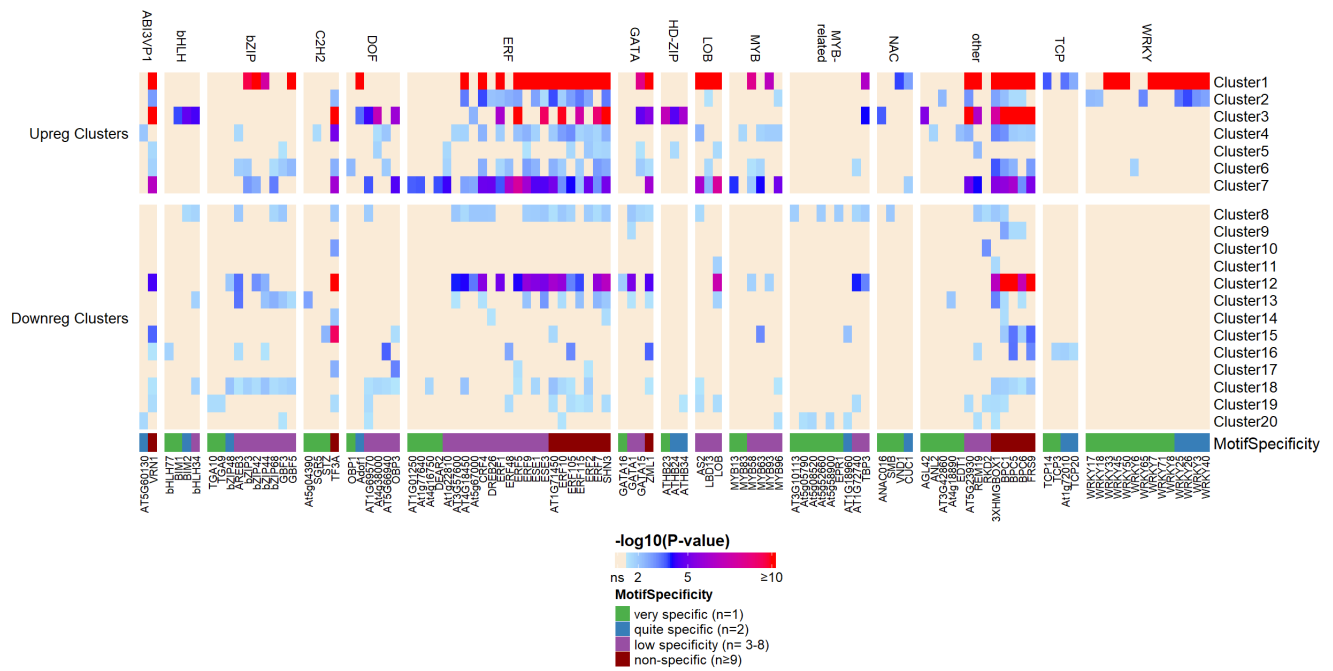


Figure 3.11: Enrichment of Arabidopsis DAP-seq DNA binding motifs in 1 Kb promoters of lettuce DEGs. Columns indicate individual motifs, which are named according to the binding TF and grouped by their respective TF family. This heatmap shows 109/265 enriched motifs. We selected motifs which were either in the top 6 enriched motifs in a module, the top 4 enriched motifs which are unique to a single module or in the top 3 enriched motifs for an individual TF family. Rows represent the time series modules. Colour indicates the significance of the enrichment ($\log_{10}$ transformation of the adjusted p-value) for a specific motif in a specific module, with white = non-significant (ns) enrichment. Significant enrichment is defined as $p_{adjust} < 0.05$ and $enrichment_ratio > 2$. “Motif Specificity” is indicated in four classes, corresponding to the number of modules a motif is enriched in.

3.3.4 Conserved transcription factor DNA-binding motifs in gene modules

We would expect genes within a module with statistically significant co-expression to be coregulated. We therefore, tested for enrichment of known DNA-binding motifs in the promoters of the lettuce DEGs in each module. DNA sequence 1 Kb upstream from the transcriptional start site of all module genes was used as the putative promoter regions, and Arabidopsis DNA affinity purification sequencing (DAP-seq) data (O’Malley et al. 2016b) used as the set of DNA binding motifs. Although this dataset characterises DNA binding motifs in Arabidopsis, not lettuce, we expect DNA-binding motifs to be conserved across species, and there is limited research on lettuce-specific DNA binding motifs.

265 unique DAP-seq motifs were significantly enriched in the promoters of at least 1 module, using shuffled promoter sequences as the comparator (adjusted $p < 0.05$ and enrichment ratio > 2). Every module had DAP-seq DNA binding motifs significantly enriched in its gene promoters. Fig 3.11 shows a subset of the enriched motifs: the 6 most significantly enriched motifs for each module, the top 4 significantly enriched motifs which are unique to a module and the top 3 enriched motifs corresponding to an individual TF family. This includes 109 of the 265 significantly enriched DAP-seq motifs. Fig 3.11 clearly shows that the ERF TFs are a dominant TF family, with many ERF DNA-binding motifs significantly enriched across both upregulated

and downregulated modules. 17/20 modules were enriched for at least 1 ERF binding motif. In contrast, WRKY DNA-binding motifs showed enrichment in either a single or two modules (mostly in modules 1 and 2). As seen in Fig 3.10, module 1 is enriched for DEGs involved in phytohormone responses (JA, SA and ET) and here we see that it is also enriched in DNA-binding motifs of TFs known to mediate these responses. The DNA-binding motif of ERF1, a key regulator of the ET/JA response, is enriched in module 1 (271 input/94 shuffled). The DNA binding motif of WRKY33, another key JA regulator, is specifically enriched in module 1 (215 input/95 shuffled) as is the WRKY50 motif (140 input/ 53 shuffled). WRKY50 is known to bind the PR1 promoter, activating SA-responsive gene expression in an NPR1-independent manner (Hussain et al. 2018; Johnson et al. 2003). Lettuce orthologues of ERF1 and WRKY33 were also identified within module 1. This data indicates that we are able to identify DNA-binding motif elements which are highly conserved from Arabidopsis to lettuce and are likely facilitating differential gene expression in response to necrotrophic fungal infection.

3.3.5 A causal gene regulatory network predicts key transcriptional regulators of the lettuce response to *B. cinerea* and *S. sclerotiorum* infection

Co-expressed modules and promoter analysis above can predict regulatory interactions for experimental testing, however, the accuracy and confidence of regulatory predictions can be strengthened by the inclusion of additional data sets, and using network inference rather than single module approaches. To this end, we constructed a gene regulatory network (GRN) using four independent datasets: the time series data from lettuce inoculated with *B. cinerea*, the time series data from lettuce inoculated with *S. sclerotiorum* (Ransom et al. 2023) and single time point expression data from 21 diverse lettuce accessions following *B. cinerea* and *S. sclerotiorum* inoculation (Pink et al. 2022). We used the random forest OutPredict algorithm (Cirrone et al. 2020) to construct the GRN with the 4362 DEGs common to the *B. cinerea* and *S. sclerotiorum* infection time series as the input genes. This includes 251 genes which were designated as TFs and hence are potential regulators of all other input genes. OutPredict tests potential regulator (in this case TF) expression profiles as predictors of the expression of all other input genes and outputs the likelihood of each TF influencing expression of each gene across all datasets.

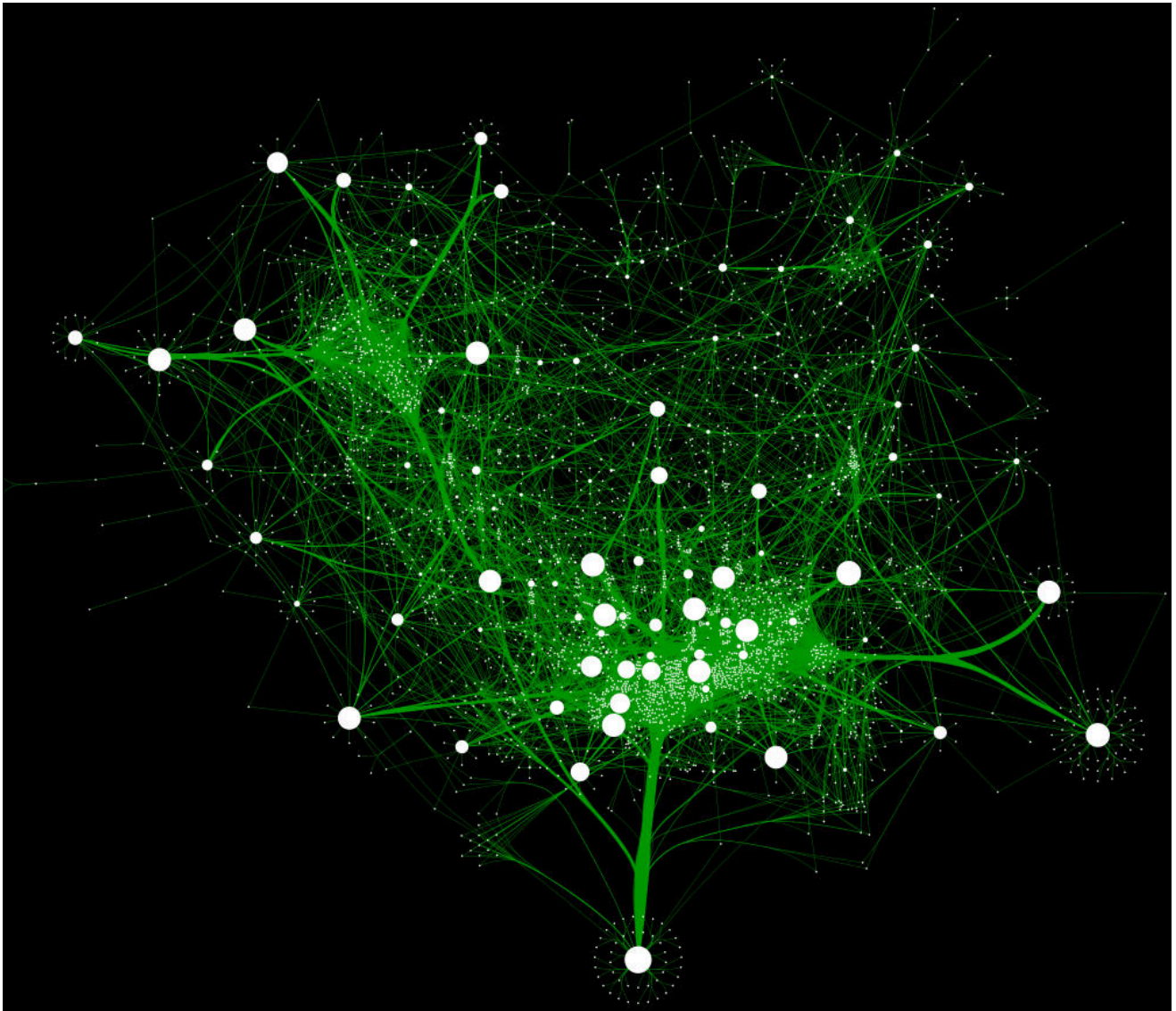


Figure 3.12: Inferred Lettuce-necrotroph gene regulatory network constructed from top 1% of high confidence TF-gene regulatory interactions. The network contains 3382 nodes (genes), 10947 regulatory edges and including 226 regulator nodes which have outdegrees (transcription factors). The size of nodes are scaled based on the number of outdegrees, providing a visual representation of the extent of regulatory influence each transcription factor possesses within the network.

The final GRN was constructed using the top 1% highest confidence TF-gene interactions and consisted of 3,382 genes (including 226 TFs) and 10,947 regulatory edges. Figure 3.12 shows the inferred lettuce-necrotroph GRN has a scale-free topology, which is common in biological networks, showing a small number of nodes "Hub genes" with a large influence on the network. The majority of TFs in the final network have a small influence on the expression of other genes in the network with 99 TFs (44%) having < 10 predicted targets and 159 TFs (70%) < 40 predicted targets. However, about a third of the TFs (hub genes) are predicted to have a very large influence on transcriptional reprogramming in response to necrotrophic fungal infection; 33 TFs (15%) have between 40 and 100 predicted targets and 34 TFs (15%) have ≥ 100 predicted targets (Figure 3.13, Table 3.1).

Table 3.1: List of hub genes (≥ 40 Outdegrees) in the lettuce-necrotroph gene regulatory network.

Hub	Outdegrees	Arabidopsis Ortholog	Symbol
Lsat_1.v5.gn.9.111840	840	salt tolerance zinc finger	STZ
Lsat_1.v5.gn.4.63220	475	SCARECROW-like 13	SCL13
Lsat_1.v5.gn.3.120520	378	myb domain protein 15	MYB15
Lsat_1.v5.gn.8.103681	348	WRKY DNA-binding protein 33	WRKY33
Lsat_1.v5.gn.3.99620	276	myb domain protein 16	MYB16
Lsat_1.v5.gn.3.90920	268	heat shock factor 4	HSF4
Lsat_1.v5.gn.7.6501	264	B-box type zinc finger protein with CCT domain	BBX15
Lsat_1.v5.gn.4.164440	257	WRKY DNA-binding protein 7	WRKY7
Lsat_1.v5.gn.3.121961	256	ethylene response factor 1	ERF1
Lsat_1.v5.gn.4.46601	250	ethylene-responsive element binding factor 13	ERF13
Lsat_1.v5.gn.4.61461	217	WRKY family transcription factor	WRKY6
Lsat_1.v5.gn.8.16081	211	C2H2-type zinc finger family protein	AT2G28710
Lsat_1.v5.gn.2.126380	208	WRKY DNA-binding protein 55	WRKY55
Lsat_1.v5.gn.6.92840	208	ethylene responsive element binding factor 1	ERF-1
Lsat_1.v5.gn.3.78100	198	GRAS family transcription factor	AT5G66770
Lsat_1.v5.gn.5.182281	197	ethylene response factor 98	ERF98
Lsat_1.v5.gn.7.85360	197	salt tolerance zinc finger	STZ
Lsat_1.v5.gn.2.103381	185	NAC domain containing protein 53	NAC053
Lsat_1.v5.gn.5.44521	183	homeobox protein 6	HB6
Lsat_1.v5.gn.4.1201	175	WRKY DNA-binding protein 75	WRKY75
Lsat_1.v5.gn.7.36701	163	bHLH DNA-binding family protein	AT5G56960
Lsat_1.v5.gn.5.48440	162	CAM binding transcription factor	CAMTA3
Lsat_1.v5.gn.5.164460	153	homeobox from Arabidopsis thaliana	HAT14
Lsat_1.v5.gn.9.14580	145	WRKY DNA-binding protein 33	WRKY33
Lsat_1.v5.gn.5.157880	131	bZIP transcription factor family protein	BZIP17
Lsat_1.v5.gn.4.176860	129	NAC domain transcriptional regulator superfamily protein	ATAF1
Lsat_1.v5.gn.4.64660	124	TCP family transcription factor	AT5G23280
Lsat_1.v5.gn.6.58420	121	bHLH DNA-binding superfamily protein	AT5G48560
Lsat_1.v5.gn.5.134441	120	Homeobox-leucine zipper family protein	ATHB-15
Lsat_1.v5.gn.9.28700	118	zinc finger (C2H2 type, AN1-like) family protein	AT2G41835
Lsat_1.v5.gn.3.31320	108	WRKY DNA-binding protein 75	WRKY75
Lsat_1.v5.gn.7.15520	107	WRKY DNA-binding protein 31	WRKY31
Lsat_1.v5.gn.2.69481	106	GRAS family transcription factor family protein	GAI
Lsat_1.v5.gn.9.8501	105	myb domain protein 14	MYB14
Lsat_1.v5.gn.3.31741	99	NAC domain containing protein 83	NAC083
Lsat_1.v5.gn.6.65141	98	bHLH DNA-binding family protein	MYC2
Lsat_1.v5.gn.6.92940	90	ethylene responsive element binding factor 1	ERF-1
Lsat_1.v5.gn.4.65921	86	GBF's pro-rich region-interacting factor 1	GPRI1
Lsat_1.v5.gn.9.19740	86	zinc finger (CCCH-type) family protein	AT5G58620
Lsat_1.v5.gn.2.3181	82	basic region/leucine zipper motif 60	BZIP60

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Table 3.1 – continued from previous page

Hub	Outdegrees	Arabidopsis Ortholog	Symbol
Lsat_1_v5_gn.4.51301	78	ethylene responsive element binding factor 2	ERF2
Lsat_1_v5_gn.4.165921	74	heat shock factor 4	HSF4
Lsat_1_v5_gn.7.35740	70	ethylene-responsive element binding factor 13	ERF13
Lsat_1_v5_gn.1.5340	65	ethylene response factor 110	ERF110
Lsat_1_v5_gn.6.56420	63	NAC domain transcriptional superfamily protein	ATAF2
Lsat_1_v5_gn.2.126880	62	WRKY DNA-binding protein 54	WRKY54
Lsat_1_v5_gn.8.147781	60	WRKY DNA-binding protein 75	WRKY75
Lsat_1_v5_gn.2.117620	59	C2H2-type zinc finger family protein	AT2G28710
Lsat_1_v5_gn.9.38680	59	WRKY DNA-binding protein 70	WRKY70
Lsat_1_v5_gn.3.51140	58	Duplicated homeodomain-like superfamily protein	FLP
Lsat_1_v5_gn.3.139241	57	WRKY DNA-binding protein 46	WRKY46
Lsat_1_v5_gn.6.107660	57	beta HLH protein 93	bHLH093
Lsat_1_v5_gn.1.46781	56	Integrase-type DNA-binding superfamily protein	TINY2
Lsat_1_v5_gn.9.41960	56	ethylene response factor 110	ERF110
Lsat_1_v5_gn.5.61301	54	Homeodomain-like superfamily protein	AT2G38250
Lsat_1_v5_gn.3.44100	53	GATA transcription factor 5	GATA5
Lsat_1_v5_gn.3.73641	50	nucleic acid binding;zinc ion binding;DNA binding	NERD
Lsat_1_v5_gn.4.65140	50	myb-like HTH transcriptional regulator family protein	AT2G01060
Lsat_1_v5_gn.6.70301	47	myb domain protein 108	MYB108
Lsat_1_v5_gn.7.60801	47	WRKY DNA-binding protein 50	WRKY50
Lsat_1_v5_gn.4.176301	46	BEL1-like homeodomain 2	BLH2
Lsat_1_v5_gn.5.175300	46	LOB domain-containing protein 15	LBD15
Lsat_1_v5_gn.8.48640	44	WRKY DNA-binding protein 3	WRKY3
Lsat_1_v5_gn.6.62960	42	GATA transcription factor 1	GATA1
Lsat_1_v5_gn.1.22221	41	WRKY DNA-binding protein 15	WRKY15
Lsat_1_v5_gn.1.27140	40	myb domain protein 98	MYB98
Lsat_1_v5_gn.3.59241	40	B-box type zinc finger protein with CCT domain	BBX15

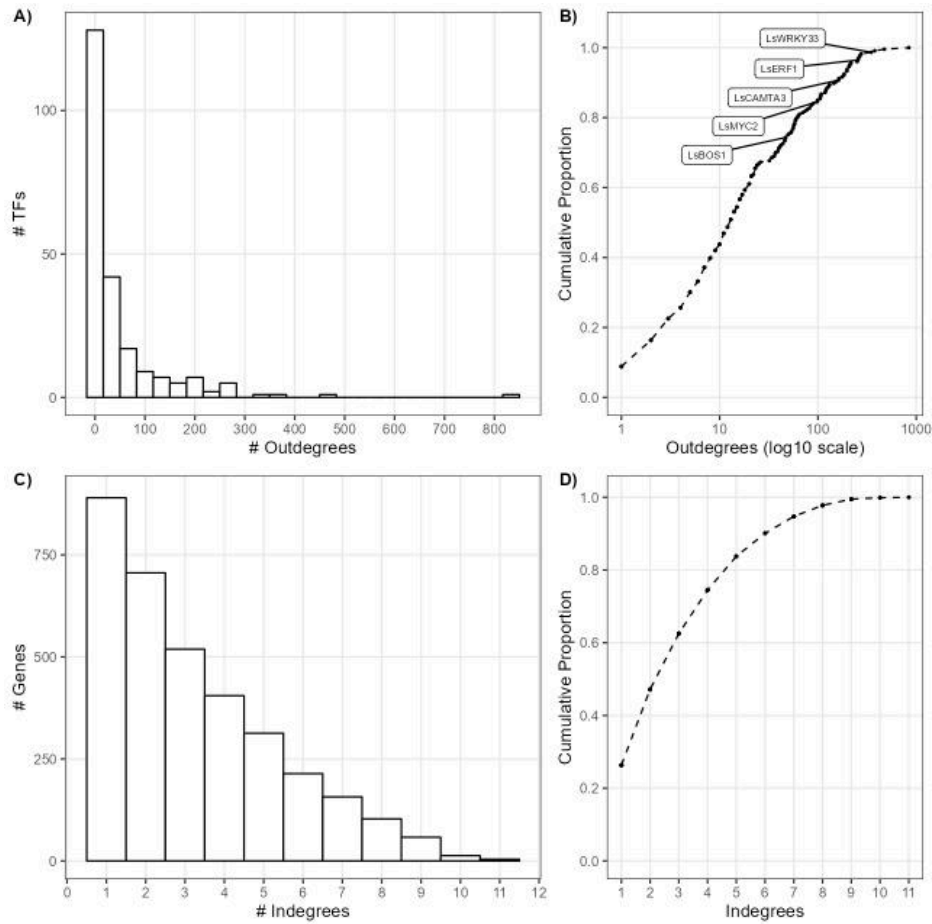


Figure 3.13: Characteristics of the gene regulatory network predicted to mediate the lettuce transcriptional response to *B. cinerea* and *S. sclerotiorum* infection. **A)** The distribution of transcription factor outdegree (i.e. number of downstream target genes); **B)** Empirical Cumulative Distribution Function (ECDF) of transcription factors outdegree, showing the proportion of transcription factors with $> X$ outdegrees. Known regulators mentioned in the text are highlighted; **C)** The distribution of indegrees for each target gene in the network (i.e. the number of transcription factors predicted to regulate a gene); **D)** ECDF of node indegree, showing the proportion of genes in the network with $> X$ indegrees.

The lettuce TFs predicted to have large numbers of downstream target genes include genes orthologous to *Arabidopsis* TFs known to impact defence against *B. cinerea*, such as WRKY33 (348 predicted targets)(Zheng et al. 2006), ERF1 (256 predicted targets)(Berrocal-Lobo et al. 2002), CAMTA3/SR1 (162 predicted targets)(Galon et al. 2008), MYC2 (98 predicted targets)(Lorenzo et al. 2004) and MYB108/BOS1 (48 predicted targets)(Mengiste et al. 2003; Cui et al. 2022)(Figure 3.13B). The prediction by the GRN of these known defence regulators having a significant impact on transcriptional reprogramming during pathogen infection, increases our confidence in the GRN to predict other (as yet unknown) regulators of the lettuce defence response against these two pathogens.

Analysis of the network node indegree distribution reveals that over a quarter of the genes in the network are predicted to be regulated by a single TF, however 2492 (74%) of the network genes are predicted to have multiple regulators (Figure 3.13C, D). Given this, we examined the extent to which TFs have shared

downstream target genes by calculating the pairwise Jaccard Index (a measure of overlap) between predicted targets of the 67 hub TFs (≥ 40 outdegrees). This highlights five groups (A-E) of hub TFs which share predicted target genes (Figure 3.14). Each group contains TFs that are differentially expressed in the same direction during infection, with the exception of a single TF in group B and four TFs in group C. Group A contains only four TFs, all WRKY TFs, three of which are in module 2, and with a large overlap in predicted target genes with each other and almost no overlap in target genes with other hub TFs. LsWRKY54 and LsWRKY70 have a pairwise Jaccard index of 0.55, the highest overlap of any hub pair. Both these TFs are orthologues of key SA regulators, WRKY70 and WRKY54 (Zhang et al. 2010; Li et al. 2004), suggesting the presence of a distinct network for SA signalling.

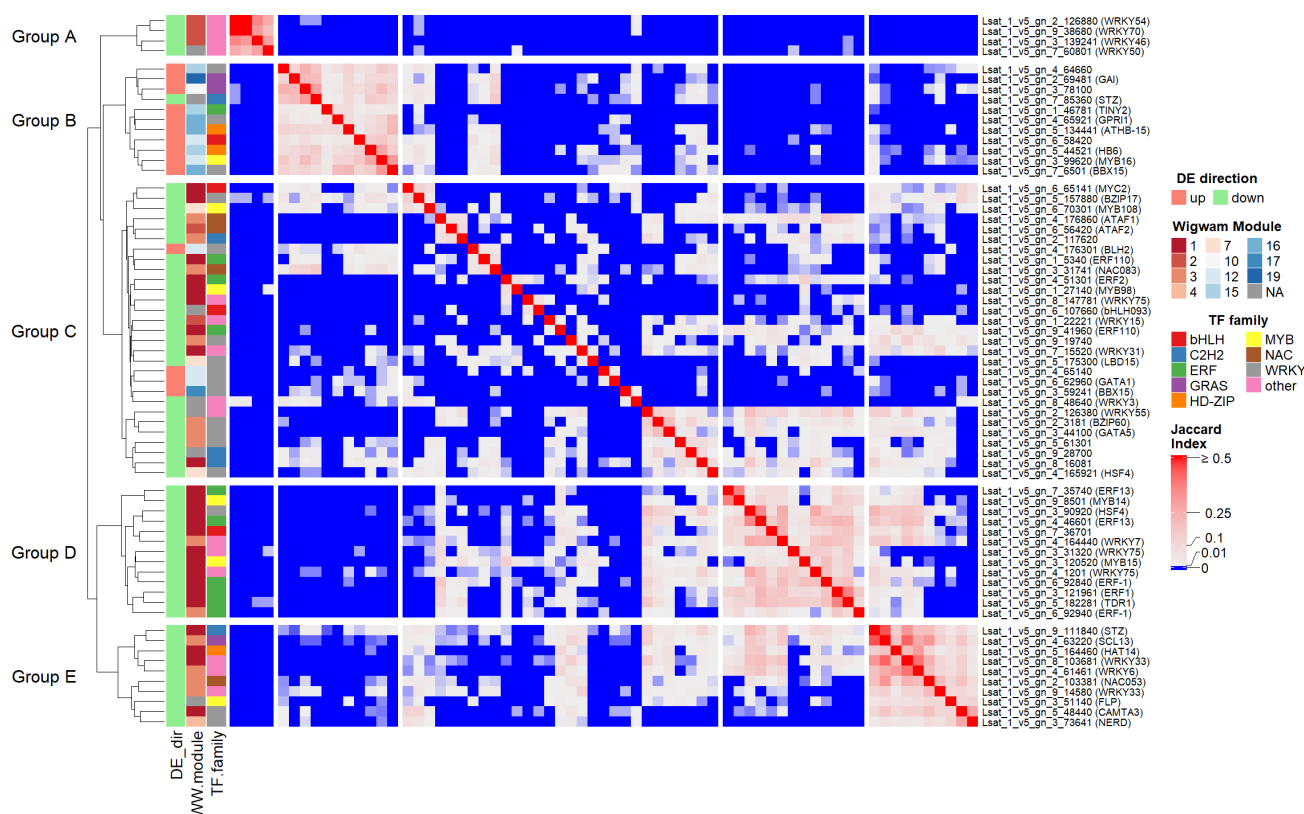


Figure 3.14: Pairwise similarity of predicted target genes of lettuce GRN TF hubs. The heatmap shows the pairwise Jaccard Index (proportion of overlap) of the predicted targets of all lettuce hub TFs (40 predicted targets). Rows and columns are clustered on Euclidian distance. Row and column annotations indicate the TF family and time-series module of each hub gene, as well as the direction of differential expression of the hub gene following infection by *B. cinerea* or *S. sclerotiorum*.

Apart from this group of WRKY TFs, the other groups of TFs with shared predicted target genes in the network contain TFs from different families. Group C is the largest but there is very little overlap between the target genes of each hub. Group D is dominated by TFs from module 1, with 6 ERF family TFs including orthologues of ERF1, ERF-1 and ERF13. This group also contains three WRKY TFs and two MYBs. Group E contains three additional WRKY TFs along with a NAC TF. This analysis therefore highlights the ability of the GRN to make distinct predictions for different members of the same TF family.

To assess the biological relevance of the specific predictions of target genes for a TF hub, we tested for GO term enrichment (using the Arabidopsis orthologues) in the genes uniquely predicted to be regulated by a TF hub, against a background of the genes expressed in the lettuce *B. cinerea* time series. 255 GO terms were significantly enriched in the unique targets of at least 1 hub, and 48 (out of all 226) lettuce TFs had at least 1 enriched GO term in their unique GRN predicted targets. “Defence response to fungus” was significantly enriched in the targets of 26 TFs. Enrichment of selected GO terms and the corresponding 39 lettuce hubs is shown in Figure 3.15.



Figure 3.15: GO-term enrichment in GRN-predicted targets of Lettuce TFs. We perform GO-term enrichment using Arabidopsis orthologs of the GRN-predicted target genes for each lettuce hub-gene. We have selected 29 key GO-terms for the heatmap, and shown all 39 lettuce hubs whose targets are significantly enriched ($p\text{-adjust} < 0.01$) for at least 1 of the selected GO-terms. Colour of the point represents the statistical significance of the enriched ($-\log_{10}$ transformed adjusted p-value), with red dots showing higher significance. Size of the point represents the “GeneRatio”, number of predicted targets whose closest Arabidopsis orthologue is attributed with the GO-term as a proportion of the total number of predicted targets.

We also see lettuce GRN hub targets being functionally enriched for GO terms that match the known function of Arabidopsis orthologues. For example, ERF1 is well-established as a key regulator of ethylene and jasmonate signalling in response to necrotrophic fungi (Berrocal-Lobo et al. 2002). The lettuce orthologue of ERF1, Lsat_1_v5_gn_3_121961 is a large network hub, and its target genes are significantly enriched for “response to jasmonic acid” and “response to ethylene” GO terms. WRKY70 is a key activator of the SA

defence response (Zhang et al. 2010; Li et al. 2004). Its lettuce orthologue, Lsat_1_v5_gn_9_38680, has unique target genes (with an Arabidopsis orthologue) enriched for “response to salicylic acid” and “response to oomycetes”.

Lsat_1_v5_gn_2_103381 (LsNAC53) is a large GRN hub with unique target genes enriched for “cell death” and “ubiquitin-protein transferase activity”, the only hub enriched for this term. LsNAC53 is a putative orthologue of AtNAC53 (also known as AtNTL4, NAC with transmembrane motif 1-like 4). AtNAC53 has been shown to regulate proteasome stress redundantly with its close homolog, NAC078 (Gladman et al. 2016). In addition, AtNAC53 is known to promote cell death and reactive oxygen species (ROS) production during drought stress, through directly activating expression of reactive burst oxidase homolog (RBOH)-encoding genes (Lee et al. 2012). In the GRN, a lettuce orthologue of RBOHD (Lsat_1_v5_gn_5_9460) is a predicted target gene of LsNAC53. LsNAC53 and LsRBOHD show very similar expression patterns in both the *B. cinerea* and *S. sclerotiorum* inoculation time series (Figure 3.16) as well as correlation of expression with LsNAC53 in the lettuce diversity panel transcriptome data, particularly after *S. sclerotiorum* infection ($R = 0.94$). Orthologues of two other genes associated with cell death in Arabidopsis (Necrotic spotted lesions 1 and 2, NSL1 and NSL2)(Noutoshi et al. 2006; Morita-Yamamuro et al. 2005) are also predicted targets of LsNAC53 in the GRN and show a similar tight co-expression pattern with LsNAC53.

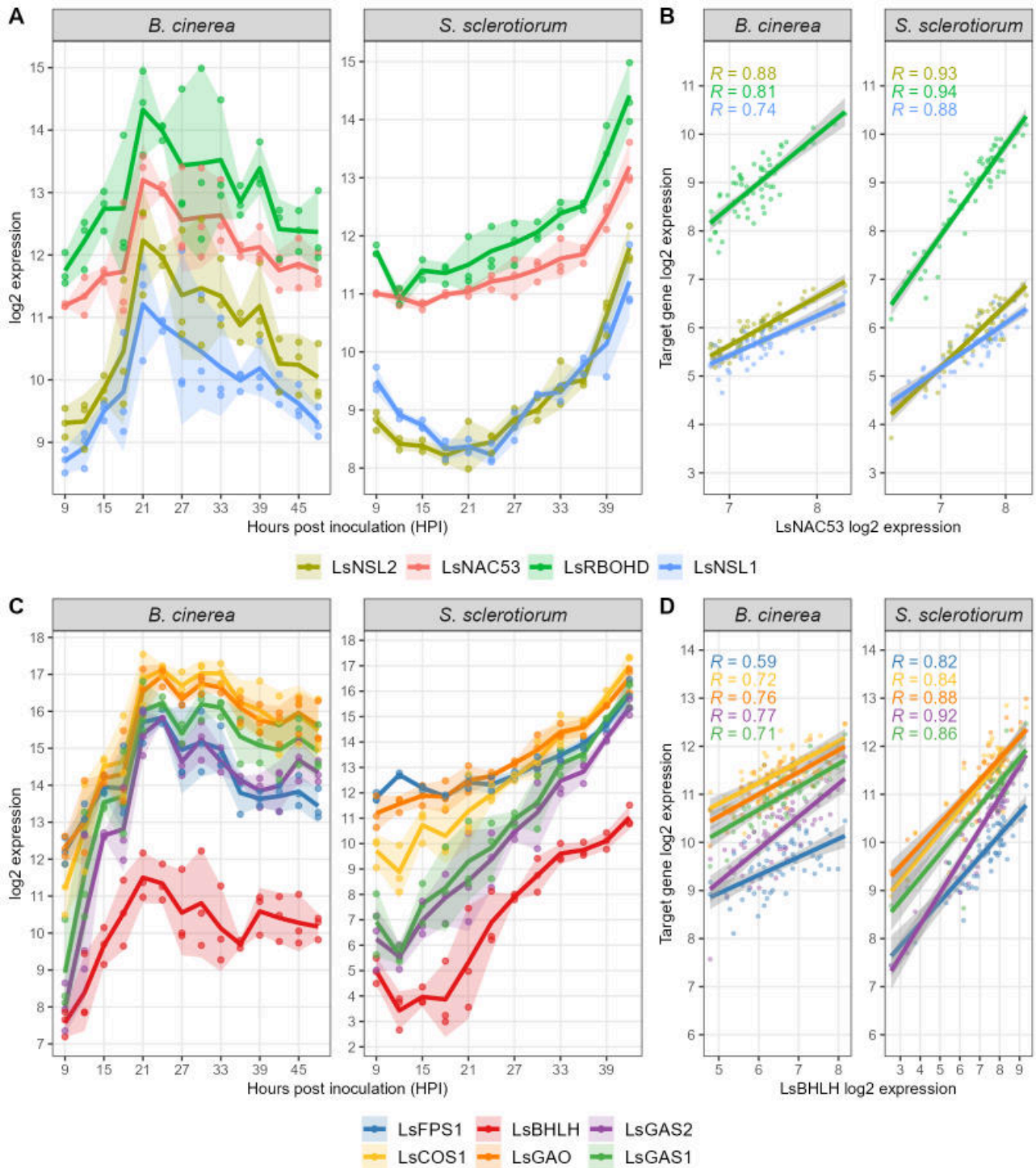


Figure 3.16: Expression profiles of the transcription factors LsNAC53 and LsBHLH and their predicted downstream targets in the gene regulatory network. The expression of LsNAC53 and its predicted targets LsRBOHD, LsNSL1 and LsNSL2 in lettuce following inoculation with *B. cinerea* (this study) and *S. sclerotiorum* (Ransom et al. 2023)(**A**) and the expression of the target genes compared to LsNAC53 expression across 21 different lettuce accessions after pathogen inoculation (Pink et al. 2022) (**B**). **C**) and **D**) show the expression profiles of LsBHLH and its predicted target genes (LsFPS1, LsGAS1, LsGAS2, LsGAO, LsCOS1) in the same data sets. R indicates the Pearson coefficient of correlation between expression of each target gene and its respective predicted regulator.

As mentioned above, lettuce synthesises a diverse range of sesquiterpene lactones (STLs), at least one of which has anti-fungal activity against *B. cinerea* in vitro (Bennett et al. 1994; Sessa et al. 2000). In the

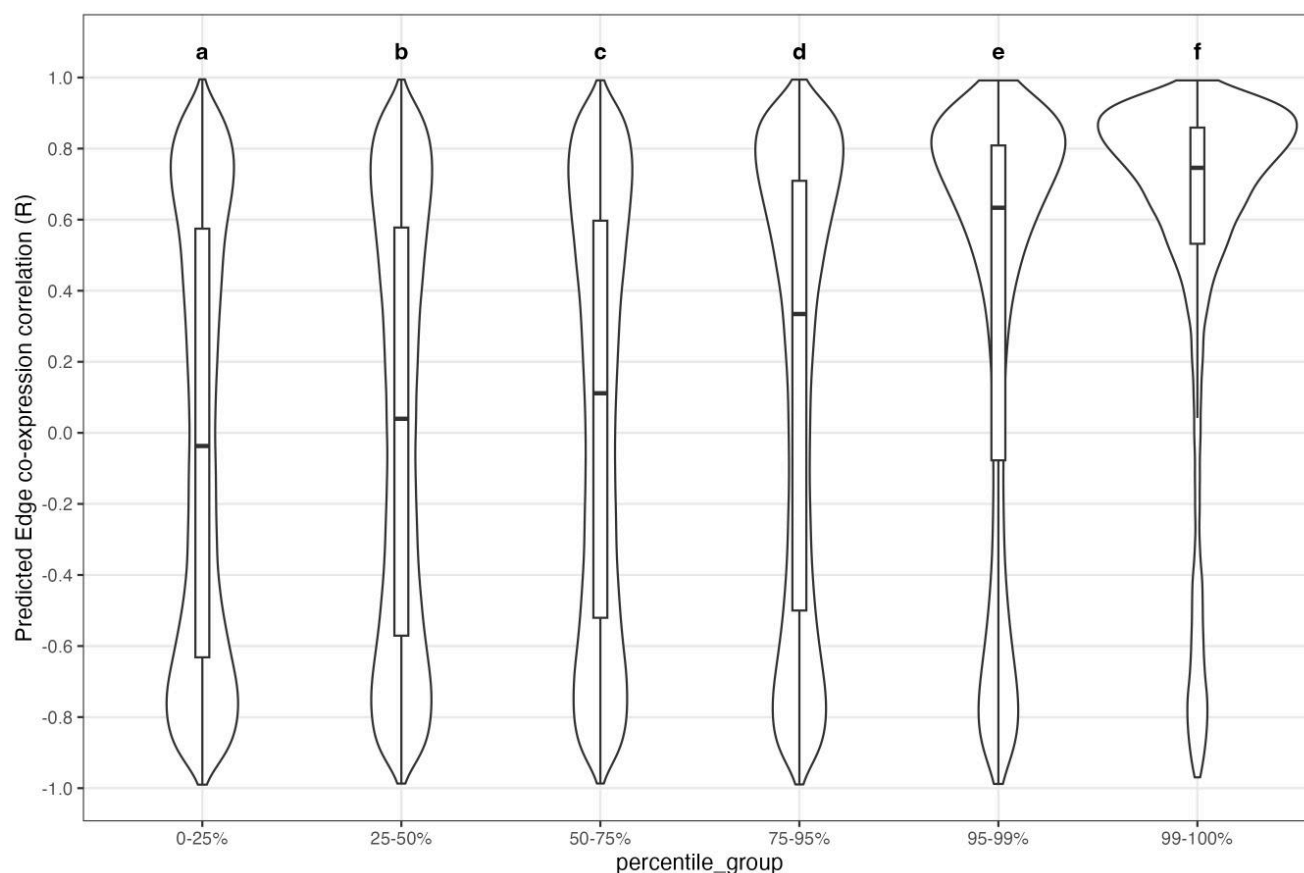


Figure 3.17: Distribution of TF-target co-expression in unseen datasets (Verwaaijen et al. 2019; Fletcher et al. 2019) grouped by the Importance percentile in our lettuce-necrotroph gene regulatory network. These interactions were then categorised into six percentile groups: 0-25%, 25-50%, 50-75%, 75-95%, 95-99%, and 99-100%. Violin plots illustrate the spread and density of the co-expression correlations within each group, with the top 1% of edges displaying the highest co-expression correlation, indicating a stronger regulatory association in the unseen datasets. Letters represent Tukey HSD statistical significance groupings ($p < 0.05$).

GRN, multiple STL biosynthetic enzyme-encoding genes are predicted to be regulated by a single lettuce β -helix-loop-helix TF (LsBHLH). These include genes encoding: a Farnesyl diphosphate synthase (LsFPS1), Germacrene A synthase (LsGAS), Germacrene A oxidase (LsGAO) and costunolide synthase (LsCOS). The expression profiles of LsBHLH and these predicted downstream targets are shown in Fig 3.16. This network prediction may not only identify a key transcriptional regulator of these specialised biosynthetic genes but also identify additional enzymes involved in the synthesis of this diverse family of compounds.

These examples highlight the ability of the lettuce GRN to not only predict TF hubs that impact disease resistance and associate these hubs with functional defence processes, but also to predict specific TF-target gene regulation that appears biologically relevant.

3.3.6 Validation of GRN edges on unseen lettuce-defence datasets

In addition, we tested the ability of our lettuce *B. cinerea*/*S. sclerotiorum* GRN to predict regulatory interactions in additional lettuce defence-related transcriptome datasets that were not used to build our GRN. We chose two publicly available data sets: i) lettuce leaves inoculated with the soil-borne fungus *Rhizoctonia solani* (Verwaaijen et al. 2019) and ii) oomycete *Bremia lactucae* (Fletcher et al. 2019). The raw reads were downloaded from relevant short-read archives and re-analysed and mapped to the lettuce v8 transcriptome and the pairwise co-expression correlation in these datasets of each TF-target gene edge in the initial OutPredict GRN (i.e. no threshold on edge importance) was calculated (1,094,611 edges). This analysis demonstrated that the top 1% of edges in our GRN had the highest correlation of TF-target gene expression in the new data sets with the median R value at 0.75 (compared to -0.04 for the 25% lowest confidence edges), Fig 3.17. This demonstrates that the highest confidence edges of our GRN perform well in predicting expression of targets in unseen defence-related datasets.

3.3.7 Opposing functions of BOS1 in lettuce and Arabidopsis

We selected two hub TF genes from the network for functional testing: Lsat_1_v5_gn_6_70301 (LsBOS1) which is orthologous to Arabidopsis BOS1, and LsNAC53 highlighted above. The Arabidopsis BOS1 gene encodes a MYB transcription factor (MYB108) that is upregulated during infection of Arabidopsis by *B. cinerea* (Windram et al. 2012; Mengiste et al. 2003). Despite the upregulation of this gene during infection, in Arabidopsis BOS1 has been shown to promote susceptibility to *B. cinerea* (Cui et al. 2022). Although a cotton orthologue of BOS1 (GhMYB108) has been demonstrated to increase resistance to both *B. cinerea* and *Verticillium dahliae* (Cheng et al. 2016). The two putative lettuce orthologues of Arabidopsis BOS1 (Lsat_1_v5_gn_6_70301 and Lsat_1_v5_gn_6_117600) were significantly upregulated in response to both *B. cinerea* and *S. sclerotiorum* infection in lettuce (Figure 3.18). Furthermore, both were hub genes in the GRN predicted to regulate 48 (Lsat_1_v5_gn_6_70301) and 39 (Lsat_1_v5_gn_6_70301) downstream target genes. To test whether the function of BOS1 is conserved between Arabidopsis and lettuce, we generated transgenic Arabidopsis lines constitutively expressing Lsat_1_v5_gn_6_70301 (named LsBOS1 and selected due to the greater number of downstream target genes) under control of the 35S promoter. Two independent p35S::LsBOS1 homozygous lines were selected from T2 lines showing Mendelian inheritance of the T-DNA selectable marker and were shown to express LsBOS1 (Figure 3.18). Both lines show increased resistance to *B. cinerea* with statistically significant reduced lesion size compared to wildtype Arabidopsis (Figure 3.19). This suggests that this LsBOS1 is acting as a positive regulator of plant defence against *B. cinerea*, in contrast to the Arabidopsis BOS1 which promotes susceptibility to this pathogen, although consistent with cotton GhMYB108. Despite these opposing functions in defence, the altered disease resistance in these transgenic Arabidopsis lines demonstrates successful prediction of defence regulators by the OutPredict GRN.

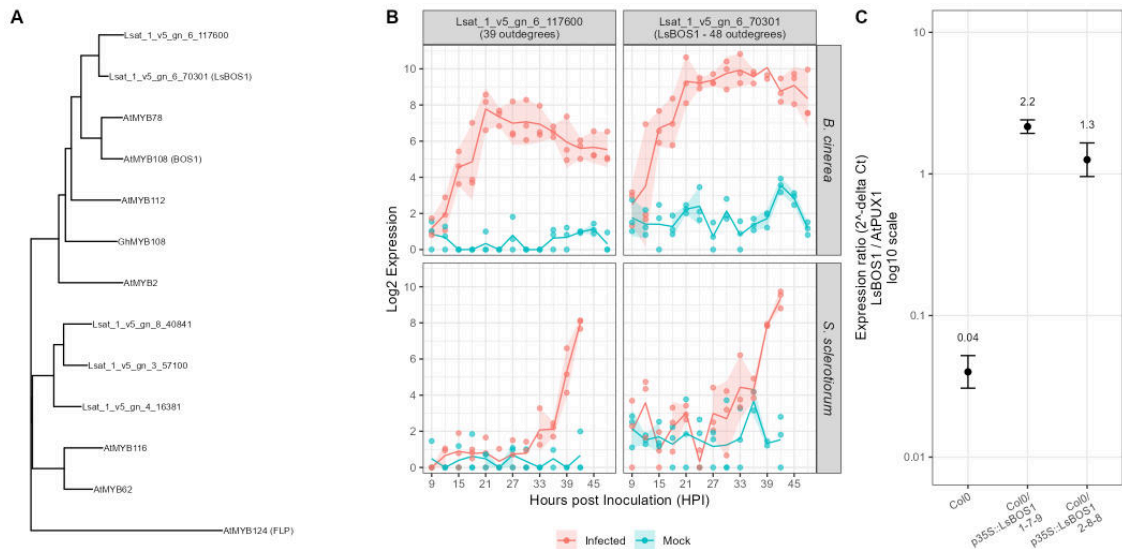


Figure 3.18: **(A)** A 3000 bootstrap maximum likelihood phylogenetic tree of Arabidopsis MYB subgroup 20 (MYB2, MYB62, MYB78, MYB108 (BOS1), MYB112 and MYB116), their predicted lettuce orthologues (Reyes-Chin-Wo et al. 2017), a cotton orthologue GhMYB108 (Genbank : ALL53614.1) and MYB124 (FLP) as an outgroup. **(B)** *B. cinerea* (top panels) and *S. sclerotiorum* (bottom panels) time series log2 expression (TPM) profiles of lettuce BOS1 orthologues (Lsat_1_v5_gn_6_70301 and Lsat_1_v5_gn_6_117600), with pathogen inoculated expression profiles shown in red, and mock inoculated expression shown in blue. Shaded region shows 95% confidence intervals. **(C)** Expression of the LsBOS1 gene (Lsat_1_v5_gn_6_70301) normalised to the AtPUX1 housekeeping gene in Col-0 wildtype Arabidopsis and two independent lines of Col-0 expressing LsBOS1 under control of the 35s promoter. Tissue was collected from pooled samples of 10 day old Arabidopsis seedlings with 3 technical replicates.

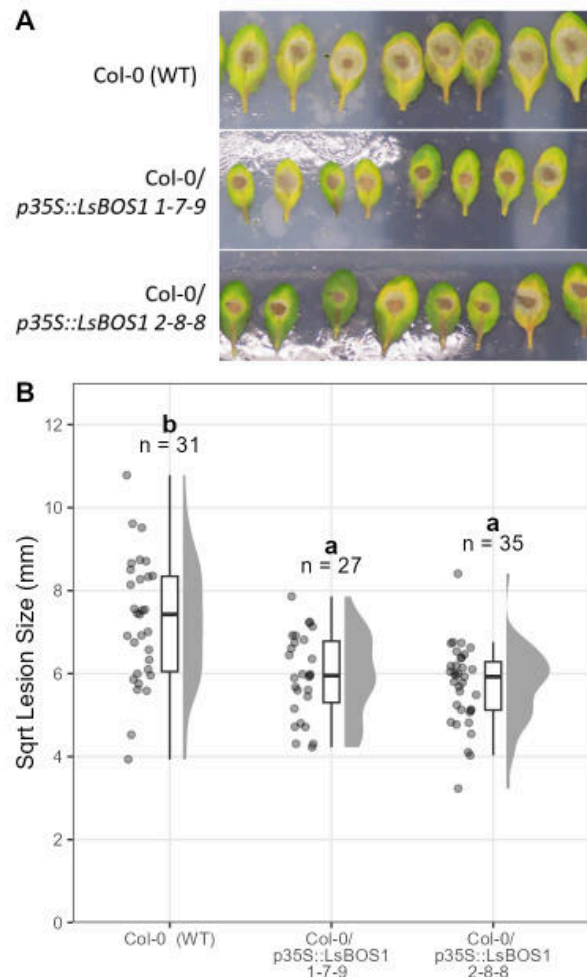


Figure 3.19: LsBOS1 acts as a positive regulator of *B. cinerea* resistance. **(A)** Representative images of Col-0 and two independent p35S::LsBOS1 transgenic lines (1-7-9 and 2-8-8) 72 hours post inoculation with *B. cinerea* “pepper” spores. Both transgenic lines exhibit stunted growth. **(B)** Quantification of (A) showing the square-root area of necrotrophic lesion, individual data points as well median (in box plot) and distributions. Letters represent statistical significance groupings – Tukey HSD $p < 0.05$. N represents the number of lesions measured per genotype.

3.3.8 LsNAC53: GRN-identification of a novel defence regulator

As outlined above, LsNAC53 is a putative orthologue of AtNAC53 (also known as AtNTL4). AtNAC53 regulates proteasome stress (redundantly with NAC078 (Gladman et al. 2016)) and ROS production/cell death during drought stress, via RBOH gene expression (Lee et al. 2012). In addition to the conserved DNA binding domain both AtNAC53 and LsNAC53 have a C-terminal transmembrane domain, which in Arabidopsis has been shown to tether the TF to the plasma membrane (Kim et al. 2010). This prevents nuclear localisation and activity of AtNAC53 until the DNA binding domain is cleaved in response to drought, enabling it to move to the nucleus and activate gene expression.

Given the ROS/cell death promoting function of AtNAC53, we hypothesised that this TF would negatively impact plant resistance to necrotrophic fungal pathogens, despite expression of both AtNAC53 and LsNAC53

being upregulated following infection with *B. cinerea* (Windram et al. 2012, Fig 3.16A) and, for LsNAC53, *S. sclerotiorum* (Ransom et al. 2023). We obtained seed of the previously characterised AtNAC53 T-DNA mutant, *nac53-1/nt14-1* (Lee et al. 2012), and tested the susceptibility of this mutant line to *B. cinerea* using our detached leaf assay. Compared to wildtype Col-0 Arabidopsis, the *nac53-1* mutant showed increased resistance (smaller lesion size) (Figure 3.20A), suggesting AtNAC53 does indeed function as a negative regulator of *B. cinerea* defence.

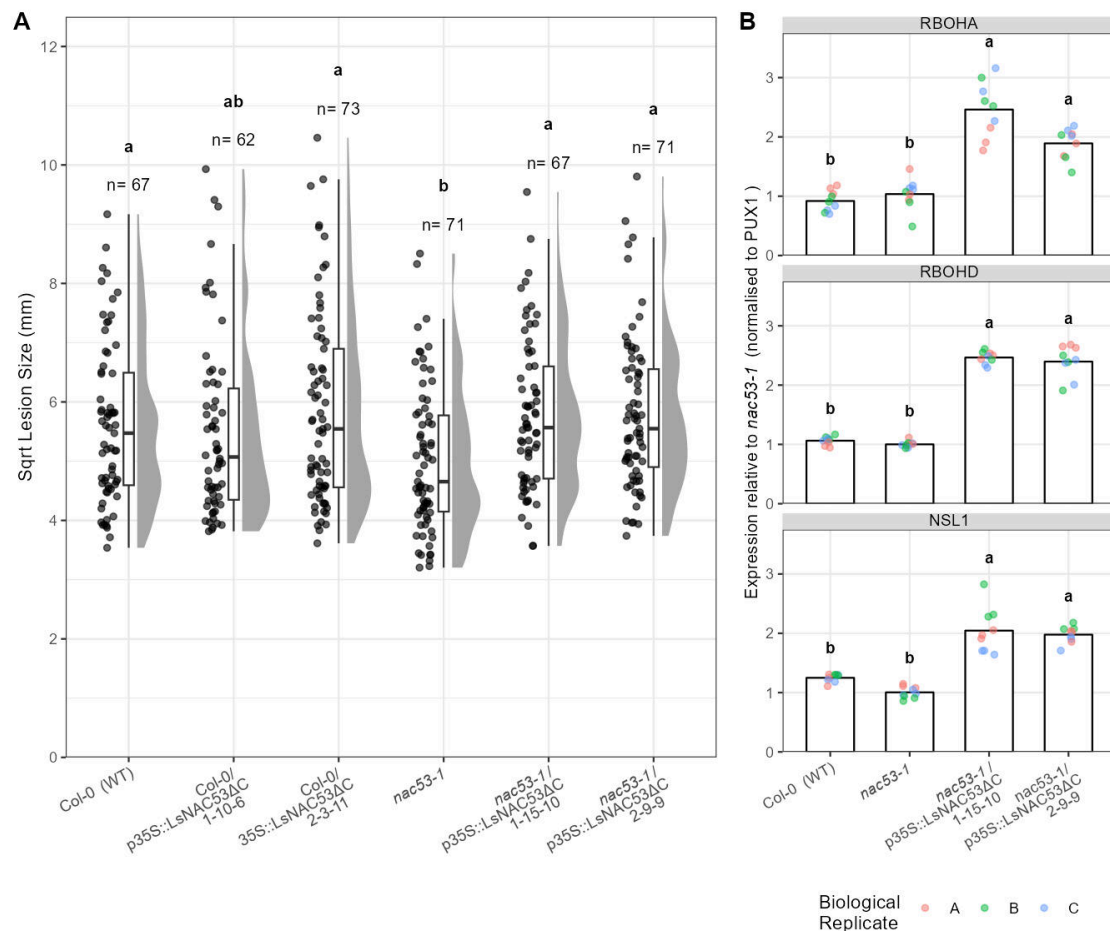


Figure 3.20: LsNAC53ΔC functionally complements *nac53-1* as a negative regulator of *B. cinerea* defence. **(A)** Lesion size after *B. cinerea* inoculation of detached leaves, 72 hours post inoculation. Arabidopsis genotypes are wildtype Col-0, *nac53-1* mutants, and constitutively expressed LsNAC53 (lacking the transmembrane domain) p35S::LsNAC53ΔC in both Col-0 and *nac53-1* backgrounds. Individual lesion sizes as well as the median and distribution of data points are shown. N = number of lesions measured, and letters indicate statistically significant groupings (Tukey HSD $p < 0.05$). **(B)** Expression of Arabidopsis genes RBOHA, RBOHD and NSL1 in wildtype Col-0, *nac53-1* mutant and transgenic Arabidopsis *nac53-1* mutants expressing truncated LsNAC53 under control of the 35s promoter (*nac53-1*/p35S::LsNAC53ΔC). Tissue was collected from non-stressed 10-day seedlings grown on 1/2 strength Murashige and Skoog (MS) media agar plates. Three technical replicates of three biological replicates are shown with expression normalised to that of AtPUX1 and shown relative to expression in *nac53-1*. letters indicate statistically significant groupings (Tukey HSD $p < 0.05$).

To determine whether LsNAC53 is also a negative regulator of defence against *B. cinerea*, we generated transgenic Arabidopsis lines constitutively expressing LsNAC53 without the transmembrane domain (p35S::LsNAC53ΔC) in both wildtype Col-0 and *nac53-1* mutant backgrounds. Two independent homozygous

lines were selected in each genetic background which show similar levels of *LsNAC53* expression (Fig 3.21).

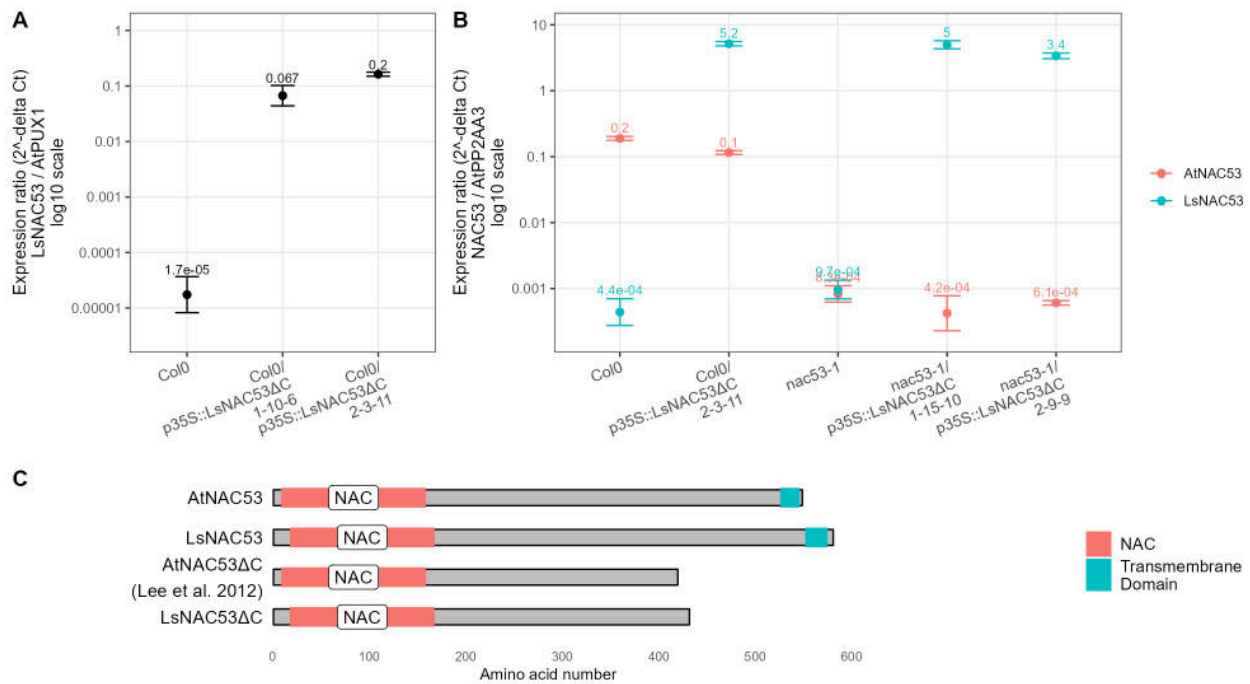


Figure 3.21: **A**) Expression of the *LsNAC53* gene (normalised to the *AtPUX1* housekeeping gene) in Col-0 wildtype Arabidopsis and two independent lines of Col-0 expressing *LsNAC53*ΔC under control of the 35s promoter. Both lines show a similar level of *LsNAC53* expression. **B**) Expression of the *LsNAC53* gene and endogenous *AtNAC53* (normalised to the *AtPP2AA3* housekeeping gene) in Col-0 wildtype Arabidopsis, one line of Col 0 expressing *LsNAC53*ΔC under control of the 35s promoter, the *nac53-1* mutant and two independent lines expressing p35s::LsNAC53ΔC in the mutant background. Endogenous *AtNAC53* expression is as expected in the wildtype and mutant lines and does not change in the presence of *LsNAC53*. All transgenic lines expressing *LsNAC53* have a similar level of expression. Samples are pooled 10 day old Arabidopsis seedlings. **C**) The domain structure of NAC53, showing the N-terminal NAC DNA binding domain and the C-terminal transmembrane domain, which prevents nuclear localisation. The truncation of *LsNAC53*ΔC is shown compared to the Arabidopsis truncated version used in Lee et al. 2012

Col-0/p35S::LsNAC53ΔC lines show no gain-of-function phenotype, with *B. cinerea* lesion size very similar to that of the wildtype Col-0. However, both independent *nac53-1*/p35S::LsNAC53ΔC lines have significantly larger *B. cinerea* lesions than *nac53-1* (Fig 3.20A), demonstrating that constitutive expression of *LsNAC53*ΔC can functionally complement the lack of *AtNAC53*. This suggests that *LsNAC53* is a functional orthologue of *AtNAC53*, and also acts as a negative regulator of *B. cinerea* defence.

As outlined above, *AtNAC53* activates expression of the RBOH genes A, C and E (Lee et al. 2012) and in the GRN, *LsNAC53* is predicted to regulate *LsRBOHD*, *NSL1* and *NSL2* (Fig 3.16A). Analysis of gene expression in these transgenic Arabidopsis lines under non-stressed conditions demonstrated that constitutive *LsNAC53* expression activates expression of two Arabidopsis RBOH genes (A and D) as well as Arabidopsis *NSL1* expression (Figure 3.20B). However, *LsNAC53*ΔC did not impact the expression of RBOH B, C, E or F in non-stressed conditions (Fig 3.22). As seen before, the *nac53-1* mutant did not reduce expression of these genes under non-stressed conditions, but clear induction of expression was seen in the presence of the

truncated LsNAC53, validating these GRN predictions.

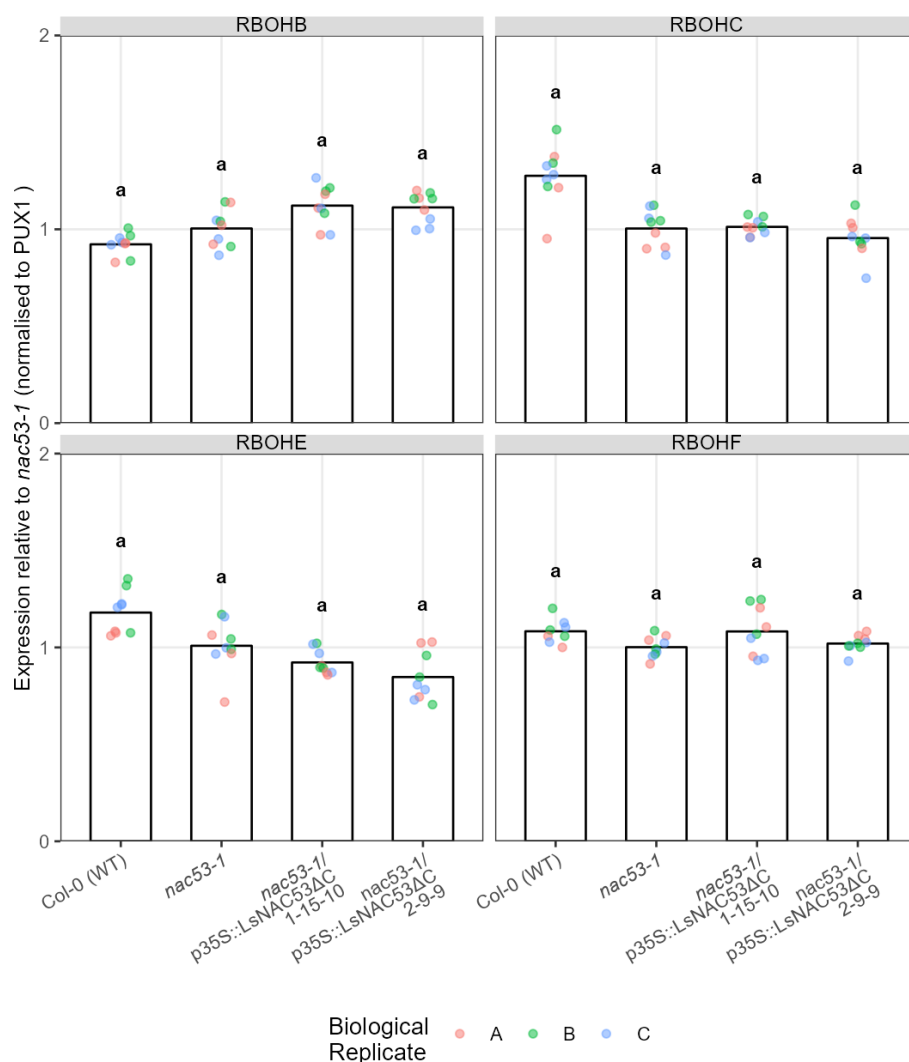


Figure 3.22: Relative expression of Arabidopsis genes *RBOHB*, *RBOHC*, *RBOHE*, *RBOHF* in wildtype Col-0, *nac53-1* mutant and transgenic Arabidopsis *nac53-1* mutants expressing truncated LsNAC53 under control of the 35S promoter (*nac53-1*/ p35S::LsNAC53 Δ C). Three technical replicates of three biological replicates are shown with expression normalised to that of AtPUX1 and shown relative to expression in *nac53-1*. No statistical difference was observed based on the threshold of Tukey's HSD $p < 0.05$ and fold change > 1.5 or fold change < 0.667 (equivalent downregulated fc).

3.4 Discussion

Here we present a high-density time series transcriptome dataset capturing gene expression after *B. cinerea* and mock inoculation of lettuce leaves. Comparing the response to a similar time series dataset following *S. sclerotiorum* infection, revealed a core set of 4362 lettuce genes that change in expression (in the same direction) in response to infection by both pathogens. An earlier lettuce *-B. cinerea* RNAseq dataset was published with just three time points (12, 24 and 48 hours post inoculation), identifying 1, 139 and 4598 DEGs at each respective time point (De Cremer et al. 2013). In contrast, the time series presented in this paper has 14 time points, one every 3 hours, and has captured significant gene expression changes from 9

hpi. As seen during *B. cinerea* infection of *Arabidopsis* (Windram et al. 2012) the majority of gene expression changes occur before significant growth of the pathogen (Fig 3.5) or lesion development (Fig 3.1). A significant proportion of the lettuce genes differentially expressed in response to *B. cinerea* are orthologous to DEGs in *Arabidopsis* (Fig 3.6) although there are some clear differences. Plant defensins play a major role in *Arabidopsis* defence against *B. cinerea* with several dramatically upregulated during infection (Windram et al. 2012; Ingle et al. 2015), but in lettuce these genes are generally not changing in expression with one member of the family downregulated (Fig 3.8). In addition, the *Arabidopsis* BOS1 TF is a negative regulator of defence against *B. cinerea* (Cui et al. 2022) whereas the lettuce gene (when expressed in *Arabidopsis*) appears to be a positive regulator (Fig 3.19). Interestingly the cotton orthologue of BOS1 (GhMYB108) is a positive regulator of resistance to *Verticillium dahlia* and *B. cinerea* (Cheng et al. 2016). These examples show the importance and value of analysing defence responses (even to the same pathogen) in different species.

The high resolution of our time series data provides insight into the timing and sequence of pathogen-induced transcriptional reprogramming. We used Wigwams (Polanski et al. 2014) to identify modules of genes that are co-expressed in response to *B. cinerea* and *S. sclerotiorum* to reduce the complexity of the data and identify groups of genes with a shared function that are similarly regulated. This did highlight known defence responses (such as JA and ET signalling, receptor signalling) as well as lettuce-specific processes such as the synthesis of sesquiterpene lactones. However, some of these modules are very large. We still observe the majority of DEGs changing in expression during a short window (21-24 hpi) and finer time points in this region could help in separating gene expression profiles (and biological processes) further. However, unlike application of hormones or defence elicitors (Hickman et al. 2017; Bjornson et al. 2021) where responses occur within minutes, the requirement for spore germination and growth of the pathogen, and the impact of the environment on this, means the first transcriptional responses can only be detected hours after inoculation and timing can vary between experiments making it hard to accurately predict the critical window for analysing more time points.

However, the availability of high-resolution time series data enables the inference of a causal GRN model of the regulatory events underlying transcriptional reprogramming during infection. The power of such network inference is increased with the availability of two such time series (lettuce inoculated with *B. cinerea* and *S. sclerotiorum*). Furthermore, the ability of the OutPredict algorithm to combine time series and static (single time point) data meant that we could also incorporate transcriptome data from 21 different lettuce accessions after infection with *B. cinerea* and *S. sclerotiorum* (Pink et al. 2022). Time series data provides information on the relative timing of a TF and target gene, whereas the diversity set data provides an independent set of data highlighting correlation in expression between the TF and target genes. Combining these different types of data has likely increased the power of OutPredict and the accuracy of the resulting GRN.

The GRN we have generated (using the top 1% high confidence edges) not only predicts key regulators but is also able to predict downstream target genes of these regulators. Our confidence in the network model comes from i) the identification as hubs of orthologues of known *Arabidopsis* TFs that impact *B. cinerea* disease resistance (Fig 3.13B); ii) the demonstration that LsBOS1 and LsNAC3 impact resistance to *B. cinerea* when expressed in *Arabidopsis* (Fig 3.19, 3.20); and iii) LsNAC53 is able to upregulate orthologues of its predicted target genes when expressed in *Arabidopsis* (Fig 3.16, 3.20). This GRN will advance our understanding of the transcriptional defence response in lettuce by identifying key regulators for experimental testing (with the resulting data able to be used to improve the model), and highlighting the network topology, network motifs and cross-talk between different signalling pathways that is driving the ultimate defence response. Future work will aim at validating the GRN in lettuce and developing GRN models with the ability to simulate the impacts of network perturbation not just on expression of GRN genes, but on disease resistance against these important pathogens.

Chapter 4

Functional *in planta* validation of predicted *Lactuca sativa* necrotroph defence regulators

4.1 Introduction

Botrytis cinerea, a devastating necrotrophic plant pathogen is able to infect over 200 species including crop species such as strawberry, grape, tomato and lettuce which causes up to \$10 billion per year in crop losses and control strategies (Williamson et al. 2007; Dean et al. 2012). Rupp et al. 2016 identified *B. cinerea* strains with resistance alleles to multiple fungicides which are used to control infection, suggesting that genetic sources of resistance may be required for sustainable crop protection. Whilst no alleles which provide complete resistance to *B. cinerea* have been identified, many small-medium effect quantitative trait loci (QTL) have been identified in *Arabidopsis* (Denby et al. 2004) and a range of crop species (Finkers et al. 2007; Zhang et al. 2016b; Fu et al. 2017; Szymański et al. 2020; Pink et al. 2022). For most of these loci, the underlying causative genes are unknown, hence time-consuming introgression is required to introduce the resistance allele into an elite cultivar. In addition, undesirable traits encoded by neighbouring genes may also be introduced (linkage drag).

Instead, targeted activation or mutation of a single gene of interest offers a more precise method to introduce a disease resistance allele. Resistant germplasm can be generated faster, as backcrossing is not required as there is no linkage drag. However, this methodology requires the ability to; **i)** identify candidate defence regulators **ii)** characterise the *in planta* defence function of candidates - ideally in a high-throughput manner and **iii)** introduce targeted loss-of-function and/or gain-of-function mutation within the species of interest.

This work will focus on i) and ii).

Selecting orthologues of known *Arabidopsis* defence genes is a common approach for identifying candidate genes (Tripathi et al. 2021; Cao et al. 2019). However, there is no guarantee that gene function will be conserved. In Pink et al. 2023, we demonstrated only two family 1 plant defensins were detectable after *B. cinerea* infection in lettuce and none were upregulated despite being key components of the *Arabidopsis* JA-response (Thomma et al. 2002). Transcriptomic analysis can be used to highlight genes responding to pathogen infection, however due to the sheer number of genes, it is often still orthologues of known regulators which are selected as candidates (Sun et al. 2018; Wang et al. 2017b). Network modelling approaches can be used for candidate prioritisation (Thompson et al. 2015; Mercatelli et al. 2020). Wan et al. 2021 generated a co-expression network from *B. cinerea*-infected time-course RNA-seq of *Vitus vinifera* (grape) and *Vitus amurensis* (wild grape), from which VaWRKY10 was identified as a defence regulator. While co-expression networks are useful tools to identify modules with highly-correlated expression profiles, they do not infer causal or directed regulatory edges.

In Pink et al. 2023 we use a machine learning approach to model transcriptional regulation across multiple temporal and steady-state lettuce necrotrophic infection RNAseq datasets. A causal gene regulatory network (GRN) with directed edges was then constructed from the top 1% of high-confidence interactions, identifying hub genes that are predicted to have a large effect on necrotroph-induced transcriptional reprogramming in lettuce. Additionally in Pink et al. 2022, we perform transcriptomic analysis and *S. sclerotiorum* disease susceptibility assessment of 21 diverse lettuce accessions. This enabled the identification of genes whose expression did not change in response to infection, but increased expression is correlated with increased or decreased *S. sclerotiorum* resistance, termed “resistance correlated” or “susceptibility correlated” respectively. Both GRN hubs and *S. sclerotiorum* resistance/susceptibility correlated genes represent candidate lettuce genes that could be used for functional testing.

Ideally, functional testing would be performed in stable lettuce overexpression or knockout lines. However, the generation of such lines requires labour-intensive and time-consuming tissue culture, reducing the number of genes that we're able to functionally validate. *Arabidopsis* transformation can be performed without the need for tissue culture (Clough and Bent 1998), allowing homozygous plants expressing a lettuce candidate gene to be generated within 9 months. The speed of gene testing can be further increased by using transient *Agrobacterium tumefaciens* infiltration (as previously demonstrated for *P. syringae* phenotyping by Buscaill et al. 2021), eliminating the need to generate homozygous lines. All these approaches use a gain-of-function approach to study gene function. In some cases, it may be desirable to investigate loss-of-function, particularly if the introduction of gene-edited plants is the end goal (Zaidi et al. 2020). Although loss-of-function testing can be more challenging, as it must be performed in species of interest and functional redundancies may

mask the phenotype (Uauy et al. 2017). Virus-induced gene silencing (VIGS) offers a transient solution to loss-of-function gene testing, but requires efficient *Agrobacterium* transformation in the crop species of interest (Robertson 2004; Liu et al. 2020).

In previous work we have characterised two lettuce GRN hubs (LsBOS1 and LsNAC53), demonstrating that both regulate *B. cinerea* defence in transgenic Arabidopsis lines (Pink et al. 2023). In this work we will use stable lettuce overexpressor lines, stable transgenic Arabidopsis and transiently infiltrated *Nicotiana benthamiana* to characterise the *B. cinerea* defence function of 6 predicted GRN hubs (identified in Pink et al. 2023) and 4 *S. sclerotiorum* resistance correlated genes (identified in Pink et al. 2022).

4.2 Methods

4.2.1 Transgenic Plant Lines and Cloning

LsERF1 (Lsat_1_v5_gn_3_121961) was cloned into p35S expression vector pGWB611 (Nakamura et al. 2010) by Dr Elspeth Ransom. Stable PI251246/p35s::LsERF1 lettuce transgenic lines were previously generated by the UC Davis Genome Centre (Ransom et al. 2023). Arabidopsis mutant lines *wrky7-1* (GK_356A10) and *drb2-1* (GK_348A09) were obtained from NASC (Alonso et al. 2003a; Rosso et al. 2003). Prof Nicole Clay (Yale) kindly sent us *myb15-1* (SALK_151976) and *myb15-1*/p35S::AtMYB15 #1 lines (Chezem et al. 2017).

LsMYB15 (Lsat_1_v5_gn_3_120520), LsWRKY7A (Lsat_1_v5_gn_4_164440) and LsWRKY7B (Lsat_1_v5_gn_4_127960) open reading frames (ORFs) were amplified from *L. sativa* cv. Saladin cDNA. Gene-specific primers with attB1 and attB2 extensions were used to generate PCR fragments that could be cloned into pDONR-zeo (Table 4.1). pDONR entry clones were verified by sequencing. In addition LsBZIP60 (Lsat_1_v5_gn_2_3181), LsBZIP17 (Lsat_1_v5_gn_5_157880), LsDRB2 (Lsat_1_v5_gn_9_69201), LsDRB4 (Lsat_1_v5_gn_1_56161), LsRDM1 (Lsat_1_v5_gn_8_21341) and LsSGS3 (Lsat_1_v5_gn_5_78841) ORFs were synthesised and cloned in pDONR221 by Invitrogen GeneArt. LsBZIP60 was synthesised as its predicted IRE1 spliced isoform (LsBZIP60s), with a 23nt deletion at positions 605-627 (Fig 4.14). LsBZIP17 was synthesised in a truncated form, position 1 - 1092, removing the C-terminal transmembrane domain hence LsBZIP17 Δ C. LsDRB2, LsDRB4, LsRDM1 and LsSGS3 were all synthesised with the addition of a HA-tag at the C-terminus.

pDONR vectors were then recombined with pB2GW7, a p35S expression vector with a BASTA selectable marker (Karimi et al. 2002; Odell et al. 1985). Floral dip transformation was carried out with LsMYB15, LsWRKY7A, LsWRKY7B, LsBZIP17 Δ C and LsBZIP60s in both Col-0 WT and T-DNA mutant backgrounds (Clough and Bent 1998). T_1 transgenic plants were grown on BASTA-treated soil. T_2 plants were tested for 3:1 Mendelian segregation on 1/2 strength Murashige and Skoog (MS) plates supplemented with BASTA (Murashige and Skoog 1962). Multiple independent transgenic lines were tested for transgene expression at T_2 or T_3 . Homozygous transgenic T_3 lines were selected for high transgene expression, and used to perform *B. cinerea* susceptibility assays.

Primer	GeneID	Forward	Reverse
LsMYB15 attB	Lsat.1.v5_gn.3.120520	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGGAGAGCACCTTGTTG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAACTCAGGTAAGCTCGGTAAT
LsWRKY7a attB	Lsat.1.v5_gn.4.164440	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCCGCCGTAGATCTAAT	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAGATGATTCTAAGATCAACCCAGAAG
LsWRKY7b attB	Lsat.1.v5_gn.4.127960	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGGTGGATTTGATGAATG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGATGACTCAAGAACATATGGCT
AtPUX1 qPCR	AT3G27310	TTTTTACCGCCTTTTGGCTA	ATGTTGCCTCCAATGTGTGA
LsMYB15 qPCR	Lsat.1.v5_gn.3.120520	TACCTGGCCGAAGTACAAAC	GTCTTTTTGGGGCATGGCTG
LsWRKY7a qPCR	Lsat.1.v5_gn.4.164440	ACGGAAAACAAGTCTCCGCC	GTAGAACCACCGCTGCACTT
LsWRKY7b qPCR	Lsat.1.v5_gn.4.127960	CGACGTTGACAGGAGACACA	ATTCGGCTGACAACCAGAGG
NbL23 qPCR	Niben101Scf00684g01002.1	AAGGATGCCGTGAAGAAGATGT	GCATCGTAGTCAGGAGTCAACC
NbBZIP60 qPCR	Niben101Scf24096g00018.1	ATTGACTCTAAGGACGGCTCT	ATACAACTTCTCCGCTCTCG
NbBLP4 qPCR	Niben101Scf08590g00005.1	TCGTTTTTCGAATCGTCCT	ATGTCCGTTCTTGACACACC
NbPDI qPCR	Niben101Scf00332g04004.1 Niben101Scf00466g04033.1	TTATTGCCAATCTTGACGCTGA	TTGCCCTTTCGAATCACGGCTA
NbERDj3B qPCR	Niben101Scf03390g09001.1 Niben101Scf01704g00002.1 Niben101Scf03349g00008.1	GAAGGGATGCCACTGCATTT	GATGTGGGGAAAAGAACCTCA

Table 4.1: Primer sequences used within Chapter 4 including those used to clone lettuce hubs (LsWRKY7A, LsWRKY7B and LsMYB15) and qPCR primers

4.2.2 *B. cinerea* susceptibility assay

4.2.2.1 Arabidopsis detached leaf assay

B. cinerea detached leaf infection assays were performed as previously described (Denby et al. 2004). Arabidopsis seeds were sown on P24 trays on Levington's F2+Sand soil in a complete randomised block design and stratified at 4°C for 3 days. Complete randomised block design layouts were computed using blocksdesign R package (Edmondson 2021). Plants were then grown for 4 weeks in a controlled environment chamber with a 16hr photoperiod, 60% relative humidity and 22°C constant temperature (day and night). A single leaf was detached from 4-week old plants and placed on 0.8% agar trays in another complete randomised block design. Detached leaves were inoculated with $10\mu\text{L} \times 10^5$ spores/mL *B. cinerea* “pepper” spores diluted in 50% filter-sterilised grape juice. Trays were sealed and placed in the growth chamber and 90% humidity (otherwise same conditions), photos of lesions were taken at 72hpi. Lesion area (mm^2) is measured using ImageJ (Abràmoff et al. 2004). The square-root of the lesion area is calculated to normalise the distribution of the data (mm^2 will have a positive skew). Linear mixed-effects models (LMMs) were used to analyse the data across experimental repeats using the lmerTest R package (Kuznetsova et al. 2017). The model formula was as followed;

$$\sqrt{\text{lesion area (mm}^2\text{)}} \sim \text{genotype} + (1|\text{exp}) + (1|\text{exp:tray}) \quad (4.1)$$

“genotype” is a fixed-effect, whereas the experimental repeat (“exp”) and the individual infection tray within each experiment (“exp:tray”) were modelled as random effects. Modelling random effects using LMMs allows for experimental variation not associated with the genotype, such as differences in fungal growth between experimental repeats, to be removed. The predictmeans R package was subsequently used to perform least-squares estimation of the lesion size for each genotype, removing random effects (Luo et al. 2022). Post-hoc Tukey HSD is performed on the model to calculate statistical significance groupings between genotypes ($p < 0.05$).

4.2.2.2 Lettuce detached leaf assay

Minor modifications were made to the Arabidopsis *B. cinerea* detached leaf assay for lettuce, as previously described in Pink et al. 2022. Seeds were sown in P15 trays using a complete randomised block design. Trays were stratified at 4°C for 3 days, and then moved to a controlled growth chamber at 20°C, 60% relative humidity and 16 hour photoperiod for 4-weeks. The third-leaf of 4-week old lettuce plants were detached and placed on 0.8% agar trays. Leaves were then inoculated with a $5\mu\text{L}$ of 3×10^4 *B. cinerea* “pepper” spores

on either side of the mid-vein. Spores were diluted in 50% potato dextrose broth (PDB) supplemented with 1% guar gum. Agar trays were sealed, and placed back in the growth chamber at 80% humidity for 64 hours. Lesions were measured using ImageJ and analysed using a linear mixed effects model, as described above.

4.2.2.3 *N. benthamiana* transient detached leaf assay

We modified the *Agrobacterium* assay (Buscaill et al. 2021) to perform transient testing of *B. cinerea* susceptibility. Wild type *Nicotiana benthamiana* seeds were sown in P15 trays on Levington's F2 + sand soil, stratified for 3 days at 4°C. After stratification, trays were moved to a controlled growth cabinet for 4-5 weeks which was set to 22°C, 60% relative humidity and 16 hour photoperiod. After 2 weeks, P15 inserts were cut into individual pots, and spaced out to 7 pots/tray to allow additional room for plant growth. *Agrobacterium tumefaciens* GV3101 (Agro hereafter) strains harbouring p35S::p19, p35S::GFP (kindly gifted by Dr Fabian Vaistij) and other genes of interest were used. Starter Agro cultures were grown for 48 hours at 28°C in 10mL LB medium supplemented with gentamicin and construct selectable marker antibiotics (kanamycin or spectinomycin). 15mL 1:50 dilutions of the starter culture were made and grown overnight until they reached stationary phase (≥ 2.0 OD₆₀₀). Cells were spun down for 20 minutes 2500 × g, and resuspended in 15mL infiltration media (10 mM MgCl₂, 10 mM MES, pH 5.7). Cells were spun down again (20 minutes 2500 × g) and resuspended in infiltration media supplemented with 150µM acetosyringone to a final concentration of 0.5 OD₆₀₀. Cells resuspended in the acetosyringone media were incubated in darkness on a shaking platform for 3 hours. Finally, cells were mixed in a 1:1 ratio of p19:GFP or a 1:1:1 ratio of p19:GFP:gene of interest with a final total concentration of 0.5 OD₆₀₀. The mixed cells were infiltrated into *N. benthamiana* leaves using a 1mL needleless syringe, after which plants were placed back into the growth chamber for 72 hours. Leaves were detached, and examined under UV lamp using GFP fluorescence as a proxy of infiltration efficacy. Well-infiltrated leaves were placed on a 0.8% (w/v) agar tray and inoculated with 4 × 15µL droplets of 2 × 10⁵ spores/mL. *B. cinerea* "pepper" spores diluted in filter-sterilised grape juice. Infection trays were sealed and placed back in the growth chamber at 90% relative humidity for 72-84 hours. Photographs were taken of lesions, and measured using ImageJ. Statistical significance in square root lesion area was calculated using Tukey HSD ($p < 0.05$). GFP infiltrated leaves were used as a control. Genes of interest were co-infiltrated with GFP and p19 unless stated otherwise.

4.2.3 RNA Sequencing

Lettuce transgenic lines and a wild-type sibling, 107-6 (wt-sib), 107-7 (LsERF1-OE #1) and 148-9 (LsERF1-OE #2), were inoculated using the protocol described above. Leaves were inoculated with 4 × 5µL droplets of either mock (50% PDB + guar) or 5 × 10⁵ *B. cinerea* spores/mL. At 24hpi, a 1cm cork borer was used to collect tissue around the inoculation site. Tissue from the 4 inoculation sites on a single leaf

was pooled into a single sample. Tissue was immediately flash-frozen in liquid nitrogen. RNA extraction was performed using NucleoSpin RNA Plus columns (Macherey-Nagel), including a gDNA removal column step. Total RNA was sent to Novogene for stranded mRNA library preparation and was sequenced using NovaSeq 6000 generating 150bp paired-end reads. Read quality was assessed with FastQC, adaptor sequences were then subsequently trimmed using fastp (Chen et al. 2018) using default arguments. Trimmed reads were then aligned to a combined *L. sativa* cv. Saladin RTD v1-*B. cinerea* transcriptome using Salmon (Patro et al. 2017), achieving a median mapping rate of 92.8%. Reference transcriptomes were obtained from Kara Mehmet et al. 2023 and Van Kan et al. 2017 respectively. Differential expression analysis was performed using limma-voom within the 3D RNA-seq app (Law et al. 2014; Guo et al. 2021). Thresholds for considering a gene differentially expressed were adjusted p-value < 0.05 and absolute log2FC $\geq$ 0.5. Principal component analysis was performed using gene-level log2 counts-per-million with prcomp in R.

4.2.4 Gene ontology enrichment analysis

As previously described in Pink et al. 2022, gene ontology (GO) term enrichment analysis used the annotations of the closest Arabidopsis orthologue for each lettuce gene. Single closest Arabidopsis orthologues were identified by performing a BLASTP for lettuce RTDv1 predicted protein sequences against Araport 11 (performed by Fatih Mehmet Kara). Arabidopsis GO-annotations were retrieved using org.At.tair.db and biomaRtr R packages (Carlson 2022; Hajk-Georg and Jerzy 2017). GO enrichment was performed using clusterProfiler R package on gene sets of Arabidopsis orthologues of differentially expressed genes, with Arabidopsis orthologues of all expressed lettuce genes as background (Wu et al. 2021b). GO-terms with adjusted p-value < 0.05 were considered significantly enriched. GO-enrichment network plots were generated using gemaplot function within enrichplot (Yu 2022).

4.2.5 GRN Model Evaluation

We evaluate the gene regulatory networks (GRN) predictive ability to correctly identify LsERF1-OE DEGs as LsERF1 GRN targets. To do this, we calculated true positives (TP), false positives (FP), true negatives (TN) and false negatives (FN). We used four metrics to assess predictive performance; precision ($\frac{TP}{TP+FP}$), recall ($\frac{TP}{TP+FN}$), F_1 -score ($2 \times \frac{\text{Precision} \times \text{Recall}}{\text{Precision} + \text{Recall}}$) (Raschka 2014) and $F_{0.25}$ -score ($\frac{(1+0.25^2) \cdot \text{precision} \cdot \text{recall}}{(0.25^2 \cdot \text{precision}) + \text{recall}}$). Precision represents the proportion of predicted targets which were identified as LsERF1-OE DEGs, whereas recall represents the proportion of actual DEGs that were predicted as LsERF1 targets. F_1 -score is a harmonic mean of the precision and recall. $F_{0.25}$ is a variant of the $F_{0.1}$ score which considers precision to be four times more important than recall.

Predictive performance was assessed at a range of edge confidence thresholds (top 0.2% - 10%) on direct (first-order targets) and extended GRN targets (first and second-order targets). In addition, we randomly shuffle targets assessing the GRNs improvement in predictive performance vs random guessing. Finally, for a more stringent comparison of predictive performance, we use all genes identified within the same time-series co-expression module as LsERF1 to predict DEGs.

4.2.6 Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed to assess transgene expression in transgenic *Arabidopsis* lines. *Arabidopsis* seedlings were sown on 1/2 strength Murashige and Skoog (MS) agar plates supplemented with BASTA (Murashige and Skoog 1962). Col-0 wild-type or T-DNA mutant seedlings were sown in parallel on 1/2 MS plates without BASTA. Plates were stratified for 3 days at 4°C, and then grown in ambient conditions for 10 days. Tissue was harvested from pooled 10-day whole-seedlings, flash-frozen in liquid nitrogen and RNA was subsequently extracted with RNeasy Plant columns (Qiagen) as per the manufacturer's instructions. An on-column RNase-Free DNase digestion was included to digest genomic DNA. cDNA synthesis was performed using SuperScript III (Invitrogen). qPCRs were performed using a SYBR master mix with three technical replicates of three biological replicates. $2^{-\Delta C_t}$ is used to calculate the expression ratio of transgene to AtPUX1 (AT3G27310), an endogenous control gene (Ingle et al. 2015). NCBI Primer-BLAST was used to design qPCR primers targeting lettuce transcripts, ensuring primers had at least 3 mismatches in the last 5bp at 3' end to unintended *Arabidopsis* transcripts (Ye et al. 2012).

qPCR was also used to perform differential expression in *N. benthamiana* post-infiltration. Leaves were infiltrated as described above. At 72 hours post infiltration, a 1cm cork borer was used to collect infiltrated tissue. Three independent biological replicate samples were collected each consisting of pooled tissue from 3 infiltrated leaves from different plants. RNA extractions, cDNA synthesis and qPCRs were performed as above. Relative expression was calculated with $2^{-\Delta\Delta C_t}$, using NbL23 (Niben101Scf00684g01002.1) as the endogenous reference gene and GFP-infiltrated leaves as the control group (Livak and Schmittgen 2001; Liu et al. 2012). NbBLP4, NbBZIP60 and NbPDI primers were taken from Li et al. 2022. NbERDj3b primers were designed by performing a BLASTP, identifying the closest hits to AtERDj3b (AT3G62600) in *N. benthamiana* (Bombarely et al. 2012). Sol Genomics Network *in-silico* PCR tool was used to test for off-target effects in all *N. benthamiana* primers.

Sequences of all qPCR primers are shown in Table 4.1.

4.3 Results

4.3.1 LsERF1 promotes increased *B. cinerea* resistance in transgenic lettuce

Lsat_1_v5_gn_3_121961 (LsERF1) was selected for *in planta* testing, as it was identified as a large gene regulatory network (GRN) hub with 256 predicted targets (Pink et al. 2023). LsERF1 has also been identified as a putative orthologue of Arabidopsis Ethylene Response Factor 1 (AtERF1) which positively regulates ethylene signalling and resistance to *B. cinerea* (Berrocal-Lobo et al. 2002). Both lettuce and Arabidopsis ERF1 orthologues exhibit rapid transcriptional upregulation following necrotrophic pathogen infection (Windram et al. 2012; Pink et al. 2023). Two independent homozygous p35S::LsERF1 overexpression lines (107-7 and 148-9, OE1 and OE2 respectively) were previously generated in PI 251246, an ancestral *L. sativa* accession. A wild-type sibling (107-6) was also isolated by segregating out the transgene from heterozygous individuals. Unlike AtERF1 overexpressor lines, which show stunted growth (Solano et al. 1998), lettuce PI252246/p35S::LsERF1 exhibits no detectable growth defects.

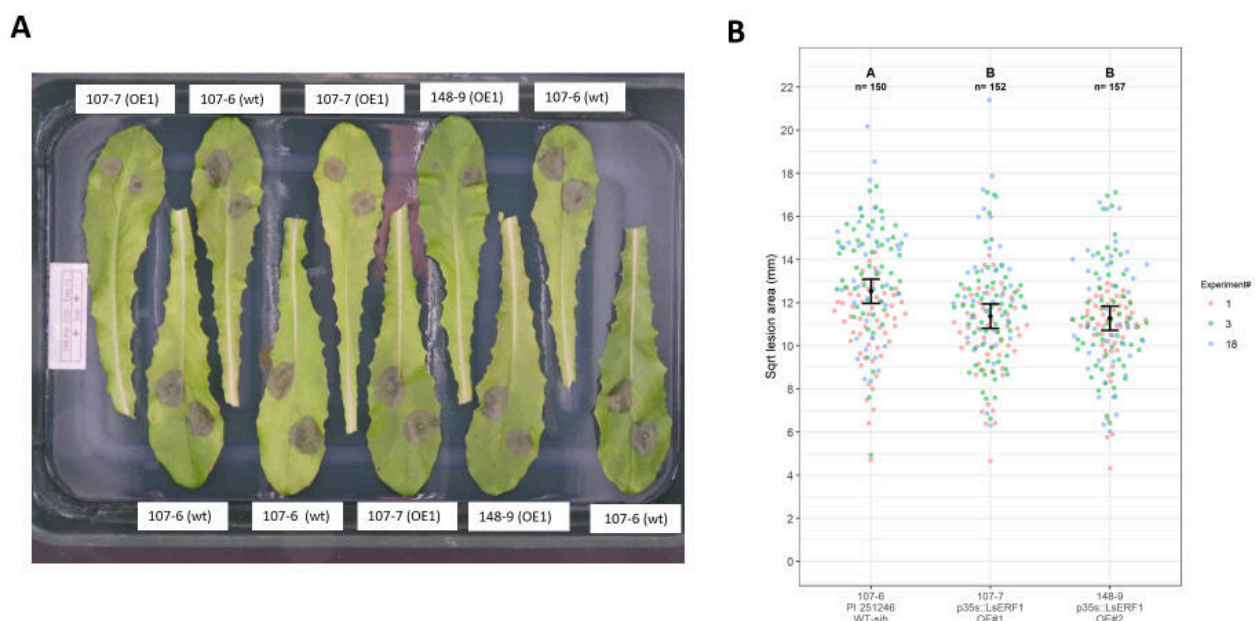


Figure 4.1: Detached leaf phenotyping assay of *B. cinerea* susceptibility on lettuce leaves across three independent experiments (#1, #3, #18). (A) Representative images of WT-sib, OE1 and OE2 leaves 64 hours post inoculation in Experiment #3. (B) Quantitative representation of (A), i.e., the square-root area of necrotrophic lesions. Raw data values from Experiments 1, 3 and 18 are represented by red, green, and blue points respectively. Black points represent the predicted least-squares mean sqrt lesion area derived from a linear-mixed effect model, with error bars indicating the REML standard error. Letters represent statistical significance groups ($p < 0.05$) determined by a post-hoc Tukey HSD test performed using lmerTest and predictmeans R packages. 'n' represents the total number of individual lesions measured across all three experiments.

Consistent with the well characterised role of Arabidopsis ERF1, we demonstrate that both independent PI252246/p35S::LsERF1 lines show increased *B. cinerea* resistance compared to a wild-type sibling across three

independent experiments (Figure 4.1). A linear mixed effects model was utilised to estimate the least-squares lesion area for each genotype, accounting for random variation between experimental repeats (Kuznetsova et al. 2017).

4.3.2 LsERF1 transcriptomics highlights a small candidate gene set which may be responsible for defence priming

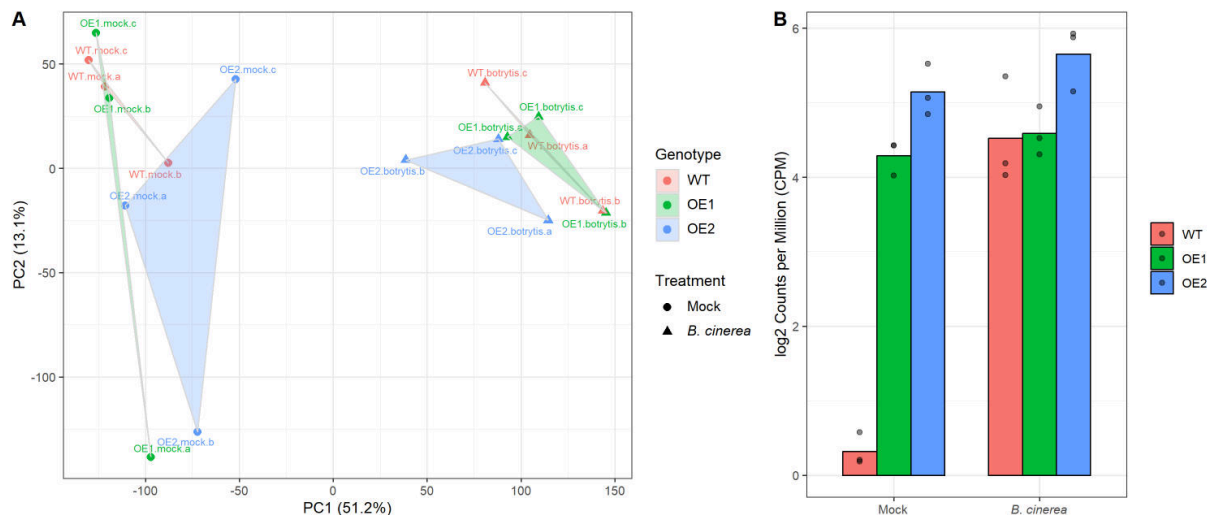


Figure 4.2: (A) Principal Component Analysis (PCA) plot of log2 counts per million (log2 CPM) expression at a gene-level. Circles represent mock samples, triangles represent *B. cinerea* infected samples. Point colour denotes the genotype of the sample; red = wt-sib (107-6), green = OE1 (107-7) and blue = OE2(148-9). (B) Log2 CPM expression of LSATv11_C03_021665 (LsERF1).

Next, in order to gain deeper insights into LsERF1's regulatory network, we carried out transcriptome profiling of both LsERF1 lettuce overexpressor lines and their wild-type sibling in mock and *B. cinerea* inoculated conditions. The third-leaf from 4-week old lettuce plants was detached and inoculated with $4 \times 5 \mu\text{L}$ droplets of either mock or 5×10^5 spore/mL *B. cinerea* suspension. At 24 hours post inoculation (hpi), a 1cm cork-borer was used to collect tissue around each inoculation site. Total RNA was extracted, mRNA was profiled using short-read Illumina sequencing and reads were aligned to a combined *L. sativa* cv. Saladin-*B. cinerea* transcriptome. Principal component analysis (PCA) was conducted, demonstrating a strong separation across PC1 between mock and infected *B. cinerea* samples. To a lesser extent, genotype separation was observed, with OE2 samples distinct from wild-type across both mock and infected (Figure 4.2A).

Figure 4.2B illustrates the expression of LsERF1 (LSATv11_C03_021665) across the RNAseq samples. Consistent with previous findings in the Saladin cultivar (Pink et al. 2023), the wild-type sibling (PI251246 background) exhibits low expression of LsERF1 under mock conditions but is upregulated upon *B. cinerea* infection. OE1 shows LsERF1 expression levels similar to that observed in infected WT under mock conditions, without any further increase upon infection. However, OE2 mock LsERF1 expression was approximately

Among the 33 DEGs identified between, OE1-mock and WT-mock, 24 were also identified as DEGs in the OE2-mock vs WT-mock comparison. These 24 genes form a high-confidence set which may be key in enhancing *B. cinerea* resistance in LsERF1 transgenic lines. This gene set includes LsERF1 itself, along with orthologues of ERF6, the chitin receptor LYK4, glyceraldehyde 3-phosphate dehydrogenase (GAPC1) and 15 non-coding RNA genes. ERF6 and LYK4 have both been demonstrated as positive regulators of *B. cinerea* resistance, while GAPC1 has known roles in redox homeostasis (Moffat et al. 2012; Ai et al. 2023; Moreno et al. 2021). Only 1 DEG was identified as downregulated in both OE1 and OE2, a PHYTOCYSTATIN family cysteine protease inhibitor (Labudda et al. 2016).

In addition to this high-confidence gene set, we identified 27 additional genes which met a reduced stringency criteria for differential expression in OE1 and were OE2 DEGs. These were all OE2-mock vs WT-mock DEGs but narrowly exceeded the OE1 p-value significance threshold (OE1 p-adjust 0.05-0.2). Despite this, they all had an absolute log2 fold change ≥ 1.5 in OE1 and exhibited the same differential expression direction as observed in OE2. This reduced stringency gene set contained orthologues of ethylene forming enzyme (EFE/ACO4), ERF4, a TIR-NBS-LRR receptor and several receptor-like kinases; receptor kinase 3 (RK3), cysteine-rich receptor-like kinase 8 (CRK8) and FERONIA (FER). Figure 4.3B shows the expression profiles of all overlapping OE1/OE2 mock DEGs and the reduced stringency gene set.

4.3.3 Higher over-expression of LsERF1 in OE2 induces up-regulation of jasmonic acid signalling and glucosinolate biosynthesis

We anticipate that the OE1 DEGs represent a minimal gene set sufficient to drive LsERF1-induced *B. cinerea* resistance. However, the additional DEGs identified in OE2 will offer further insights into genes which LsERF1 is able to directly or indirectly regulate at high expression levels. To understand whether these DEGs were enriched for specific biological functions, gene-ontology (GO) term analysis was performed using annotations of the Arabidopsis orthologues of each lettuce gene.

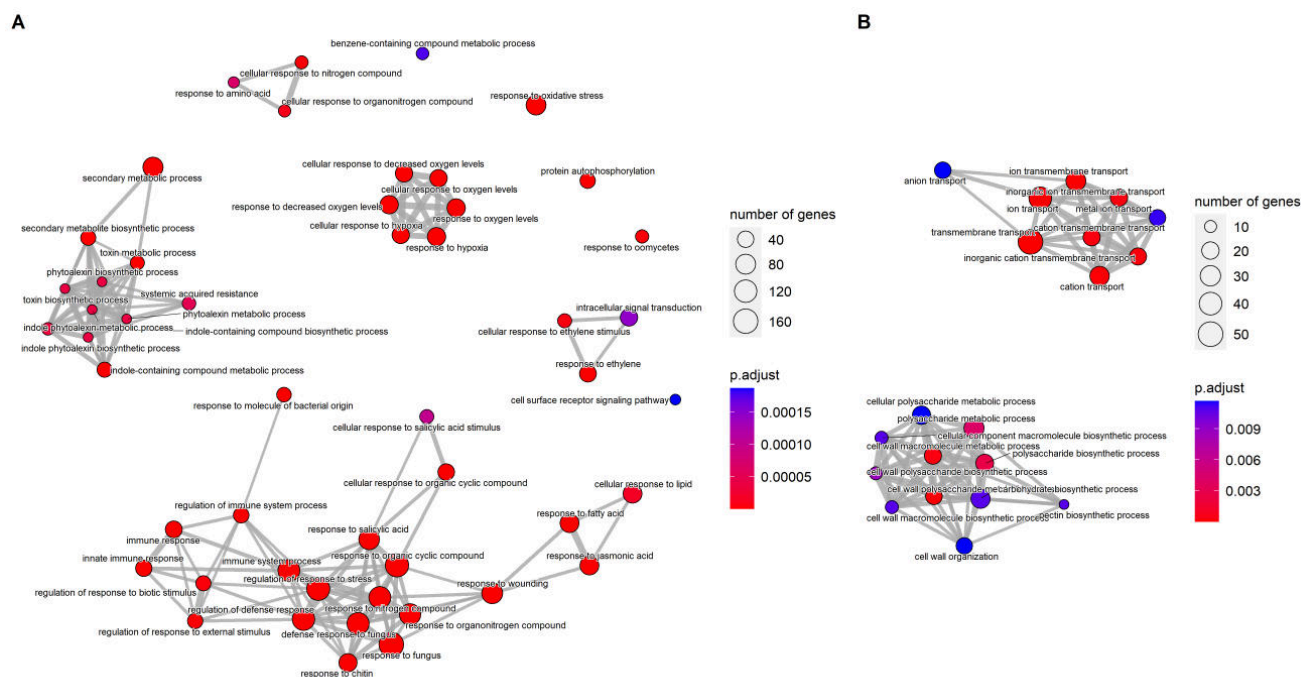


Figure 4.4: LsERF1 OE2-mock vs WT-mock GO-enrichment network plot. Network edges represent GO-terms containing $\geq 33\%$ overlapping Arabidopsis orthologues of DEGs. The size of the node represents the number of unique Arabidopsis orthologues of DEGs. Colour represents the statistical significance, of the enrichment, with red nodes showing higher significance. A) GO-enrich network for upregulated DEGs, B) GO-enrich network for downregulated DEGs

GO enrichment was performed separately on the unique Arabidopsis orthologues of up-regulated and down-regulated OE2-mock vs WT mock DEGs, with all Arabidopsis genes that had at least one expressed lettuce orthologue as the background. A GO-enrichment network plot for the top 50 OE2-mock up-regulated terms and top 20 OE2-mock down-regulated terms is shown in Figure 4.4A and Figure 4.4B respectively. Network edges are drawn between enriched GO-terms that share over 33% of annotated genes.

Figure 4.4A shows that OE2.mock-vs-WT.mock up-regulated DEGs are enriched for GO-terms such as "cellular response to ethylene stimulus" and "response to jasmonic acid". DEGs annotated with these GO-terms include orthologues of ACC synthase 6 (ACS6), ethylene insensitive 3 (EIN3), lipoxygenase 1 and (LOX1/3), jasmonate-zim domain 2 (JAZ2), WRKY33 and 21 ERF family transcription factors. These findings are consistent with the well-established role of AtERF1 in integrating jasmonic acid and ethylene (JA/ET) signalling (Lorenzo et al. 2003).

We also observed enrichment of "secondary metabolite biosynthetic process", "indole-containing compound metabolic process" and "toxin metabolic process" GO-terms in OE2.mock-vs-WT.mock up-regulated DEGs. These results suggest that LsERF1 may regulate the production of defence metabolites. These DEGs include 61 putative Cytochrome P450s, 3 orthologues pleiotropic drug resistance 12, and a camalexin secretion transporter (He et al. 2019). Orthologues of High Indolic Glucosinolate 1 (HIG1/MYB54) and Superroot 1 (SUR1), both

of which are involved in glucosinolate biosynthesis, were also upregulated in OE2 (Gigolashvili et al. 2007; Mikkelsen et al. 2004).

Interestingly "cellular response to salicylic acid stimulus" was also found to be an enriched term among the LsERF1-OE2 upregulated DEGs. This was unexpected due to the well-characterised reciprocal inhibition between JA/ET and SA signalling (Thaler et al. 2012; Caarls et al. 2015). Notably, five lettuce orthologues of WRKY70, a "convergence node" activating SA and repressing JA signalling (Li et al. 2004; Li et al. 2017), were up-regulated. Orthologues of other key SA regulators such as Nonexpressor of PR1 (NPR1), CaM-binding protein 60-like g (CBP60g), SAR Deficient 1 (SARD1) and Enhanced Disease susceptibility 1 (EDS1) were also up-regulated (Peng et al. 2021). However, neither SA marker gene PR1 nor SA biosynthetic genes isochorismate synthase 1/2 (ICS1/2) were not identified as DEGs, this may be due to an ET/JA-SA cross-talk mechanism. In Arabidopsis EIN3 has been shown to repress expression of PR1 and ICS2 (Chen et al. 2009).

Figure 4.4B identifies two clear groups of LsERF1-OE2 down-regulated enriched GO-terms; ion transporters and cell wall polysaccharide biosynthesis. Arabidopsis nitrate transporter mutants (*nrt1.5-5*) show upregulation of JA signalling genes (e.g. PDF1.2b, TPS4, VSP2, LOX2) (Drechsler et al. 2015), suggesting negative feedback of nitrate accumulation of JA signalling. Lettuce orthologues of NRT1.5 and NRT1.1 were identified as downregulated in OE2.

In (Pink et al. 2023) we reported that five sesquiterpene lactone biosynthetic enzymes (LsFPS1, LsGAS1, LsGAS2, LsGAO and LsCOS1) (Sessa et al. 2000; Nguyen et al. 2010; Ikezawa et al. 2011; Kwon et al. 2022)) were all identified in a single co-expression module (Module 1). LsERF1 and 39 additional cytochrome p450s were also identified in Module 1. Here we identify that all five STL biosynthetic enzymes and 27 out of the 39 uncharacterised cytochrome P450s from Module 1 were upregulated in OE2 mock, none were downregulated, none were differentially expressed in either OE1 or OE2 after *B. cinerea* infection (Fig 4.5). Though, despite all STL genes biosynthetic being upregulated in OE2, they were all further upregulated upon *B. cinerea* infection, demonstrating LsERF1-independent regulation is required to reach full activation. Interestingly, a GRN-hub that was also predicted to regulate all five Module 1 STL biosynthetic genes (LsbHLH) was not differentially expressed by LsERF1 overexpression and therefore may be the additional LsERF1-independent component required for full STL biosynthetic enzyme activation.

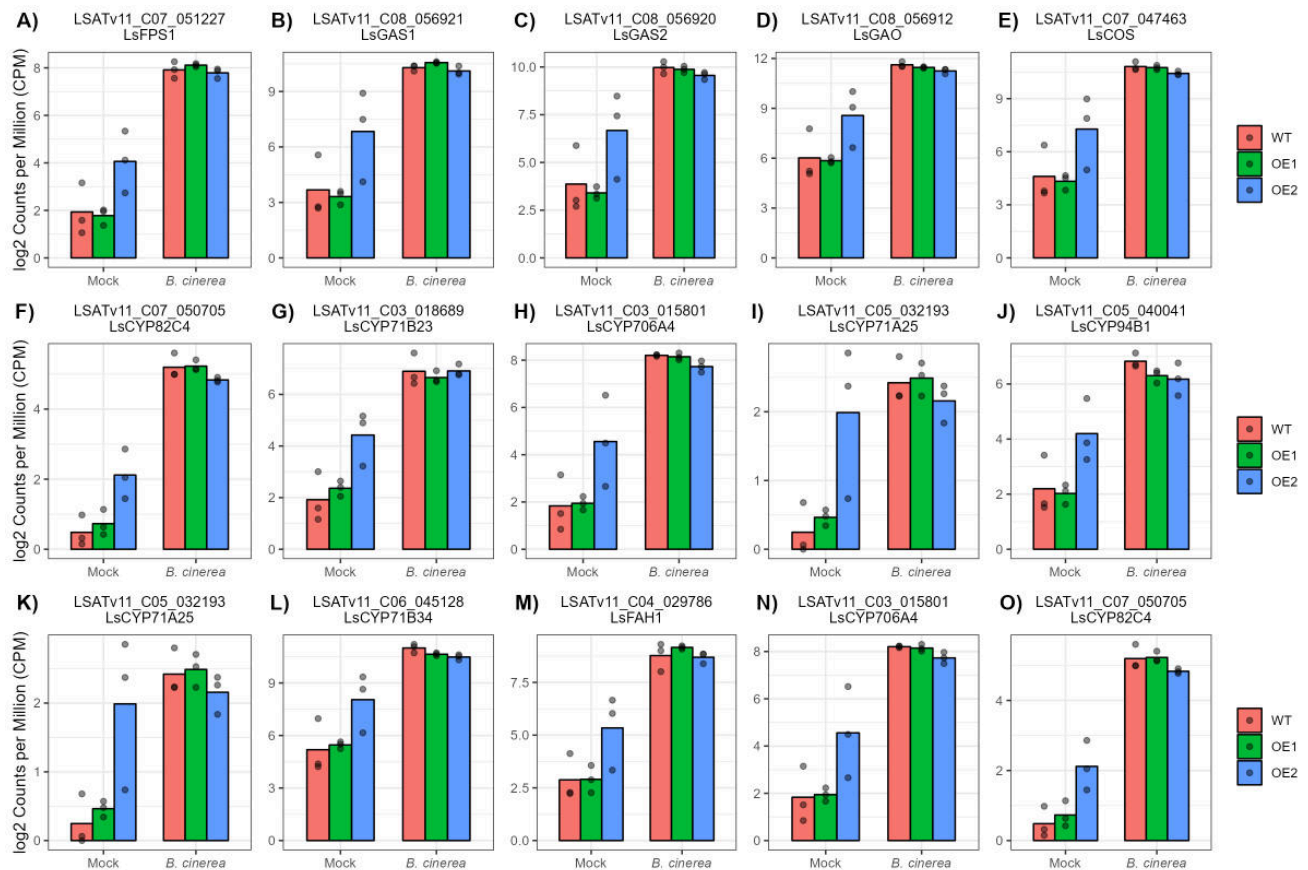


Figure 4.5: LsERF1-OE expression of lettuce sesquiterpene lactone (STL) biosynthetic enzymes (A-E) and the top 10 most significantly upregulated uncharacterised cytochrome p450s from Wigwam Module 1 (Pink et al. 2023) (F-O)

4.3.4 Lettuce necrotroph gene regulatory network performs well as a predictor of LsERF1 DEGs

Previously we constructed a GRN, modelling transcriptomic regulation in response to *B. cinerea* and *S. sclerotiorum* infection in lettuce (Pink et al. 2023). Having used a random-forest based modelling approach, we obtained importance scores quantifying the relative confidence in all pairwise TF-Target gene interactions. A stringent threshold of just the top 1% of high-confidence edges was used to construct the final GRN.

The GRN was modelled on the expression of 4362 lettuce genes, of these 4010 (out of 23811) had detectable expression in the LsERF1-OE RNAseq dataset, and 842 of these were identified as an LsERF1 DEG. We, therefore, assessed the model's ability to distinguish the 842 DEGs from the other 3168 genes that were present in the network with detectable expression in the LsERF1 RNAseq, but not differentially expressed.

Firstly, we utilised a binomial generalised linear model (GLM), modelling the probability of a gene being a DEG using solely the Importance percentile of the genes' predicted regulatory by LsERF1 (Figure 4.6). The importance percentile was shown to be a highly significant term ($p = 7.5 \times 10^{-15}$).

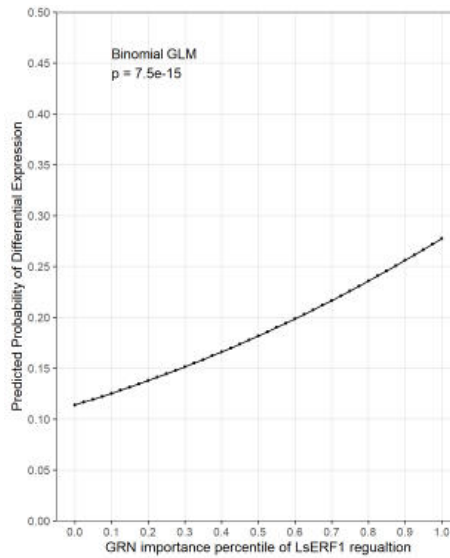


Figure 4.6: Model-predicted probability of a given gene being identified as a differentially expressed gene (DEG) in p35S::LsERF1 transgenic lines based on GRN importance score of its interaction with LsERF1. X-axis represents the importance score percentile of the predicted regulation by LsERF1. Y-axis shows the GLM predicted probability of a gene being differentially expressed. Points show GLM predictions made at specific percentile values.

Three key metrics are used to evaluate GRN predictions; precision (proportion of predicted targets that were DEGs), recall (proportion of DEGs that were predicted targets) and F1 score (harmonic mean of precision and recall).

Unlike a DNA-binding experiment such as yeast-1-hybrid or chromatin-immunoprecipitation (ChIP) which identify direct TF-targets, we expect that many of the expression changes observed in our RNA-seq experiment are not caused by LsERF1 directly. Instead, they may be driven by other transcription factors that are themselves regulated by LsERF1. For this reason, we considered two categories of GRN-predicted targets for LsERF1; “direct only” (first-order) and “including indirect” (first and second-order). The latter group includes targets of other transcription factors that are predicted to be under the direct regulation of LsERF1 as well as direct targets.

In Pink et al. 2023, an outdegree threshold of the top 1% highest confidence edges was used to construct the final GRN. To determine if this threshold is optimal, we evaluate predictive performance of the GRN when applying outdegree thresholds from the top 0.2-10% of highest confidence edges. Figure 4.7A shows the number of predicted GRN targets of LsERF1 (direct only or including indirect) at each outdegree threshold. As the threshold becomes more stringent, the number of direct-only targets decreases steadily, but targets including indirect edges remain high until the top 6% threshold, after which they decrease rapidly.

Next we, analyse the precision of our GRN's DEG prediction compared to random prediction (Figure 4.7B). Direct-only target predictions consistently out-performed random predictions across all outdegree thresholds. By contrast, targets including indirect edges are indistinguishable from random guessing at outdegree thresholds over 6%, but precision improves significantly at thresholds under 6% and competes with direct-only precision at under 2%.

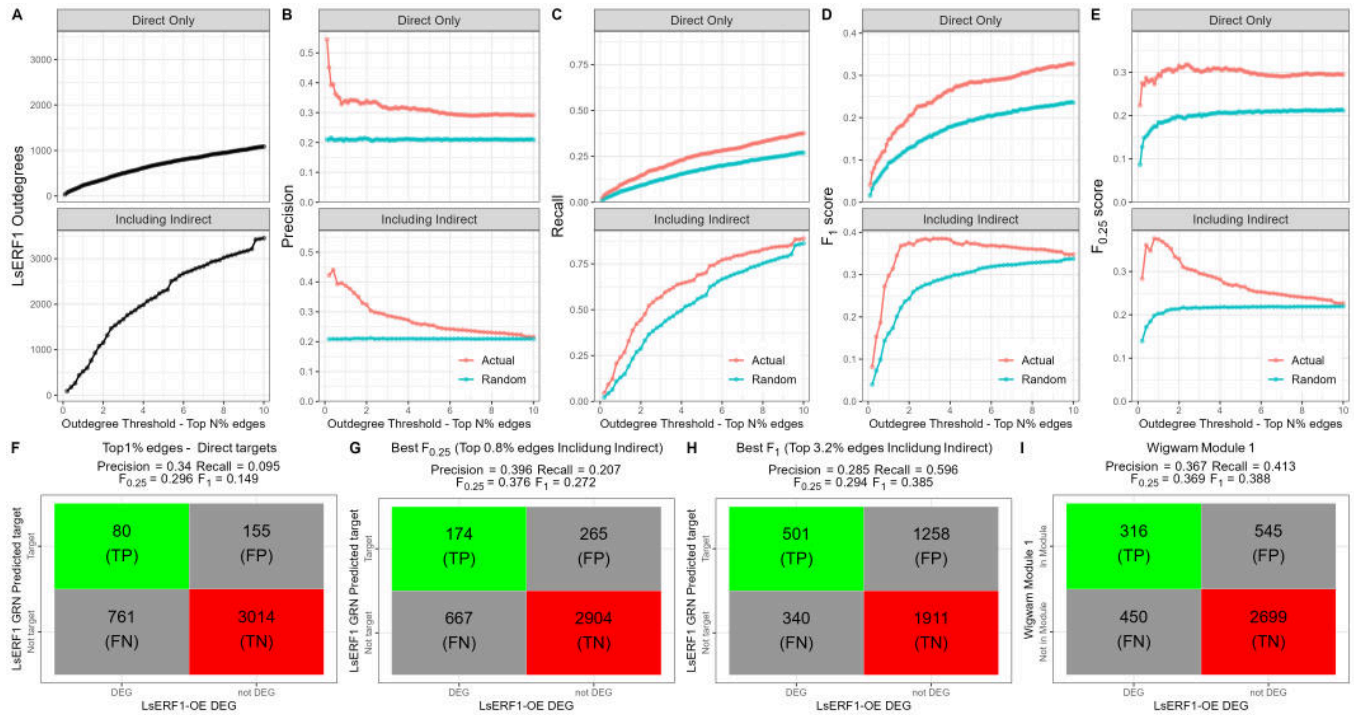


Figure 4.7: **(A-D)** Top panel evaluates the network using solely direct LsERF1 targets. The bottom panel evaluates network performance considering both direct targets and second-order indirect targets (targets of a predicted LsERF1-regulated TF) as LsERF1 targets. These are designated as "Direct and Indirect targets" **A)** Number of predicted LsERF1 GRN targets. X-axis shows the Outdegree threshold, reflecting the upper percentage limit for an edge to be considered an Outdegree. **B)** Evaluation of GRN Precision (proportion of predicted targets that were identified as DEGs). The Outdegree threshold is shown on the X-axis, and Precision score is shown on the Y-axis. Actual predictions are shown in red, while randomly shuffled predictions are displayed in blue. **C)** Evaluation of GRN recall (proportion of DEGs which were predicted as GRN targets), Outdegree threshold on X-axis, recall value on Y-axis (actual predictions = red, random = blue). **D)** Evaluation of F1 score (harmonic mean combining precision and recall). Outdegree threshold on X-axis, F1 on Y-axis (actual predictions = red, random = blue). **E)** Evaluation of F_{0.25} score, an F1 variant that considers precision four times more important as recall **F-I)** Confusion matrices visualising DEG predictive performance - showing the number of true positives (TP), false positives (FP), false negatives (FN) and true negatives (TN). X-axis shows whether a gene was identified as DEG or not, Y-axis whether a gene was a predicted target or not. **E)** Confusion matrix showing prediction of DEGs from top 1% of LsERF1 direct GRN targets. **F)** Confusion matrix showing prediction of DEGs from top 0.8% of including indirect targets (optimal F_{0.25}-score. **G)** Confusion matrix showing prediction of DEGs from top 3.2% of including indirect GRN targets (optimal F1 score). **H)** Prediction of DEGs using genes identified in the same co-expression module as LsERF1.

As expected, recall declines as threshold stringency increases. However, both direct-only and including-indirect edges have better recall than random guessing even at stringent thresholds. The top 2% of including-indirect edges are able to recall 54% of all DEGs, whereas random guessing at a 2% threshold only

identifies 36% of DEGs (Figure 4.7C).

To identify the optimal threshold which reduces both false positives (FP - predicted targets not DEG in LsERF1 RNAseq) and false negatives (FN - not predicted as a target, but was DEG in RNAseq), we use the F1-score. F1-score provides a trade-off between precision and recall, penalising both FPs and FNs (Figure 4.7D). The direct-only targets F1-score is penalised by lower recall values, as the precision gains at more stringent thresholds do not out-weight the increased FN's. That being said, direct-only F_1 at all thresholds outperforms random guessing. Interestingly, including indirect F_1 is highest using the top 3% edges - this offers an optimal balance where both precision and recall are maximised.

In addition to F_1 , we compute the $F_{0.25}$ score, a variant of the F1 score that considers precision four-times as important as recall. This is because we wish to place a higher emphasis on reducing FPs (genes that we call a target, but were not identified as a DEG). We do not expect our current model to be capable of identifying all DEGs, therefore, we place less importance on FN's (DEGs that we didn't identify as a target). Again, our GRN consistently outperforms random guessing for this metric, but at high-confidence thresholds, including-indirect targets performs very well (Figure 4.7E).

Confusion matrices provide a breakdown of the number of true positives (TPs), false positives (FPs), true negatives (TNs) and false negatives (FNs) respectively. Figure 4.7F-I shows confusion matrices for the prediction of LsERF1 DEGs using the top 1% direct edges, top 0.8% including indirect edges (optimal $F_{0.25}$), top 3.2% including-indirect edges (optimal F_1) and Wigwam Module 1 genes. "Wigwam Module 1" refers to a co-expression module of 942 genes with highly similar co-expression profiles across both *B. cinerea* and *S. sclerotiorum* infection and includes LsERF1 (Pink et al. 2023). Genes within this co-expression module predict LsERF1-OE DEGs with a precision of 0.367, an F_1 score of 0.388 and an $F_{0.25}$ of 0.369, backed by 316 TPs. The top 0.8% of include-indirect targets outperform Module 1 for both precision and $F_{0.25}$. These results demonstrate that our GRN outperforms both random guessing and co-expression modules at precisely inferring *in vivo* transcriptomic regulatory events.

4.3.5 WRKY7: GRN mediated selection functional orthologues

Given that our GRN has now been validated as a reliable resource for the identification of lettuce defence regulators, such as LsERF1, in addition to robust prediction of *in vivo* targets. We then used the network to identify further defence regulators.

Closely related lettuce group II-d WRKY transcription factors, Lsat_1_v5_gn_4_164440, Lsat_1_v5_gn_4_127960, Lsat_1_v5_gn_1_22221 (LsWRKY7A, LsWRKY7B and LsWRKY15 respectively) were all identified as differentially expressed in response both *B. cinerea* and *S. sclerotiorum* (Pink et al. 2023). They exhibit a strikingly similar time-series expression profile, and demonstrate moderate co-expression across a diverse lettuce panel, albeit not as tight as the time-series (Fig 4.8a-b),

A maximum likelihood phylogenetic tree of all Arabidopsis and Lettuce group II-d WRKYs (Fig 4.8c) revealed low bootstrap values on branches which split AtWRKY7, AtWRKY15 and their lettuce orthologues. Therefore, we're unable to confidently assign a single lettuce orthologue of AtWRKY7 or AtWRKY15 using phylogenetics alone.

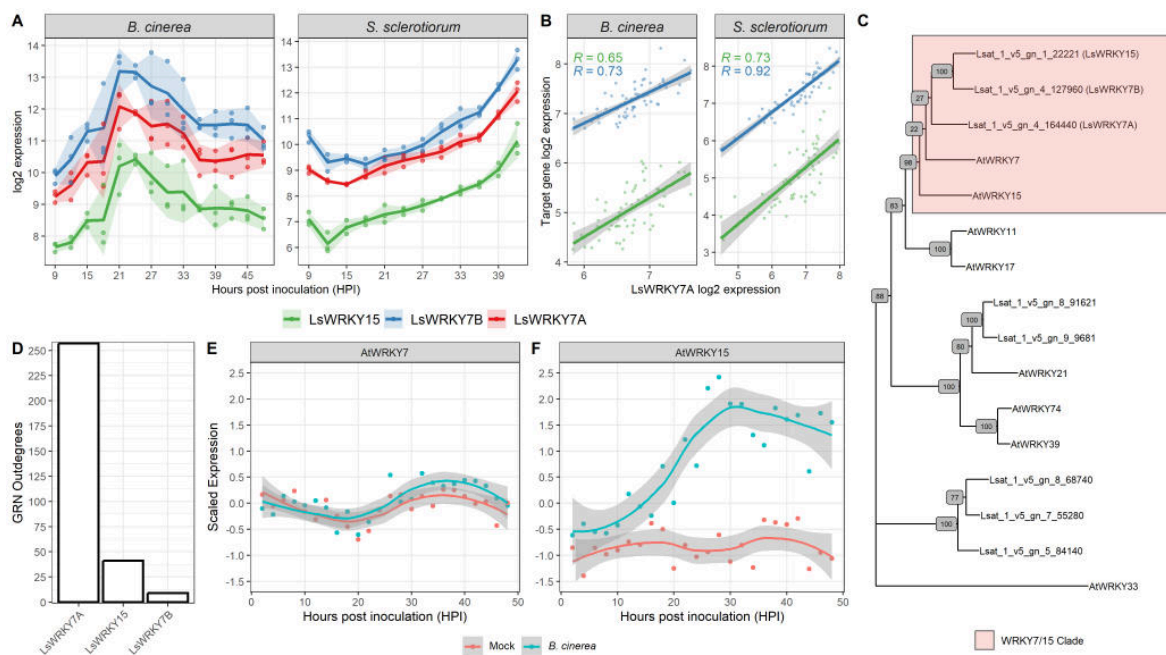


Figure 4.8: **A)** Lettuce time-series expression profiles of Lsat_1_v5_gn_4_164440 (LsWRKY7A; red), Lsat_1_v5_gn_4_127960 (LsWRKY7B; blue) and Lsat_1_v5_gn_1_22221 (LsWRKY15; green) in response to *B. cinerea* (left-panel) and *S. sclerotiorum* (right-panel) hours post inoculation (HPI) shown on the X-axis and log2 counts per million expression shown on the y-axis. **B)** Co-expression of LsWRKYs across diverse lettuce accessions after infection with *B. cinerea* (left-panel) or *S. sclerotiorum* (right-panel) (Pink et al. 2022). log2 LsWRKY7A expression shown on the x-axis, log2 expression of either LsWRKY7B (blue points) or LsWRKY15 (red points) are shown on the Y-axis. Pearson's correlation coefficient (R) for gene-coexpression is displayed, similarly colour coded as the points. **C)** A 2000-bootstrap maximum likelihood phylogenetic tree of Arabidopsis group IId WRKYs and their putative lettuce orthologues (Eulgem et al. 2000; Reyes-Chin-Wo et al. 2017), using AtWRKY33 (group I) as an outgroup. Bootstrap values are shown. **D)** Bar graph displaying the number of predicted GRN targets for each LsWRKY7/15 orthologues. **E-F)** Time-series expression of AtWRKY7 and AtWRKY15 in response to mock (red) or *B. cinerea* inoculation (Windram et al. 2012). Hours post inoculation (HPI) shown on the X-axis and scaled expression is shown on the y-axis. Individual biological replicates are shown with a loess-smoothed regression line.

However, these lettuce WRKYs exhibit large differences in predicted GRN targets (Fig 4.8d). Notably, LsWRKY7A emerged as a large hub with 257 putative targets compared to 41 and 9 targets in LsWRKY15

and LsWRKY7B respectively. These results suggest that LsWRKY7A may function as the primary defence regulator within this clade during lettuce necrotrophic fungal infection.

In Arabidopsis, AtWRKY7 and AtWRKY15 are both implicated in plant-pathogen interactions with *B. cinerea*. AtWRKY7 transcripts are targeted by a fungal small RNA causing increased susceptibility, whereas plants overexpressing AtWRKY7 show increased *B. cinerea* resistance (Wang et al. 2017c). AtWRKY15 is transcriptionally up-regulated after *B. cinerea* infection (Fig 4.8e-f; data from Windram et al. 2012). These results along with our lettuce GRN predictions, suggest that the WRKY7/15 clade TFs activate the expression of unknown defence genes to enhance resistance, and LsWRKY7A may act as the main regulator of these genes in lettuce.

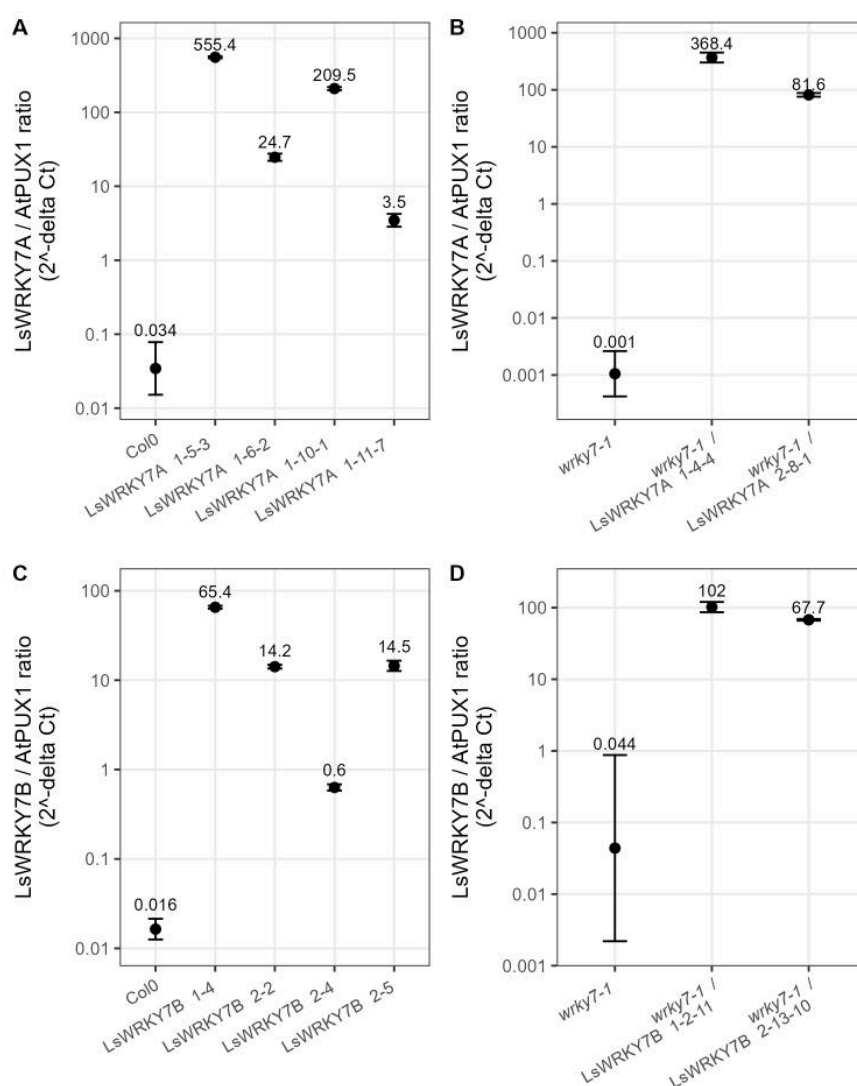


Figure 4.9: delta Ct transgene expression ratio compared to an endogenous reference (AtPUX1) in LsWRKY7 transgenic lines. Y-axis is on a log10 scale. **A)** Col-0/p35S::LsWRKY7A homozygous T3's, **B)** *wrky7-1*/p35s::LsWRKY7A homozygous T3's, **C)** Col-0/p35s::LsWRKY7B BASTA-selected T2 lines and **D)** *wrky7-1*/p35s::LsWRKY7B homozygous T3's

To test this hypothesis, we generated transgenic Arabidopsis lines constitutively expressing LsWRKY7A or LsWRKY7B under the p35s promoter in both Col-0 and the *wrky7-1* mutant background. Independent transgenic lines were selected for Mendelian segregation of herbicide resistance in the T₂ generation were profiled for transgene expression (Fig 4.9).

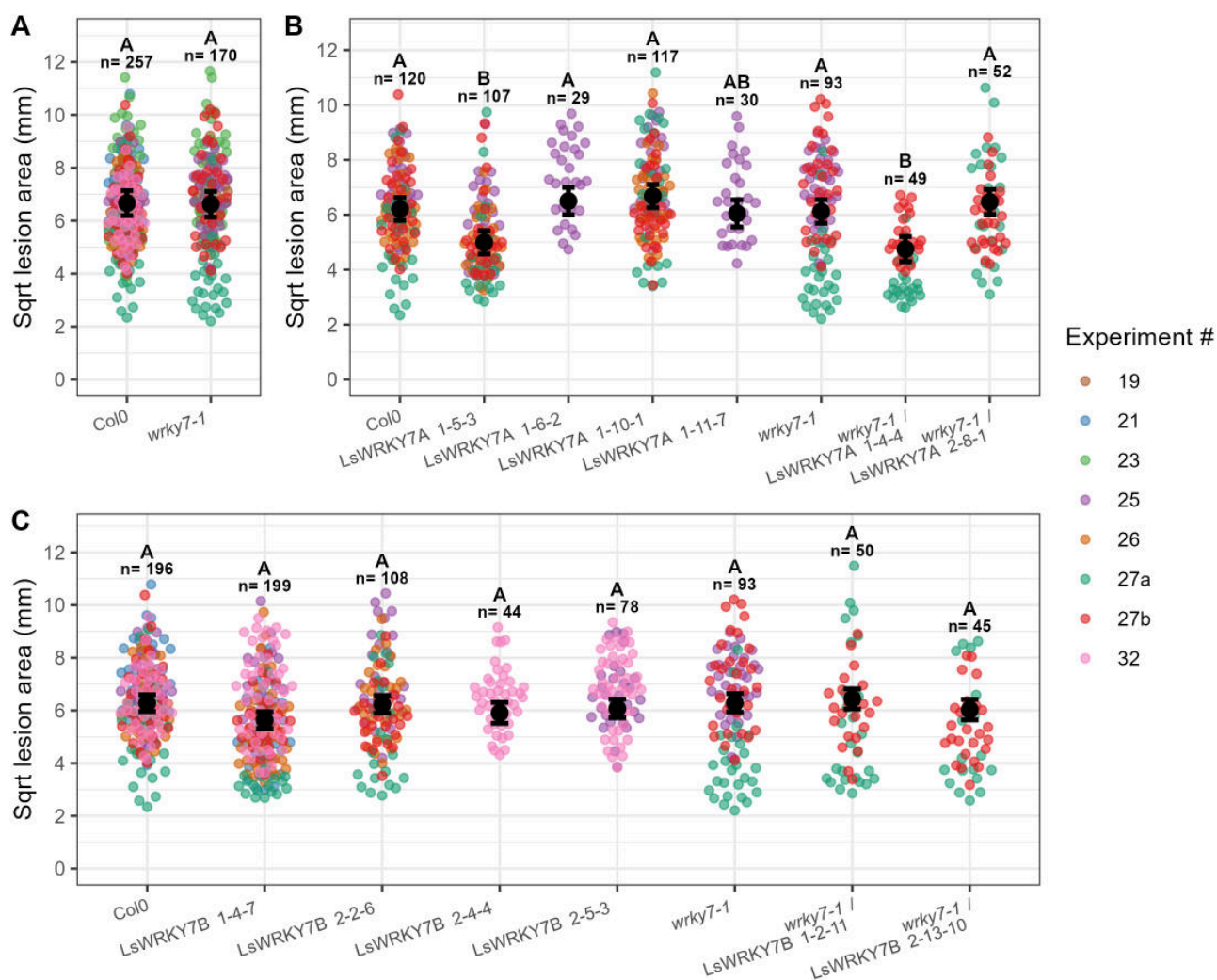


Figure 4.10: *B. cinerea* detached leaf assay susceptibility testing of LsWRKY7 expressing transgenic Arabidopsis lines. Raw data values are shown by coloured points, with colour denoting the experimental replicate. Black points represent the predicted least-squares mean sqrt lesion area derived from a linear-mixed effect model (REML), with error bars indicating the REML standard error. Letters represent statistical significance groups (p < 0.05) determined by a post-hoc Tukey HSD test performed on the REML model using lmerTest and predictmeans R packages. 'n' represents the total number of individual lesions measured across all experiments. **A)** Col-0 and *wrky7-1* mutant only, **B)** LsWRKY7A transgenic lines and **C)** LsWRKY7B transgenic lines

Detached leaf assays were used to quantitatively measure *B. cinerea* susceptibility of different Arabidopsis lines. We were unable to identify any differences in *B. cinerea* susceptibility between Col-0 and *wrky7-1* mutants across many independent experiments (Fig 4.10a). These results are contradictory to previous work which reported increased susceptibility in *wrky7-1* (Wang et al. 2017c). However, the two highest LsWRKY7A expressing transgenic lines, LsWRKY7A 1-5-3 and *wrky7-1*/LsWRKY7A 1-4-4, both display enhanced *B.*

cinerea resistance compared to their respective backgrounds across multiple experimental repeats (Fig 4.10b). No LsWRKY7B expressing lines displayed significantly different lesion sizes from either Col-0 or *wrky7-1* (Fig 4.10c). These findings suggest that our GRN was able to identify LsWRKY7A as the functional orthologue, which phenocopies AtWRKY7 overexpression lines, demonstrating enhanced *B. cinerea* resistance (Wang et al. 2017c). However, LsWRKY7B which was not predicted as a GRN does not show this phenotype.

As we were unable to isolate a second Col-0/LsWRKY7A transgenic line which showed altered *B. cinerea* resistance, we used a modified detached leaf assay in *N. benthamiana* leaves to further probe the defence function of LsWRKY7A. 5-week *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* harbouring p35S::GFP, p35S::LsERF1 or p35S::LsWRKY7A constructs. Leaves were detached 3 days post-infiltration and inoculated with *B. cinerea*. Both LsERF1 and LsWRKY7A infiltrated leaves show dramatically reduced lesion size compared to GFP (Fig 4.11), providing further evidence that high expression of LsWRKY7A increases *B. cinerea* resistance.

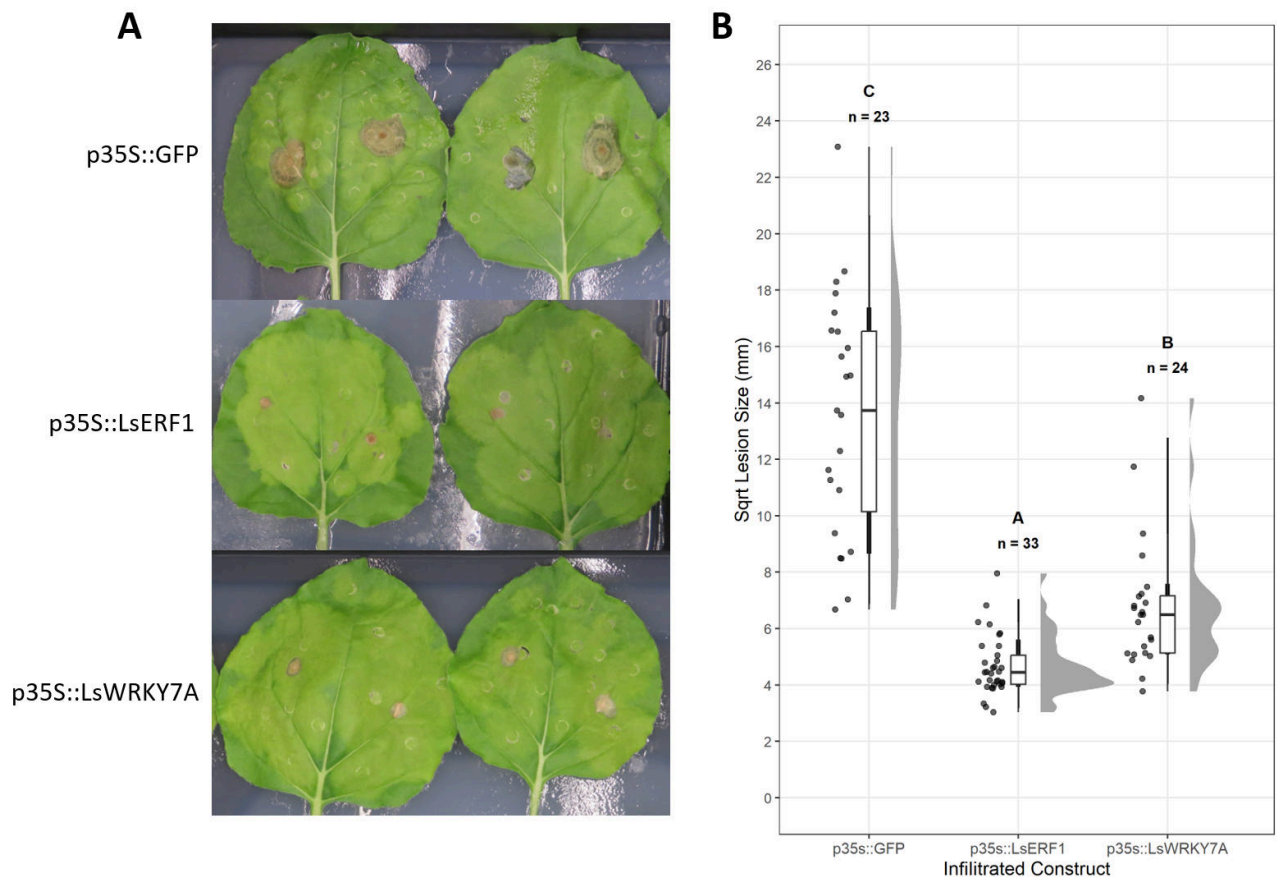


Figure 4.11: Transient *B. cinerea* susceptibility assay in *N. benthamiana* leaves. 5-week *N. benthamiana* leaves are infiltrated with 0.5OD₆₀₀ total *Agrobacterium* using a needleless syringe (0.25OD₆₀₀ each p19/GFP or 0.167 OD₆₀₀ each p19/GFP/gene of interest). 3 days post infiltration, GFP is inspected under UV light, well-infiltrated leaves are detached, and infected with 2 × 15 μL droplets of 2 × 10⁵ spores/mL of *B. cinerea* inoculum diluted in 50% grape juice. 4 days post *B. cinerea* infection, lesions area is measured using ImageJ. Representative photos shown in (A). (B) Quantification of (A), infiltrated construct on X-axis, Square-root *B. cinerea* lesion size on Y-axis. Individual measurements are shown as points, as well as the distribution curve. N represents the number of individual lesions measured. Letters represent statistical significance grouping (Tukey HSD $p < 0.05$).

4.3.6 LsMYB15: a putative lignin regulator does not impact *B. cinerea* resistance in Arabidopsis

Lsat_1_v5_gn_3_120520 (LsMYB15) was identified as a large network hub with 378 predicted targets, and is a putative ortholog of AtMYB15 (Reyes-Chin-Wo et al. 2017, Fig 4.12a). AtMYB15 is required for of flg22-induced lignification and enhances resistance to *Pseudomonas syringae* DC3000 (Chezem et al. 2017). MYB15 orthologues in an Asteraceae hybrid flower, *Chrysanthemum × morifolium*, CmMYB15 and CmMYB15-like have both been shown to positively regulate lignin biosynthesis and aphid resistance (An et al. 2019; Li et al. 2023a). Predicted GRN targets of LsMYB15 include cinnamyl-alcohol dehydrogenase orthologues (LsCAD4, LsCAD8) and basic peroxidase 52 (LsPRX52) all of which are involved in lignin biosynthesis in Arabidopsis (Kim et al. 2004; Kim et al. 2007b; Fernández-Pérez et al. 2015). LsMYB15 shows tight co-expression with these lignin biosynthetic enzymes across the time-series, but less so across

diversity set panels (Fig 4.12b). Therefore, we hypothesise that LsMYB15 may be driving lignification in response to necrotroph infection in lettuce, which may act as a barrier for pathogen infection.

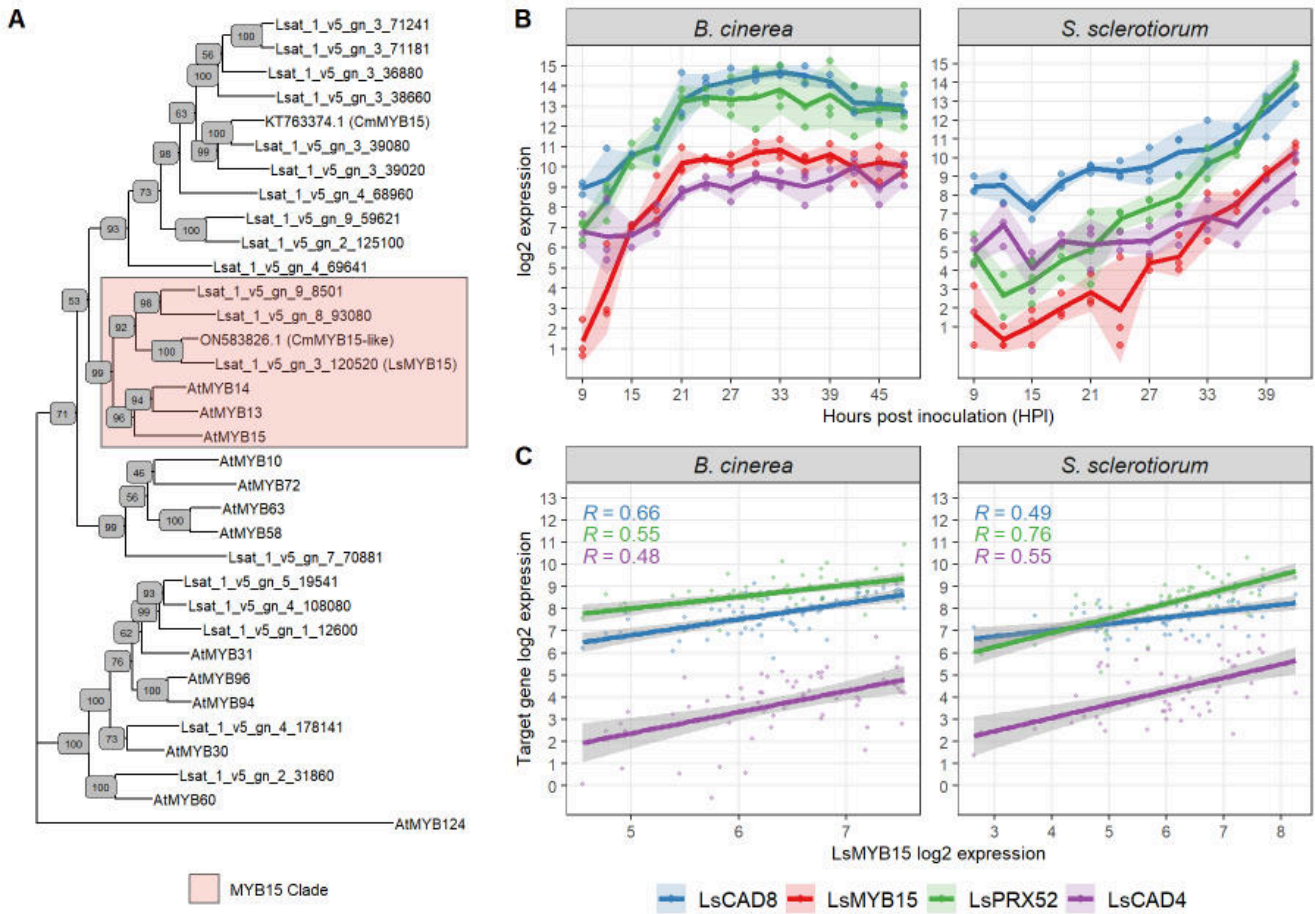


Figure 4.12: **A**) 2000-bootstrap maximum likelihood phylogenetic tree containing Arabidopsis R2-R3 MYB subgroups 1-3, their putative lettuce orthologues, *Chrysanthemum* MYB15 orthologues and AtMYB124 as an outgroup. **B**) Expression of Lsat_1_v5_gn_3_120520 (LsMYB15 - red), Lsat_1_v5_gn_9_111281 (LsCAD4 - purple), Lsat_1_v5_gn_2_69560 (LsCAD8 - blue) and Lsat_1_v5_gn_5_175321 (LsPRX52 - green) after *B. cinerea* (left panel) and *S. sclerotiorum* (right panel) infection, mock-expression not shown. Hours post inoculation on X-axis, log₂ expression on Y-axis. Points show individual biological replicates, line is drawn through time-point mean, shaded area shows the 95% confidence interval. **C**) Lettuce diversity-panel co-expression (data from Pink et al. 2022), LsMYB15 log₂ expression is shown on X-axis, log₂ expression of putative target genes is shown on Y-axis (same genes and colours as Panel B). Expression after *B. cinerea* infection on left panel, and expression after *S. sclerotiorum* infection on right panel. Pearson's correlation coefficient for gene expression shown, with colour denoting which target gene the co-expression refers to.

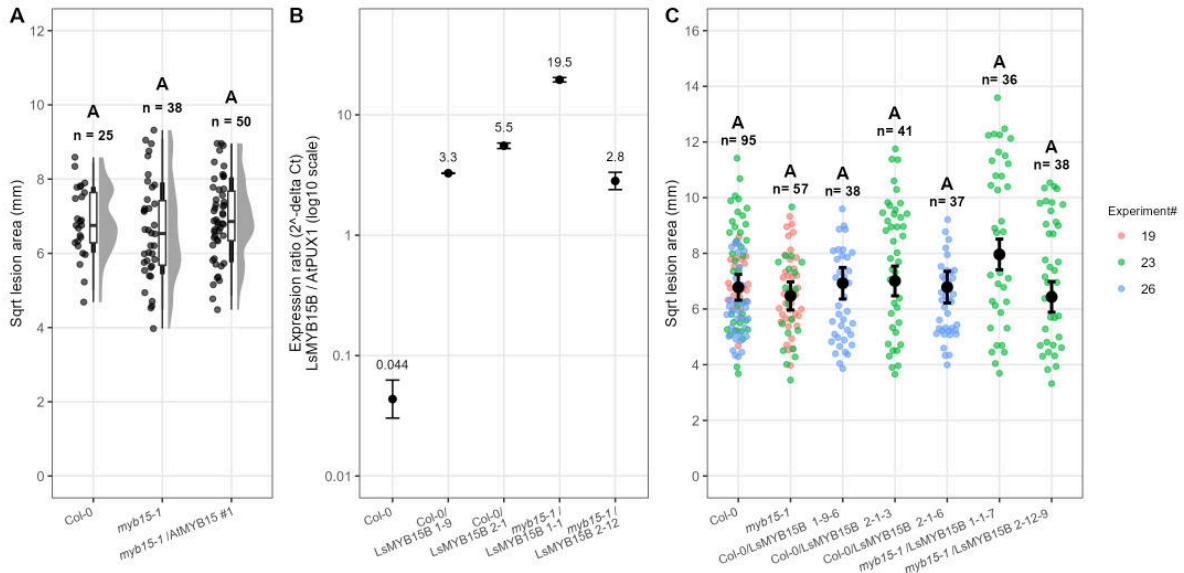


Figure 4.13: **A)** *B. cinerea* detached leaf assay of Col-0, *myb15-1* and *myb15-1/AtMYB15 #1* (generated by Chezem et al. 2017). Photos were taken of developing lesions at 72hpi, and measured with ImageJ. Raw data points, boxplots, and distribution of individual measured lesions are shown. Letters represent statistical significance groupings ($p < 0.05$) **B)** delta Ct transgene expression ratio compared to an endogenous reference (AtPUX1) in Col-0/p35S::LsMYB15 and *myb15-1*/p35S::LsMYB15. Tissue was collected from pooled samples of 10-day T₂ seedlings grown on 1/2 MS + BASTA plates. Col-0 seedlings were grown in parallel on 1/2 MS plates without BASTA. **C)** *B. cinerea* detached leaf assay susceptibility testing of LsMYB15 expressing transgenic Arabidopsis lines. Raw data values are shown by coloured points, with colour denoting the experimental replicate. Black points represent the predicted least-squares mean sqrt lesion area derived from a linear-mixed effect model (REML), with error bars indicating the REML standard error. Letters represent statistical significance groups ($p < 0.05$) determined by a post-hoc Tukey HSD test performed on the REML model using lmerTest and predictmeans R packages. 'n' represents the total number of individual lesions measured across all experiments.

To test whether MYB15-dependent PTI-induced lignification impacts necrotrophic defence, we carried out *B. cinerea* detached leaf assays on *myb15-1* mutants and Arabidopsis complement lines (*myb15-1*/p35S::AtMYB15#1) (Chezem et al. 2017). No significant differences in *B. cinerea* defence were observed between Col-0, *myb15-1* mutants, or the complement lines (Fig 4.13a). These results suggest that either AtMYB15-induced lignification does not occur in response to *B. cinerea* or it does not affect pathogen growth.

Next, we generated p35S::LsMYB15 transgenic Arabidopsis lines in Col-0 and *myb15-1* mutant background. Independent transgenic lines showing 3:1 Mendelian segregation in the T₂ generation were profiled for transgene expression (Fig 4.13b). *B. cinerea* susceptibility assays were conducted on homozygous T₃ plants, which showed no differences in lesion area between Col-0, *myb15-1* and any of the LsMYB15-expressing lines (Fig 4.13c).

These results indicate that neither orthologue of MYB15 impacts *B. cinerea* defence in Arabidopsis. Despite this, LsMYB15 may still act as a functional orthologue of AtMYB15 or CmMYB15-like (Fig 4.12a). Both AtMYB15 and CmMYB15-like positively regulate defence against *P. syringae* DC3000 and aphid attack respectively by inducing lignification (Chezem et al. 2017; Li et al. 2023a).

4.3.7 Putative lettuce unfolded protein response regulators are network hubs

Lsat_1_v5_gn_5_157880 (LsBZIP17) and Lsat_1_v5_gn_2_3181 (LsBZIP60) have been identified as putative orthologues of unfolded protein response (UPR) regulators AtBZIP17 and AtBZIP60 (Nawkar et al. 2018). Both lettuce orthologues were identified as large GRN hubs with 131 and 82 predicted targets respectively. The UPR is activated by environmental stresses such as heat shock or pathogen infection, leading to an accumulation of misfolded proteins in the endoplasmic reticulum (ER). UPR then results in the activation of protein-folding chaperones.

A group of conserved BZIP transcription factors with inactivating C-terminal transmembrane regions control UPR activation. Upon accumulation of misfolded proteins BZIP17/BZIP28 (orthologous to human ATF6) undergo proteolytic cleavage, releasing the N-terminal DNA-binding domain, allowing translocation to the nucleus (Ye et al. 2000a; Iwata et al. 2017). BZIP60 (orthologous to human XBP1 and yeast HAC1) undergoes cytoplasmic splicing of a stem-loop 23bp intron. This splicing event induces a frameshift mutation in the spliced variant, resulting in the loss of the transmembrane domain (Yoshida et al. 2001; Nagashima et al. 2011). BZIP60 is highly conserved across distant species, such that AtBZIP60 expression in *hac1* mutant yeast strains rescues the UPR-deficiency phenotype (Zhang et al. 2015b), suggesting that BZIP60 function will likely be conserved between Arabidopsis and lettuce.

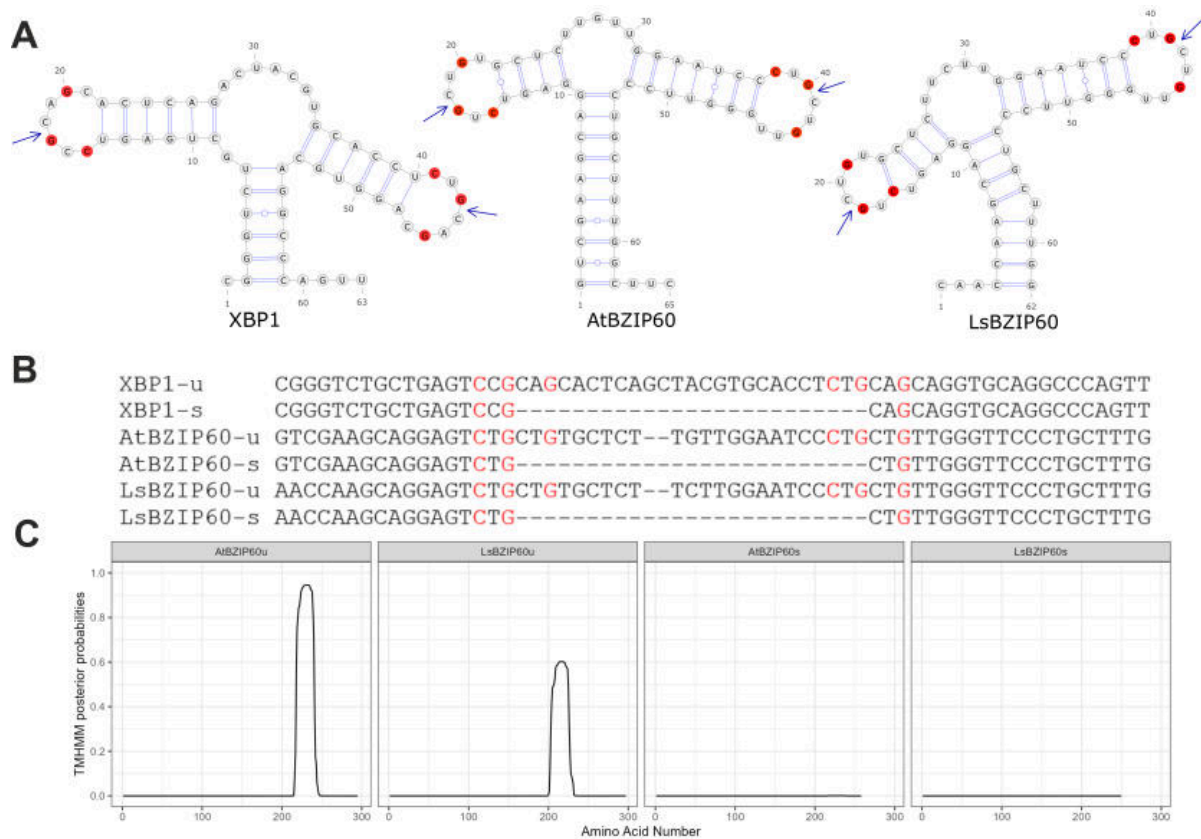


Figure 4.14: **A**) RNA secondary structure predictions of XBP1/BZIP60 splices introns and flanking sequences, structure prediction by RNAfold (Mathews et al. 2004), visualised using VARNA (Darty et al. 2009). Red nucleotide denote the conserved residues within the CNGNNG consensus sequence. Blue arrows denote the IRE1 cut-site. **B**) Multiple sequence alignment of Human XBP1, Arabidopsis BZIP60 and lettuce BZIP60 unspliced (-u) and spliced (-s) variants. Conserved residues of the CNGNNG sequence are coloured in red. **C**) TMHMM (transmembrane hidden markov model; Krogh et al. 2001) predicted transmembrane probability of residues along AtBZIP60 and LsBZIP60 (spliced and unspliced).

Inositol-requiring enzyme 1 (IRE1) performs BZIP60 splicing, recognising a specific stem-loop structure and cutting at CNGNNG consensus sites. These consensus sites are conserved in LsBZIP60 suggesting it may also be spliced by IRE1 (Fig 4.14a-b). The unspliced LsBZIP60 variant (LsBZIP60u) contains predicted transmembrane domains towards the C-terminus, which is absent in the spliced variant (LsBZIP60s) (Fig 4.14c). These results suggest that IRE1 splicing will facilitate nuclear translocation of LsBZIP60.

To evaluate the role of LsBZIP17 and LsBZIP60 in plant defence, we attempted to generate transgenic Arabidopsis constitutively expressing active variants (LsBZIP17 Δ C and LsBZIP60s) under the p35S promoter. Although we were able to isolate transgenic lines which segregated for the BASTA selectable marker in a Mendelian ratio, none of these lines show detectable transgene expression (data not shown). This suggests that constitutive expression of either UPR regulator active variant, LsBZIP17 Δ C or LsBZIP60s, is lethal.

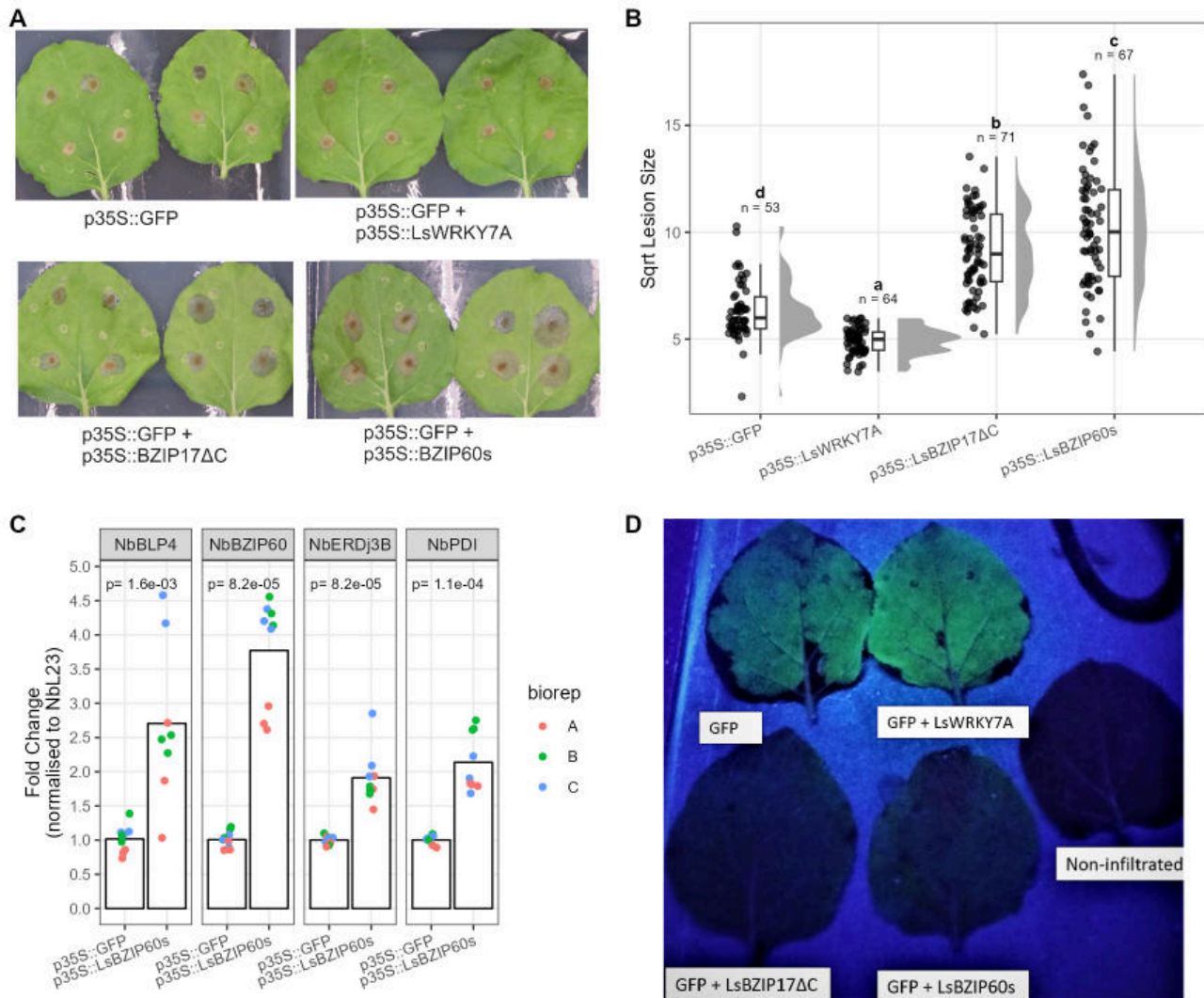


Figure 4.15: Functional characterisation of lettuce unfolded protein response (UPR) regulators Lsat_1.v5_gn_5.157880 (LsBZIP17ΔC) and Lsat_1.v5_gn_2.3181 (LsBZIP60s). **(A)** Representative leaves from transient *B. cinerea* susceptibility assay. 5-week *N. benthamiana* leaves are infiltrated with 0.5OD₆₀₀ total *Agrobacterium* using a needleless syringe (0.25OD₆₀₀ each p19/GFP or 0.167OD₆₀₀ each p19/GFP/gene of interest). 3 days post infiltration, leaves are detached, and infected with 4 × 15 μL droplets of 2 × 10⁵ spores/mL of *B. cinerea* inoculum diluted in 50% grape juice. 4 days post *B. cinerea* infection, lesions area is measured using ImageJ. **(B)** Quantification of (A). Square-root lesion area (mm) shown on the y-axis, individual lesion sizes, boxplots and distribution are shown. N represents the number of individual lesions measured. Letters represent statistical significance groups (Tukey HSD $p < 0.05$). **(C)** Expression of *N. benthamiana* UPR genes, NbBZIP60, NbBLP4, NbERDj3b and NbPDI after GFP or LsBZIP60s. Tissue is collected 4 days post infiltration (not infected with *B. cinerea*). Three biological replicate samples were collected from independent leaves from different plants. Three technical replicate qPCRs were performed for each biological replicate. $2^{-\Delta\Delta C_t}$ fold change shown on the y-axis with NbL23 used as the endogenous reference, and 35S infiltrated leaves as the control group. Wilcoxon rank-sum test p-values corrected for multiple testing are shown (Wilcoxon 1945). Gene IDs and primer sequences are listed in Table 4.1. **(D)** GFP fluorescence under UV-light of representative leaves 4-days post infiltrated with p19+GFP, p19+GFP+LsWRKY7A, p19+GFP+LsBZIP17ΔC, p19+GFP+LsBZIP60s and non-infiltrated leaves are shown. Infiltration procedure was performed the same as (A), with 0.5OD₆₀₀ total used, having each construct mixed in a 1:1 or 1:1:1 ratio.

We therefore used *N. benthamiana* to conduct functional characterisation of LsBZIP17ΔC and LsBZIP60s in transient assays. Unexpectedly, *N. benthamiana* leaves infiltrated with LsBZIP17ΔC or LsBZIP60s showed increased *B. cinerea* susceptibility compared to GFP-infiltrated leaves (Fig 4.15a-b). Although, BZIP60

silencing in *Nicotiana attenuata* has been previously reported to also promote susceptibility to *Alternaria alternata*, a necrotrophic fungal pathogen (Xu et al. 2019). This is contradictory as both endogenous silencing and transient overexpression is producing the same phenotype.

Endogenous *N. benthamiana* UPR chaperones (Li et al. 2022) such as BiP-like protein 4 (NbBLP4), endoplasmic reticulum DNAj-homolog 3B (NbERDj3B), protein disulphide isomerase (NbPDI), and endogenous NbBZIP60 were all upregulated in LsBZIP60s infiltrated leaves, compared to those infiltrated with GFP (Fig 4.15c). These results demonstrate that LsBZIP60s functions as an activator of the UPR.

When co-infiltrated with GFP, both LsBZIP17 Δ C and LsBZIP60s demonstrated a marked reduction in GFP fluorescence compared to leaves infiltrated with either GFP alone or co-infiltrated with LsWRKY7A and GFP (Fig 4.15d). This suggests that these lettuce UPR regulators are somehow inhibiting GFP accumulation. One possible explanation is that LsBZIP17 Δ C and LsBZIP60s are triggering ER-associated degradation (ERAD), (Meusser et al. 2005; Chen et al. 2020b). UPR is part of a larger ER quality control (ERQC) network. Under mild ER stress, UPR is activated to refold proteins, if misfolded proteins persist in the ER, ERAD is activated to clear them. In case of severe or prolonged ER stress, programmed cell death (PCD) is induced (Strasser 2018; Simoni et al. 2022).

From these results, we hypothesise that different ERQC pathways have different effects on necrotrophic pathogen defence. *N. attenuata* BZIP60 silencing data suggests that normal BZIP60 function, likely UPR activation, promotes increased necrotroph resistance. However, the very high expression levels of LsBZIP17 Δ C or LsBZIP60s during transient infiltration may signal to the cell that severe ER stress is present, activating ERAD, illustrated by reduced GFP accumulation, promoting susceptibility against necrotroph pathogens.

4.3.8 Characterisation of post-transcriptional gene silencing in lettuce necrotrophic pathogen defence response

In addition to our GRN hubs, we have previously identified 3724 lettuce genes whose expression was significantly correlated with *S. sclerotiorum* lesion size across a diversity panel (Pink et al. 2022). These genes, most of which were not differentially expressed in response to infection (hence not present in our GRN), represent a fresh pool of candidate defence genes. Notably, an enrichment of post-transcriptional gene silencing (PTGS) gene ontology (GO) terms was observed among the 1580 'resistance-correlated genes', i.e., genes with higher expression in resistant accessions.

Plant small RNAs (sRNAs) are involved in PTGS, an sRNA is processed by dicer-like (DCL) proteins, and loaded onto agonaute (AGO) complexes to target mRNAs for degradation or translation inhibition (Borges and Martienssen 2015). Several classes of plant sRNA exist which are generated through different biogenesis pathways, microRNAs (miRNAs) are transcribed from their own genetic loci by RNA polymerase II. Secondary short-interfering RNAs (siRNAs), also known as phasiRNAs, are produced by RNA-dependent RNA polymerases (RDRs) from RNA templates (Yoshikawa et al. 2005). siRNAs can also direct epigenetic silencing through the action of RNA-directed DNA methylation (Matzke and Mosher 2014b).

Several lettuce orthologues of notable genes involved in sRNA-mediated gene silencing were identified as highly correlated with *S. sclerotiorum* resistance (Fig 4.16a). These included orthologues of dsRNA-binding protein 2 (DRB2) and dsRNA-binding protein 4 (DRB4), RNA-directed DNA methylation 1 (RDM1) and Suppressor of gene silencing 3 (SGS3). SGS3 is known to participate in phasiRNA biosynthesis by interacting with RNA-dependent RNA polymerase 6 (RDR6) (Yoshikawa et al. 2005; Kumakura et al. 2009). DRBs are able to bind dsRNA and to specific DCL homologues (Hiraguri et al. 2005), with DRB4-DCL4 interactions required for the processing of tasiRNAs (Nakazawa et al. 2007). DRB2 is involved in a non-canonical miRNA biogenesis pathway, regulating the levels of a specific subset of miRNAs (Eamens et al. 2012).

Previously it has been demonstrated that Arabidopsis PTGS mutants such as *dcl4-2*, *rdr6-11*, *rdr2-4* *ago9-1* and *sgs3-1* have increased susceptibility to necrotrophic pathogens such as *S. sclerotiorum* and *V. dahliae* (Ellendorff et al. 2009; Cao et al. 2016a). Suggesting that PTGS genes promote necrotrophic disease resistance, which is consistent with our findings where lettuce accessions with higher expression of PTGS genes demonstrated greater resistance to *S. sclerotiorum*. However, there are no previous reports of DRBs having a role in necrotroph resistance. Although both DRB2 and DRB4 have been shown to positively regulate resistance against *P. syringae avrRPM1* (Lim et al. 2019).

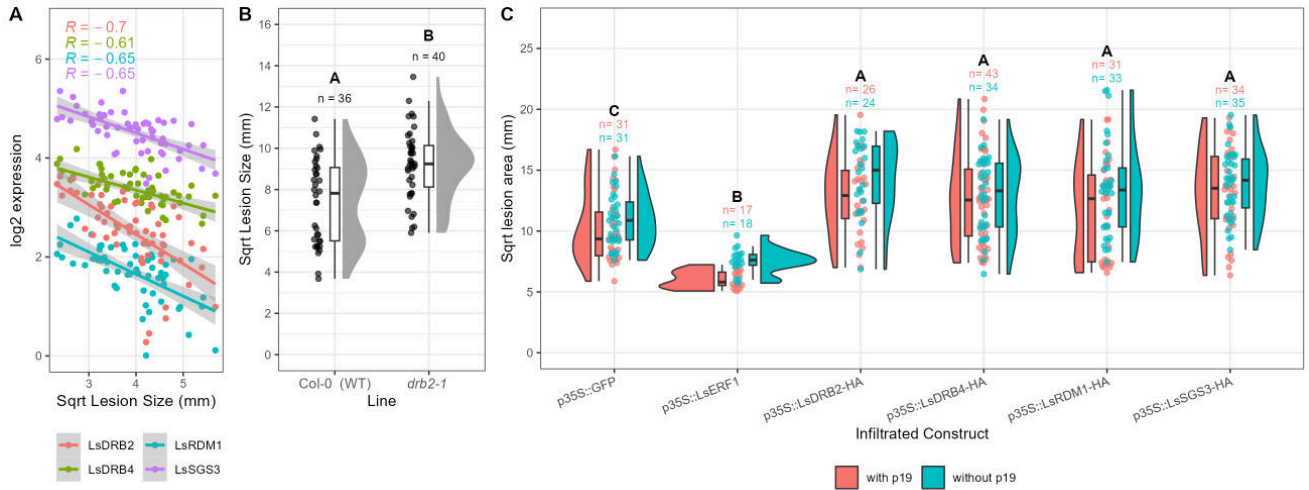


Figure 4.16: **A**) Lettuce diversity panel gene expression - *S. sclerotiorum* lesion size correlation for Lsat_1_v5_gn_9_69201 (LsDRB2 - red), Lsat_1_v5_gn_1_56161 (LsDRB4 - green), Lsat_1_v5_gn_8_21341 (LsRDM1 - blue) and Lsat_1_v5_gn_5_78841 (LsSGS3 - purple). *S. sclerotiorum* square-root lesion size on X-axis, and log₂ expression on Y-axis. Pearson's correlation coefficients (R) are shown, with colour denoting the gene. **B**) *B. cinerea* detached leaf assay on Col-0 wild-type Arabidopsis and *drb2-1* T-DNA mutant. Square root lesion size at 72hpi shown on Y-axis, individual data points shown as well as boxplot and distribution curve. Letters represent statistical significance groupings (Tukey HSD $p < 0.05$), n represents the number of lesions measured. **C**) *B. cinerea* detached leaf infection assay on *Agrobacterium*-infiltrated *N. benthamiana* leaves. The infiltrated construct is shown on X-axis, square-root *B. cinerea* lesion size is shown on Y-axis. Red points denote lesion sizes from p35S::p19 + construct_x co-infiltration's, blue points denote just construct_x infiltration's. Total infiltration = 0.4 OD₆₀₀ (0.4 OD₆₀₀ construct_x or 0.2 OD₆₀₀ construct_x + 0.2 OD₆₀₀ p19). Letters represent statistical significance groupings (Tukey HSD $p < 0.05$)

Firstly, we examined necrotroph defence in Arabidopsis DRB2 mutant, *drb2-1*, which displayed increased susceptibility to *B. cinerea* (Fig 4.16b), providing further evidence that PTGS promotes increased necrotroph resistance.

To investigate the *in planta* functions of lettuce PTGS orthologues, we utilised the transient *N. benthamiana* assay. In this experiment, leaves were infiltrated with a gene of interest either in the presence or absence of p19 silencing suppressor and subsequently infected with *B. cinerea* two days post infiltration (Fig 4.16c). p19 increases transgene expression by binding and sequestering 21-nt siRNA, leading to inhibiting transgene silencing in an RDR6-dependent manner (Jay et al. 2023). As observed previously, p35S::LsERF1 infiltrated leaves show increased *B. cinerea* resistance. Unexpectedly all tested lettuce PTGS genes; p35S::LsDRB2-HA, p35S::LsDRB4-HA, p35S::LsRDM1-HA and p35S::LsSGS3-HA all promote increased *B. cinerea* susceptibility compare to GFP-infiltrated leaves. Furthermore, p19 co-infiltration had no effect on the susceptibility of PTGS-genes, but further increased the resistance of LsERF1-infiltrated leaves.

These experiments provide contradictory results. The increased susceptibility displayed in mutants such as *drb2-1* (this work) and *sgs3-1* (Ellendorff et al. 2009) suggests that PTGS genes promote resistance

to necrotrophic pathogens. However, transient overexpression of lettuce PTGS orthologues also results in increased necrotroph susceptibility. This may be due to an over-saturation of the PTGS machinery, disrupting a finely controlled process, leading to uncontrolled RNA degradation. We note that these lettuce PTGS genes function in an p19-independent manner, although the extent to which p19 suppresses the silencing of endogenous transcripts remains largely unknown.

4.4 Discussion

GeneID	Name	<i>B. cinerea</i> phenotype			Ref
		Lettuce	Arabidopsis	<i>N. benthamiana</i>	
Lsat_1.v5_gn_6.70301	LsBOS1		R	NS ^{*1}	Fig 3.19
Lsat_1.v5_gn_2.103381	LsNAC53		S		Fig 3.20A
Lsat_1.v5_gn_3.121961	LsERF1	R	R ^{*2}	R	Fig 4.1,4.11, Fatih Kara
Lsat_1.v5_gn_4.164440	LsWRKY7A		R	R	Fig 4.10B,4.11
Lsat_1.v5_gn_4.127960	LsWRKY7B		NS		Fig 4.10C
Lsat_1.v5_gn_3.120520	LsMYB15		NS		Fig 4.13C
Lsat_1.v5_gn_5.157880	LsBZIP17ΔC			S	Fig 4.15B
Lsat_1.v5_gn_2.3181	LsBZIP60-s			S	Fig 4.15B
Lsat_1.v5_gn_9.69201	LsDRB2			S	Fig 4.16C
Lsat_1.v5_gn_1.56161	LsDRB4			S	Fig 4.16C
Lsat_1.v5_gn_5.78841	LsSGS3			S	Fig 4.16C
Lsat_1.v5_gn_8.21341	LsRDM1			S	Fig 4.16C

Table 4.2: Summary of characterisation of putative lettuce defence regulators against *B. cinerea*. R = Increased resistance against *Botrytis cinerea*, S = Increased susceptibility to *Botrytis cinerea*, NS = Non-significant effect on *Botrytis cinerea* infection, blank = Not tested against *Botrytis cinerea* in that host species.

^{*1} *N. benthamiana* leaves transiently infiltrated with LsBOS1 induced necrosis-like symptoms, and no significant differences in *B. cinerea* lesion sizes were detected between GFP and LsBOS1 infiltrated leaves. Data not shown, as were not included in Pink et al. 2023.

^{*2} Arabidopsis LsERF1 *B. cinerea* phenotyping performed by Fatih Mehmet Kara

In Pink et al. 2023, we inferred a lettuce-necrotroph gene regulatory network (GRN) which identified candidate hub genes, including LsBOS1 and LsNAC53. We were then able to validate that LsBOS1 positively regulates *B. cinerea* defence in Arabidopsis, and that LsNAC53 complements AtNAC53 in negatively regulating *B. cinerea* resistance. LsNAC53 not only complemented the defence phenotype of AtNAC53, but was also shown to positively regulate Respiratory Burst Oxidase Homolog (RBOH) genes, which AtNAC53 had previously been shown to regulate (Lee et al. 2012).

In this work, we characterise the *B. cinerea* defence function of a further six GRN hubs and four *S. sclerotiorum*-resistance correlated genes (identified in Pink et al. 2022). *B. cinerea* defence characterisation was carried out using quantitative detached leaf assay on transgenic Arabidopsis, transgenic Lettuce or transiently infiltrated *N. benthamiana* leaves. The results from which are summarised in Table 4.2.

Across Pink et al. 2023 (Chapter 3) and this work, we have tested the *in vivo* defence response of six lettuce TFs in stable transgenic plant lines (either lettuce or Arabidopsis), of which three showed increased resistance, one showed increased susceptibility and two showed no significant differences in *B. cinerea* susceptibility (compared to transformation background, WT or T-DNA mutant). One TF that showed no significant difference, LsWRKY7B, is not considered a hub due to only having 9 predicted GRN targets, therefore 4 out of 5 (80%) of GRN hubs tested in stable transgenic lines showed altered resistance to *B. cinerea*. In addition, we tested the defence function of an additional 6 genes using transiently infiltrated *N. benthamiana* leaves, two of which were GRN hubs, the other 4 were selected from genes whose expression correlated with increased resistance in lettuce diversity panel from Pink et al. 2022. All 6 genes showed increased susceptibility to *B. cinerea* (compared to GFP infiltrated leaves). So, out of all 12 candidate lettuce defence regulators that we tested, 10 (83%) show an altered *B. cinerea* resistance phenotype. These results clearly demonstrate that both GRN hubs and diversity panel lesion-size correlated genes can be used as high-confidence candidates to identify lettuce defence regulators.

However in order to perform a high-throughput assessment of many candidate defence regulators, only 1 hub gene, LsERF1, has been tested in lettuce. Therefore, it is possible that we miss species-specific regulators which alter *B. cinerea* defence in lettuce, but not in Arabidopsis or *N. benthamiana* (false negative). Of course the opposite may also be true, where we identify false positives, TFs that act as defence regulators in Arabidopsis but not lettuce. Therefore, the next steps would be to the defence function of additional candidate genes in lettuce, such as LsWRKY7A and LsNAC53.

LsERF1 was identified as a large network hub, and was subsequently shown to positively regulate *B. cinerea* defence in both lettuce (Fig 4.1) and *N. benthamiana* (Fig 4.11). Transcriptomic analysis of two independent LsERF1 lettuce transgenic lines suggested that only small set of 33-60 (dependent on stringency criteria) genes were required for LsERF1-induced *B. cinerea* resistance. Although higher levels of LsERF1 expression induced large-scale transcriptional reprogramming, including the differential expression of genes associated with "response to ethylene stimulus" and STL biosynthetic enzymes. Our GRN outperforms random guessing in the prediction of LsERF1 using precision, recall, F_1 or $F_{0.25}$. Additionally, combined first and second-order LsERF1 GRN targets (at top 0.8% or top 1% edges threshold) outperform co-expression modules at predicting LsERF1-OE DEGs (using precision or $F_{0.25}$). Therefore our GRN is able to more precisely predict genes that are regulated by LsERF1 *in vivo* than conventional co-expression modelling. Together with the initial network

validation performed in Pink et al. 2023, these results establish high confidence in the GRN's ability to identify defence regulators and their *in vivo* targets.

Given this established confidence, we use the GRN to identify additional defence regulators. Two putative lettuce orthologues of AtWRKY7, LsWRKY7A and LsWRKY7B are present in the GRN with 257 and 9 predicted targets respectively. Therefore LsWRKY7A is identified as a "large hub", whereas LsWRKY7B is not considered a network hub. LsWRKY7A conferred increased *B. cinerea* resistance in both transgenic Arabidopsis and infiltrated *N. benthamiana* (Fig 4.10B, 4.11B). We did not observe any significant differences in transgenic Arabidopsis lines expressing LsWRKY7B. These results suggest that our GRN has been able to identify the functional lettuce orthologue of AtWRKY7 also which confers *B. cinerea* resistance (Wang et al. 2017c). Although, we do note that only high-expressing LsWRKY7A transgenic lines showed *B. cinerea* resistance, and LsWRKY7B transgene expression levels were lower than that observed in the LsWRKY7A lines.

LsMYB15 was also identified as a major hub gene with 378 predicted targets and is a putative orthologue of known lignin biosynthesis regulators AtMYB15 and CmMYB15-like (Chezem et al. 2017; Li et al. 2023a) (Fig 4.12A). Overexpressors of both AtMYB15 and CmMYB15-like show increased resistance to biotic stresses; *P. syringae* DC3000 and *Macrosiphoniella sanborni* (aphid) feeding respectively (Chezem et al. 2017; Li et al. 2023a). Despite this, neither AtMYB15 mutants, AtMYB15 overexpressors, nor LsMYB15 overexpressors had differing *B. cinerea* susceptibility from Col-0 (Fig 4.13). These results indicate MYB15-induced lignification does not impact *B. cinerea* defence in Arabidopsis. It is then difficult to assess any role of LsMYB15 as it could still regulate lignification in lettuce with lignification not important in Arabidopsis defence, or it may also not function in this manner in lettuce. The GRN predicts LsMYB15 may act as a regulator of lignin biosynthesis, as its GRN predicted targets include CAD4, CAD8 and PRX52 all of which are involved in lignin biosynthesis (Kim et al. 2004; Fernández-Pérez et al. 2015). Hence further characterisation of this gene requires gain or loss of function analysis in lettuce.

In addition to detached leaf assays performed on transgenic plants, we also modified this protocol for use on transiently infiltrated *N. benthamiana*. This dramatically reduces the time taken to characterise a gene from 10 months to 5 weeks, allowing more genes to be tested. Protein accumulation may be orders of magnitude larger than what is observed in transgenic plants. This appeared to exacerbate the LsERF1 and LsWRKY7A phenotypes that were observed in lettuce and Arabidopsis respectively. However, the relationship between expression and defence phenotype may be non-linear, and high-level over-accumulation may introduce erroneous protein activity and/or targets. We believe this to be the case for the tested PTGS and UPR regulators. LsDRB2, LsDRB4, LsSGS3, LsRDM1, LsBZIP17 Δ C and LsBZIP60s all promoted increased susceptibility to *B. cinerea* compared to GFP-infiltrated *N. benthamiana* leaves. This increase in susceptibility from overexpression is in contrast to numerous Arabidopsis PTGS mutants and tobacco plants with silenced

UPR regulators that also show increased susceptibility to necrotrophic pathogens, suggesting their wild-type function promotes defence (Fig 4.16A, Ellendorff et al. 2009; Cao et al. 2016a; Cai et al. 2018; Xu et al. 2019). Therefore, we hypothesise that these results may be artefacts of the high-level overexpression that does not represent the wild-type function. Loss-of-function approaches such as viral-induced gene silencing (VIGS) or CRISPR knockout would therefore be required to study the wild-type function of these regulators.

These results suggest that our transient *N. benthamiana*-*B. cinerea* detached leaf susceptibility assay is able to accurately profile the effect of high-level overexpression on pathogen resistance. Whether these results are representative of wild-type functions depends on whether erroneous behaviour is introduced by the overexpression.

N. benthamiana is more closely related to lettuce (both asterid) than *Arabidopsis* (rosid), however, we still expect that there may be differences in gene function when expressed in non-endogenous hosts. High-efficiency *Agrobacterium* vacuum-infiltration of lettuce has been previously reported (Negrouk et al. 2005; Chen et al. 2016; Yamamoto et al. 2018). Therefore, it may also be possible to perform transient *B. cinerea* susceptibility assays, allowing the defence function of candidate lettuce genes to be tested within their endogenous host. However, when following the protocol outline in Chen et al. 2016 using the visible RUBY marker on several lettuce cultivars only small patches of RUBY were visible on < 5% of infiltrated leaves (He et al. 2020b).

The Cauliflower Mosaic Virus (CaMV) 35S promoter has been used to test the effect of constitutive hub gene expression in transgenic plants (Odell et al. 1985). However, the expression of these genes are very tightly-controlled in non-infected conditions as they often regulate growth-defence. As we observed in transient *N. benthamiana*, it may be undesirable to test the function of some genes under high-level overexpression. This is particularly true in the case of LsBZIP60s or LsBZIP17 Δ C, where we attempted to express consecutively active forms of these genes under the 35S promoter which was lethal. In order to study these genes, it is crucial to induce expression in a more controlled manner, such as the use of estradiol-inducible promoters (Zuo et al. 2000). In addition, naturally occurring pathogen inducible promoters can be used such as *pBnGH3.17^{D7}*, which has been engineered to drive expression specifically in response to *S. sclerotiorum* infection, and is functional in both *Arabidopsis* and *Brassica napus* leaves (Lin et al. 2022).

The work highlights the value of computational prediction and gene regulatory network modelling in order to identify candidate defence regulators from high-throughput transcriptomic profiling. "Hub genes" with many predicted targets are postulated to be key defence regulators within the network. Our findings suggest that many hub genes do indeed act as defence regulators. Further testing of hub genes identified by our network

may uncover further defence regulators.

Chapter 5

Final Discussion

5.1 Summary of Key Findings

The overarching aims of this work were to identify candidate genes that could be utilised for the generation of disease-resistant cultivars. To achieve this we have looked to further our understanding of the lettuce defence response to necrotrophic fungal pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Extensive datasets generated by the Denby Lab and collaborators were essential in order to do this. Using these datasets, I have applied numerous analytical methods to identify candidate lettuce defence genes and performed functional characterisation on a short-list of selected candidates

Prior to my project, previous members of the Denby lab generated 284 RNA-sequencing datasets across 5 key experiments; lettuce-*B. cinerea* time series, lettuce-*S. sclerotiorum* time series, lettuce-*B. cinerea* diversity panel, lettuce-*S. sclerotiorum* diversity panel and PI251246 x Armenian *L. serriola* (PlxArm) mapping population parents. In addition, quantitative necrotroph susceptibility phenotyping was also performed on the lettuce diversity panel and PlxArm RILs had also been performed by the Denby Lab. The Micheltore Lab (UC Davis) also genotyped the PlxArm RILs, identifying 2677 genetic markers (Han et al. 2021). The combinatorial analysis performed on these datasets is summarised in Figure 5.1.

5.1.1 Identification of lettuce genes associated with *S. sclerotiorum* susceptibility across a genetically diverse population

In Chapter 2 (Pink et al. 2022), we profiled the *S. sclerotiorum* susceptibility and transcriptomes of 55 individual lettuce leaves from 21 diverse accessions. This approach focuses on individual leaves rather than average expressions from each accession, therefore including intra-accession variability when correlating pathogen susceptibility with gene expression. Prior to this, a broader phenotyping experiment was conducted,

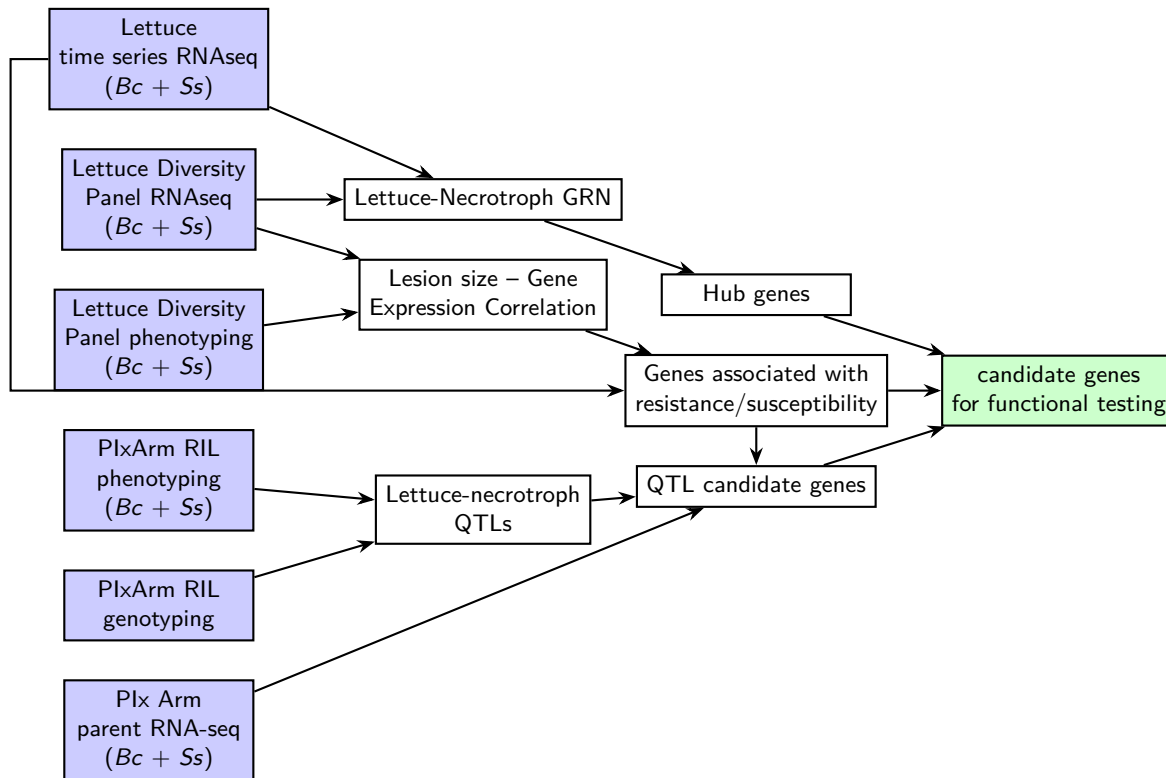


Figure 5.1: Flow diagram of the integrative analysis to identify candidate genes in lettuce for necrotrophic pathogen resistance. Input datasets are listed on the left; **Lettuce-necrotroph time series RNAseq**: high-density time series gene expression data during *B. cinerea* (*Bc*) or *S. sclerotiorum* (*Ss*) infection (Pink et al. 2023; Ransom et al. 2023); **Lettuce Diversity Panel RNAseq**: gene expression after *B. cinerea* and *S. sclerotiorum* infection in 21 diverse lettuce accessions (Pink et al. 2022); **Lettuce Diversity Panel necrotroph phenotyping**: quantitative detached leaf assay phenotyping of *B. cinerea* and *S. sclerotiorum* resistance in diverse lettuce accessions (Pink et al. 2022); **PI x Arm RIL necrotroph phenotyping**: quantitative detached leaf assay phenotyping of *B. cinerea* and *S. sclerotiorum* resistance in 236 F₆ recombinant inbred lines (RILs) from the PI251246 x Armenian *L. serriola* mapping population (PlxArm) (Pink et al. 2022); **PI x Arm RIL genotyping**: 2677 markers for the PlxArm population were previously described by Han et al. 2021; **PlxArm parent RNA-seq**: gene expression in mapping population parents PI251246 and Armenian *L. serriola* (Pink et al. 2022).

Summary of analysis performed to select candidates from these datasets. **Lettuce-Necrotroph GRN**: a single gene regulatory network (GRN) modelling transcriptional regulation of time series DEGs using four RNAseq datasets - time series and diversity panel RNAseq after *B. cinerea* and *S. sclerotiorum* infection (Pink et al. 2023). **Hub genes**: transcription factors with many GRN targets, hence predicted to have a high influence in necrotroph-induced transcriptional reprogramming. **Lesion size – Gene Expression Correlation**: association of quantitative disease severity with gene expression across diversity accessions (Pink et al. 2022). **Genes associated with resistance/susceptibility**: we notice that differential expression in response to infection is a confounding factor for whether a gene will be resistance or susceptibility correlated, therefore we use “Lesion size – Gene Expression Correlation” and “Lettuce-necrotroph time series RNAseq” to determine whether a gene is associated with resistance or susceptibility (Pink et al. 2022). **Lettuce-necrotroph QTLs**: identification of genetic loci that confer differences in *B. cinerea* or *S. sclerotiorum* susceptibility between PI251246 and Armenian *L. serriola*. **QTL candidate genes**: genes located within QTL loci and differentially expressed between mapping population parents.

Candidate genes for functional testing: hub genes, genes associated with diversity set resistance/susceptibility or QTL candidates are all possible defence regulators which could be short-listed for functional testing.

evaluating *S. sclerotiorum* susceptibility across the same accessions with a higher level of replication over multiple independent trials. Despite the more extensive dataset, the method of comparing individual leaf susceptibilities to their respective transcriptomes proved to be more informative.

Many studies profile just a single resistant and susceptible accession in order to identify genes linked with the disease sensitivity phenotype (Wu et al. 2016; Wang et al. 2017a; Chittem et al. 2020; Wan et al. 2021). Although these are sometimes able to hand-pick some DEGs that play a role in the phenotype, they are unable to disentangle the differences that are linked to the disease phenotype and those that are not at a transcriptome-level. However, our method enabled us to pinpoint genes whose expression was linked with defence across 55 individual leaves from 21 diverse accessions. As such, we identified 3724 genes whose expression correlated with increased *S. sclerotiorum* resistance, and a further 1580 correlated with increased susceptibility. As far as we are aware this is the most extensive *S. sclerotiorum* screening of this type (coupled pathogen susceptibility and transcriptomic profiling) in a crop species.

Crucially, our approach allows genes whose expression is not differentially expressed in response to infection, but where a higher constitutive expression of the gene affects pathogen resistance either positively or negatively to be identified. We identified multiple orthologues of COI1 whose expression were strongly correlated with *S. sclerotiorum* resistance, and a MAP Kinase Substrate 1 (MKS1) orthologue correlated with increased susceptibility. In Arabidopsis, COI1 is the JA receptor which activates JA signalling and *B. cinerea* defence (Feys et al. 1994; Thomma et al. 1998), whereas MKS1 directly binds WRKY33 inhibiting JA response and reducing *B. cinerea* defence (Andreasson et al. 2005; Petersen et al. 2010). Among others, the COI1 and MKS1 results demonstrate the value of this approach to identify genes associated with both resistance and susceptibility to *S. sclerotiorum* across the lettuce diversity panel. Thus, other resistance or susceptibility correlated genes can be used as candidate genes for functional testing.

Interestingly, we identified that *S. sclerotiorum* resistance-correlated genes were enriched for both orthologues of Arabidopsis genes annotated with post-transcription gene silencing (PTGS) GO-terms and genes with Pentatricopeptide Repeat Domains (PPRs). Howell et al. 2007 previously demonstrated that PPR transcripts are a major source of secondary short-interfering RNAs (siRNAs). siRNAs derived from PPR transcripts (PPR-siRNAs) have been implicated in plant-pathogen interactions with *Phytophthora capsici* expressing an effector that suppresses PPR-siRNAs (Hou et al. 2019). Furthermore, orthologues of key proteins involved in this siRNA biogenesis pathway are also correlated with *S. sclerotiorum* in the lettuce diversity such as an RNA-dependent RNA polymerase (RDR2), dicer-like 4 (DCL4), suppressor of gene silencing 3 (SGS3) and dsRNA-binding protein 4 (DRB4) (Peragine et al. 2004; Yoshikawa et al. 2013; Adenot et al. 2006). Together these data suggest that an increased expression of the secondary siRNA biosynthesis machinery may increase *S. sclerotiorum* resistance, possibly due to an increased ability to generate PPR-siRNAs. These findings highlight

the ability of a high-throughput transcriptomic to uncover some novel biological findings using genetic variation across diverse accessions, as well as identifying lettuce orthologues of known *Arabidopsis* defence regulators.

5.1.2 Integrative analysis to identify high-confidence genes within QTLs conferring necrotroph resistance in lettuce

Also in Chapter 2, five necrotroph resistance QTLs were identified in a wild lettuce mapping population (*L. sativa* PI251246 × Armenian *L. serriola*), two of which conferred resistance to *B. cinerea* and three conferred resistance to *S. sclerotiorum*. These were the first reported QTLs conferring resistance to *B. cinerea* or *S. sclerotiorum* in lettuce, although several *S. minor* QTLs have previously been reported in lettuce (Mamo et al. 2019). These QTL loci could potentially be introgressed into elite breeding cultivars to increase pathogen resistance, however many backcrossing generations would be required to remove undesirable genes that are in linkage disequilibrium with the favourable allele. It may be faster to look to identify causative variants and directly introduce these into an elite cultivar.

To this end, we perform comparative transcriptomics between the mapping population parents after *S. sclerotiorum* infection, revealing 96 genes that were located within a QTL region and were differentially expressed between the parents. Of these QTL DEGs, 5 genes showed higher expression in the parent harbouring the resistance allele and were correlated with resistance across the entire diversity panel, this includes an orthologue of pleiotropic drug resistance 12 (PDR12) which is involved in the secretion of camalexin (He et al. 2019). A further 11 genes showed higher expression in the parent harbouring the susceptible allele and were correlated with increased susceptibility across the diversity set. Hence by implementing an integrative analysis workflow, we have reduced the list of candidate genes by > 99.5% from a total of 3264 genes located within QTLs to a short-list of 16 high-confidence candidates that may underlie the differences in necrotroph resistance in these QTL regions.

5.1.3 Utilising high-resolution temporal transcriptomic of the lettuce defence response to infer a causal gene regulatory network

The utilisation of time series RNAseq and subsequent construction of gene regulatory networks (GRNs) have emerged as powerful methodologies for high-throughput gene discovery in crop science, particularly beneficial for species where generating transgenics presents significant challenges. This approach has been instrumental in decoding the complex temporal dynamics of plant-pathogen interactions, providing unparalleled insights into the regulatory mechanisms underlying disease resistance. For crops like lettuce, where transgenic approaches are time-consuming and technically demanding, the accessibility of RNAseq offers a viable alternative for understanding genetic responses to environmental stresses. When integrated into GRNs, these

data facilitate the prediction of causal relationships between genes, highlighting potential targets for enhancing disease resistance. This methodology not only circumvents the limitations associated with traditional genetic manipulation in less tractable crop species but also accelerates the discovery of critical genes involved in stress responses. Consequently, time series RNAseq and GRNs represent a significant advancement in crop gene discovery, offering a pathway to improve crop resilience and productivity through a deeper understanding of genetic regulation.

In Chapter 3 we present high-resolution temporal transcriptomics after *B. cinerea*'s infection of lettuce leaves with 14 time points from 9 to 48 hours post inoculation (hpi). In addition, Ransom et al. 2023 previously produced a 12 time point time series of lettuce-*S. sclerotiorum* infection (9 to 42 hpi). These datasets provide unprecedented insight into the dynamics of pathogen-induced transcriptional reprogramming previously only available in Arabidopsis (Windram et al. 2012; Lewis et al. 2015). Previous necrotrophic pathogen infection temporal transcriptomic datasets available in crop species has been limited to 3-6 time points (De Cremer et al. 2013; Zambounis et al. 2020; Xu et al. 2021; Wang et al. 2021; Wan et al. 2021).

Through an integrated analysis of both temporal transcriptomic datasets, we identified a core set of 4362 lettuce genes that exhibited differential expression in response to both pathogens in the same direction. This enabled dual time series co-expression analysis, revealing modules of genes with highly similar expression profiles in response to both pathogens. In Module 1 we identified orthologues of known JA/ET regulators including ERF1, WRKY33 and MYC2 (Lorenzo et al. 2003; Birkenbihl et al. 2012; Lorenzo et al. 2004) and enzymes involved in costunolide biosynthesis, a sesquiterpene lactone (STL) precursor in lettuce (Ikezawa et al. 2011). Alongside 37 uncharacterised cytochrome P450s, this module suggests a mechanism where STL biosynthesis, activated by JA/ethylene signalling, may contribute to *B. cinerea* resistance (Zhang et al. 2022; Bennett et al. 1994). These findings were supported further in Chapter 4 through transcriptomic analysis of transgenic lettuce overexpressing LsERF1, which showed increased expression of JA/ET signalling genes and STL biosynthetic enzymes in mock conditions.

One of the most significant advantages to generating high-resolution temporal transcriptomic datasets is the enhanced ability to model causal gene-regulatory networks (GRNs) (**time series-GRN-annrev**). Unlike co-expression networks, which draw undirected edges based on correlation, the use of time series data allows for the inference of causal, directed connections between regulators (TFs) and targets (Opgen-Rhein and Strimmer 2007). time series GRNs are also able to incorporate the chronology of expression profiles, giving a more accurate representation of the underlying biological system. Using this approach a causal GRN of the lettuce-necrotroph defence response was modelled, incorporating both pathogen time series and static diversity panels. This enables directed edges to be identified where a TF and target have highly similar expression profiles in both time series, and their expression is tightly correlated across diverse lettuce accessions.

5.1.4 The lettuce-necrotroph GRN accurately identifies defence regulators

It should be first stated the machine-learning models used are not directly identifying “defence regulators”. Instead, they predict the expression of a gene at an unseen time point based on the expression of transcription factors (TFs). This is analogous to the way large language models such as ChatGPT are simply trained by predicting the next word/character on large volumes of text, but by doing so they are able “learn” the underlying complexities of human language. In a similar way, by attempting to predict expression of a gene using TF expression, the random-forest is able to predict which TFs may be important in regulating that gene.

It is then possible to generate GRNs by drawing causal-directed edges between target genes and their most informative TFs. Hub genes, TFs with many causal connections, are therefore predicted to regulate a significant portion of the network. Since our lettuce-necrotroph GRN consists only of genes differentially expressed in response to both *B. cinerea* and *S. sclerotiorum* infections, the GRN hubs are presumed to direct a large proportion of the transcriptional reprogramming common to infection by both necrotrophic fungi. As a result, these hub genes can be used as candidate “defence regulators”, even though the models do not test for this directly.

In chapters 3 and 4, we conduct an extensive validation of the gene-regulatory network (GRN) for both the identification of defence regulators and the accuracy of inferred regulatory connections (edges). We assessed the *B. cinerea* susceptibility of transgenic plants constitutively expressing six lettuce TFs in the GRN, out of which three increased resistance (LsERF1, LsBOS1, LsWRKY7A), one increased susceptibility (LsNAC53ΔC), and two displayed no significant differences (LsMYB15 and LsWRKY7B). Five of these TFs were identified as GRN hubs, LsWRKY7B was not identified as a hub and was only included to determine whether our GRN could identify the function LsWRKY7 orthologue from two closely related paralogues. Additionally, two GRN hub genes tested in transiently infiltrated *N. benthamiana* leaves both revealed increased susceptibility (LsBZIP17ΔC and LsBZIP60s). Therefore 6 out of 7 GRN hubs tested (86%) exhibited altered *B. cinerea* defence, clearly demonstrating that a high proportion of GRN hubs function *in vivo* as necrotroph defence regulators.

In addition to the ability to identify defence regulators, we are interested in the predictive accuracy of individual edges within the GRN. Accurate prediction of a regulator’s targets not only sheds light on the potential function of that regulator but also the likely consequences on the network of overexpressing or mutating the regulator. Arabidopsis orthologues of LsNAC53’s predicted GRN targets, RBOHD and NSL1, were upregulated in Arabidopsis lines expressing LsNAC53ΔC. Since AtNAC53 has previously been identified as a regulator of RBOH genes (Lee et al. 2012), this observation suggests that the GRN correctly identified a conserved regulatory module without access to prior data. Beyond small regulatory modules, our results

also demonstrate the GRN's capability to predicting transcriptome-wide targets of a TF. For instance, the GRN-predicted targets of LsERF1 significantly outperformed both random guessing and coexpression modules as predictors of 35S::LsERF1 differentially expressed genes (LsERF1-OE DEGs). We also note that including second-order targets (i.e., targets of TFs that are themselves targets of LsERF1) further increased the predictive performance in detecting LsERF1-OE DEGs. This outcome aligns with expectations, as RNAseq does not discriminate between direct and indirect targets.

The above results give us reasonable confidence that the GRN we inferred has accurately captured the complex dynamic regulatory interactions that occur in response to pathogen infection in lettuce. As such, this GRN represents a significant improvement in our ability to accurately predict direct regulatory edges in a non-model species. Therefore, the GRN can now be used to identify additional defence regulators and their putative targets.

5.2 Challenges and Limitations

Throughout the course of my PhD, I encountered a variety of challenges and limitations that impacted the scope and direction of my research.

I attempted to generate Arabidopsis transgenic lines expressing constitutively active forms of LsBZIP17 and LsBZIP60 under the control of the 35S promotor. These genes act as cell death activators, which I was unaware of at the time of selection, and high expression was toxic to the plants, resulting in the death of those expressing high levels of these genes. Although 35S is a very convenient promotor to indiscriminately drive high-level expression, this is not always desirable, particularly when studying stress-responsive genes whose expression is tightly controlled. As a result, I had to pivot to using transient expression in *Nicotiana benthamiana* to study these genes. However, this approach significantly limited the experiments I could perform and impacting the conclusions I was able to draw from my data.

Given that my the species of interest for this work is lettuce, we ideally want to study the function of our candidate genes in lettuce. However, generating lettuce transgenics is very time-consuming which would limit the amount of candidate genes I would have been able to test. Therefore the majority of my work utilised Arabidopsis as a model for high-throughput testing. Although this has enabled us to test the defence function of many genes, we only have data for 1 gene (LsERF1) in lettuce, it is unclear whether other defence regulators identified in this work (LsNAC53, LsWRKY7A, LsBOS1, etc) function as defence regulators in lettuce.

Another limitation is that due to the time constraints of the project, I began cloning candidate genes towards the end of my first year. At this point, I produced an earlier version of the GRN, and I had not yet performed an extensive analysis of the inferred network. Throughout, the following years of my PhD I produced further iterations of the network, and performed more extensive analysis. These improvements included modifying the train-test split, modifying the trees in the random forest, and updating the genes which were designed as potential regulators (transcription factors). As a result, two of the genes I selected to generate transgenic lines from (LsBOS1 and LsWRKY7B) were later deemed to have much less influence in the final version of the network. Additionally, the final version of the network highlighted some interesting candidates which could have been tested, which were not hub genes in earlier iterations of the GRN.

In Chapter 2, I analysed an RNA-seq dataset from a diversity-panel of lettuce after pathogen infection. The aim of this dataset was to correlate gene expression with lesion size (a measurement of pathogen resistance). However, the analysis of this dataset was complicated by having samples post-infection of lettuce varieties with different levels of susceptibility to pathogen infection. For example, the genes whose expression correlated most strongly with increased susceptibility (had higher expression in more susceptible varieties), were genes that were known to promote increased resistance (e.g. LsERF1). This was puzzling at first, as we'd expect accessions that express more LsERF1 to be more resistant to pathogen infection. However, this turned out to just be an artefact of the experimental design. LsERF1, like many genes that promote pathogen resistance, is rapidly upregulated in response to infection. Susceptible lettuce varieties have a much faster disease progression than resistant varieties, hence genes such as LsERF1 are more likely to have been upregulated, making it appear in our simple correlation that LsERF1 was linked with disease susceptibility, which we know to be false. As a result, we had to discard genes whose expression changes in response to infection from these analyses. By performing additional preprocessing steps, we were still able to draw valuable insights from this dataset on genes where a higher constitutive level of expression impacts disease resistance (positively or negatively). However in hindsight, we may have got greater value from this dataset if it was collected from un-inoculated samples.

5.3 Future Directions and Next Steps

Building upon the extensive insights gained through the integrative analysis of high-throughput transcriptomic datasets and the construction of a predictive gene regulatory network (GRN), this work sets the stage for several promising avenues of research aimed at enhancing crop resilience against necrotrophic pathogens. The challenges and limitations encountered not only highlight the complexity of plant-pathogen interactions but also outline specific areas where further research can yield significant advancements. Future work should focus on refining the predictive accuracy and resolution of the lettuce-necrotroph GRN, exploring

innovative approaches to gene function validation, and translating these findings into practical strategies for breeding disease-resistant lettuce cultivars. By addressing these objectives, this work can contribute to the development of sustainable agricultural practices that can mitigate the impacts of plant diseases, thereby ensuring food security and agricultural productivity in the face of changing environmental conditions.

5.3.1 Enhancing the resolution and predictive performance of the lettuce-necrotroph GRN

Our GRN has successfully identified lettuce defence regulators and offers a significant enhancement in predicting directed regulatory edges. Compared to what was previously available in non-model organisms, our time series datasets, with 3-hour resolution (3 hour gaps between time points), represent a considerable step forward (De Cremer et al. 2013). Yet, to accurately model the chronological hierarchy of transcriptional regulation and establish a chronological order of events in the reprogramming, there are still gaps to fill.

In elicitor or hormone treatment time series, the onset of transcriptional reprogramming is predictable (5min-1hr post-treatment) (Hickman et al. 2017; Bjornson et al. 2021). However, in fungal inoculations such as our time series, which is far less predictable due to the variable speed of pathogen growth between experiments. In our *B. cinerea* time series 60% of genes were first differentially expressed between 21-24 hours post inoculation (hpi), whereas in *S. sclerotiorum*, this large-scale transcriptional programming occurred at 39-42 hpi. Therefore, despite having ≥ 12 time points, the initial change in transcription (which is vital for identifying putative regulators) occurs at 1-2 time points for the majority of differentially expressed genes.

Despite simultaneous differential expression (at our 3 hour resolution), the *B. cinerea* time series still provides good data on the dynamic expression profiles, with many time points captured after initial gene expression changes. Conversely, in the *S. sclerotiorum* time series, most genes are first differentially expressed in the last two time points due to slower pathogen growth, resulting in less information on these dynamic profiles. Hence, it is challenging to "pull apart" the chronological hierarchy of transcriptional reprogramming and understand the direct regulatory edges. As a result, in co-expression analysis, we have a single module of 942 genes (Module 1) whose expression profiles across both time series are very difficult to separate. As a result, it is challenging to assign direct TF-Target edges within this module.

A possible solution could come from additional time series with < 30 minute resolution, for example time series after jasmonic acid (JA) and/or 1-aminocyclopropane-1-carboxylic acid (ACC, an ethylene precursor) at 10 min, 30min, 45min, 1 hr, 2hr post-treatment. Such a data set would provide a higher-resolution view within the early critical window of transcriptional reprogramming. With this increase in resolution, we could more accurately determine the time lag between the activation of a regulator and its putative targets, and observe

whether intermediary regulators are activated during the lag period. Enabling the complex transcriptional cascades to be deciphered.

We do not expect that the full pathogen-induced transcriptional reprogramming will occur in response to hormone treatment, for example Windram et al. 2012 identified 9534 *B. cinerea* DEGs in Arabidopsis and of these only 1550 were also differentially expressed in response to JA treatment (Hickman et al. 2017). Thus demonstrating that a significant proportion of *B. cinerea*-induced transcriptional reprogramming is JA-independent. The recognition of pathogen- or damage associated molecular patterns (PAMPs/DAMPs) also triggers a rapid transcriptional response during pathogen infection. Bjornson et al. 2021 conducted a time series after oligogalacturonide (plant cell wall fragments, DAMP) treatment in Arabidopsis, identifying 3973 DEGs, 1839 of which were identified as Arabidopsis-*B. cinerea* DEGs by Windram et al. 2012. The majority of these DAMP-regulated *B. cinerea* DEGs (n=1187) were not differentially expressed by JA treatment in Hickman et al. 2017 and include key regulators such as WRKY33, CAMTA3, TGA3 and WRKY70. Therefore, if we wish to utilise additional time series experiments to gain a higher-resolution insight into the early window of transcriptomic reprogramming, multiple treatments may be required such as a hormone (JA+ACC) and a PAMP/DAMP. This approach would maximise the number of lettuce-necrotroph DEGs that may be differentially expressed in these additional time series, uncovering high-resolution data on early transcriptional reprogramming events.

As seen with the Arabidopsis EXPLICIT model that utilised > 20,000 publicly available datasets to generate an expression predictor (Geng et al. 2021), the addition of relevant data sets may improve our model performance. Publicly available lettuce transcriptomic datasets include *L. sativa* cv. Tizian-*Rhizoctonia solani* infection (Verwaaijen et al. 2019), *L. sativa* cv. Cobham Green-*Bremia lactucae* infection (Fletcher et al. 2019), chitin-treated lettuce roots (Li et al. 2023b) and a 240-accession *Lactuca spp* diversity panel (Zhang et al. 2017b). Although using all data from these studies may saturate our model, a subset of their samples could be incorporated where our hubs show differing expression profiles.

To further reinforce confidence in the regulatory edges inferred by the network, more extensive *in vivo* target validation should be conducted. Considering the challenges in generating transgenic lettuce lines, higher throughput assays like the transient transformation of protoplasts or infiltration in mature leaves may present a viable path. Subsequently, if transcriptome-wide datasets are generated for validation, they might also contribute to training new, improved models.

While our GRN has made significant strides in capturing the regulatory dynamics in lettuce defence mechanisms, a future challenge would be to develop network models that allow *in silico* simulation of

perturbation effects. By not only identifying edges but also simulating the GRN's response to altering regulator expressions or modifying network structures (like adding or removing edges), attempt to re-wire the network.

5.3.2 Network predictions to the field: future challenges to generate disease-resistant lettuce cultivars

This work has constructed a lettuce-necrotroph defence GRN, and validated it as a reliable resource for the identification of lettuce defence regulators. The next significant step is to apply this knowledge in improving the pathogen resistance of cultivars - "GRN to field". This will need to be achieved without negatively impacting other important traits like yield, taste, and resistance to other pathogens/stresses and ensuring that the molecular biology techniques used are not legislated against in the country of intended use.

Defence regulators' expression is typically tightly controlled, with constitutive expression often leading to undesirable effects. This includes growth-defence trade-offs, SA-JA antagonism leading to contrasting biotrophic-necrotrophic resistance phenotypes, and altered taste profiles due to the continuous expression of defence metabolites (Belkhadir et al. 2014; Thomma et al. 1998; Agrawal et al. 2002). This was observed with our GRN hubs, as *Arabidopsis* lines expressing 35S::LsBOS1 showed reduced growth, suggesting that the constant expression of a hub gene is likely not to be desirable under field conditions.

Instead, the use of a cis-regulatory element (CRE) which induces high-level expression, rapidly in response to pathogen infection, but low expression in normal-growth conditions would be favourable. An 188bp CRE within a *Brassica napus* promoter, *pBnGH17^{D7}*, was shown to drive high expression specifically in response to *S. sclerotiorum*, but not in normal growth conditions, nor after treatment with defence hormones JA and ET (Lin et al. 2022). Using our lettuce-necrotroph transcriptomic datasets, lettuce promoters which drive high-level expression rapidly in response to necrotrophic pathogen infection could be identified (e.g. *pLsat_1_v5_gn_3.82701* or *pLsERF1*) and engineered to remove CREs that drive expression in non-infected tissue. Additionally, it may be possible to further engineer promoters by adding logic gates that can be activated and repressed in specific conditions (Brophy et al. 2022).

Utilising *pBnGH17^{D7}* or lettuce promoters with similar expression profiles could enable the conditional knockdown of genes that suppress pathogen defence, such as *Lsat_1_v5_gn_0_1380* (LsBIR1). We identified LsBIR1 to be strongly correlated with *S. sclerotiorum* susceptibility in our diversity panel (Pink et al. 2022). *Arabidopsis bir1-1* mutants show constitutively active defence responses, but have an extreme reduced growth phenotype (Gao et al. 2009; Liu et al. 2016; Ma et al. 2017). While LsBIR1 mutants might suffer from a significant yield reduction, we hypothesise that expressing a LsBIR1-RNAi construct under a

pathogen-responsive promoter (e.g. *pBnGH17^{D7}* or *pLsERF1*) could increase pathogen resistance without impacting yield. In addition, designing RNAi constructs to silence multiple orthologues could help overcome any functional redundancies.

However, these approaches involve the addition of foreign DNA via *Agrobacterium*-mediated transformation and therefore fall under the genetically modified (GM) classification. This imposes significant regulatory constraints in several parts of the world, including the UK and Europe (Turnbull et al. 2021). However, the recent passing of the Precision Breeding Act (2023) in England permits the development and marketing of gene edited (GE) crops (Coe and Ares 2023; Caccamo 2023). Consequently, technologies such as CRISPR-Cas9, which can induce targeted mutations without introducing foreign DNA, could become the focus of future breeding programmes.

The ideal candidates for gene editing would be genes that negatively regulate pathogen defence, increase resistance when mutated, and do not adversely impact other traits. Chapter 3 (Pink et al. 2023) identifies LsNAC53 as one such candidate. We've shown that *nac53-1* Arabidopsis mutants exhibit increased resistance to *B. cinerea*, and that LsNAC53ΔC expression within this mutant background complements AtNAC53, restoring the susceptibility to wild-type level. Interestingly, AtNAC53 mutants have demonstrated improved resistance to drought and heat-stress (Lee et al. 2012; Lee et al. 2014). Furthermore, AtNAC53 mutation does not impact *P. syringae* DC3000 resistance, suggesting independence from JA-SA antagonism (Hickman et al. 2019). These findings suggest that a CRISPR knockout mutant of LsNAC53 might improve resistance to necrotrophic pathogens and environmental stresses, while maintaining defence against biotrophic pathogens.

LsCAMTA3, a prominent hub in our GRN is another candidate for gene-editing. AtCAMTA3 mutants have been demonstrated to increase resistance to *B. cinerea*, *S. sclerotiorum* and *P. syringae* DC3000 (Galon et al. 2008; Du et al. 2009; Rahman et al. 2016). Although some defects like increased drought susceptibility and reduced growth have been associated with AtCAMTA3 mutations (Zeng et al. 2022; Du et al. 2009). Interestingly, these defects are not observed in warmer conditions (25-27°C). While AtCAMTA3 is transcriptionally downregulated in response to *B. cinerea*, LsCAMTA3 shows an upregulation upon *B. cinerea* and *S. sclerotiorum* infection, suggesting its mutation might considerably influence pathogen resistance. If identified, CREs within the LsCAMTA3 promotor responsible for pathogen-induced expression could be targeted for CRISPR mutation, potentially minimising adverse effects on other traits such as yield or drought tolerance.

Having validated a candidate gene as an *in vivo* defence regulator, the next steps involve manipulating this gene in lettuce plants using any of the methods discussed and then conducting field trials. A successful

variety must outperform existing cultivars for necrotrophic pathogen resistance in a field environment, ideally across several field sites in successive years. Moreover, it is crucial to ensure that the new variety maintains a comparable performance in yield, nutritional content and taste as the existing elite cultivars before it can be considered for commercialisation.

5.4 Concluding Remarks

The work presented within this thesis represents a significant improvement in our understanding of the lettuce defence response to necrotrophic fungal pathogens. By utilising several high-throughput transcriptomic datasets, it has been possible to:

- Identify over 5000 lettuce genes whose expression is correlated with *S. sclerotiorum* susceptibility
- Identify 16 high-confidence candidate genes located within five lettuce-necrotroph QTLs that may underly the disease resistance phenotypes
- Reveal a core set of 4362 genes which are differentially expressed in the same direction in response to both *B. cinerea* and *S. sclerotiorum*
- Infer a causal-directed gene regulatory network (GRN) which models transcriptional regulation during pathogen infection, and is able to identify candidate defence regulators
- Demonstrate that our GRN outperforms both random guessing and co-expression modules in prediction the downstream targets of a key lettuce defence regulator (LsERF1)

Through extensive validation of the GRN (performed in Chapters 3 and 4), we now have high-confidence in its ability to accurately identify both candidate defence regulators and their downstream targets. Thus, this GRN could ultimately be used to identify further lettuce defence regulators and to inform the generation of disease-resistant lettuce cultivars. However, careful manipulation of these regulators' expression may be required to limit pleiotropic effects that result from crude constitutive over-expression or knockout.

Data Availability

Supplemental Datasets:

Supplemental datasets for published and pre-printed chapters are available online

- Chapter 2 (Pink et al. 2022):
<https://link.springer.com/article/10.1007/s00122-022-04129-5#Sec20>
- Chapter 3 (Pink et al. 2023):
<https://www.biorxiv.org/content/10.1101/2023.07.19.549542v1.supplementary-material>

RNAseq data deposited on NCBI short reads archive (SRA):

- Diversity set and mapping population parent RNAseq data (PRJNA804213)
<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA804213>
- Lettuce-*B. cinerea* time-series RNAseq (PRJNA1013336)
<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1013336>
- LsERF1 overexpression RNAseq (PRJNA1022321)
<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1022321>

Lettuce Data Explorer (Shiny App):

A shiny app that allows users to view, plot or download data from the key data sets presented in this thesis for genes of interest through a web app (Figure A). The shiny app is available from University of York server (https://shiny.york.ac.uk/lettuce_transcriptomics/), or on shinyapps.io (<https://hpink97.shinyapps.io/Lettuce-Data/>). Source code for the app is available at https://github.com/hpink97/lettuce_data_app. Example outputs generated by the Shiny App are shown; time-series expression of lettuce orthologues of selected Arabidopsis genes (Figure B), time-series expression heatmap for DEGs associated with a specific GO-term (Figure C) and hub genes predicted to regulate genes involved with a specific GO-term (Figure D)

Lettuce Data Explorer

Citations:

Pink, H., Talbot, A., Graceson, A. et al. Identification of genetic loci in lettuce mediating quantitative resistance to fungal pathogens. *Theor Appl Genet*, 135, 2481-2500 (2022).

Pink, H., Talbot, A., Carter, R., et al. Identification of *Lactuca sativa* transcription factors impacting resistance to *Botrytis cinerea* through predictive network inference. *bioRxiv* (2023).

Select a dataset to analyse
Time-Series Expression

Gene selection method
Lettuce GeneID

Enter lettuce GeneIDs as comma separated values
LSaT_1_v5_gn_7_33721, LSaT_1_v5_gn_8_115261, LSaT_1_v5_gn_8_116421, LSaT_1_v

Do you wish to filter gene based on differential expression
Only display DEGs

Plot time-series expression in response to which fungi?
B. cinerea AND S. sclerotiorum

Maximum of number of genes to plot (filtered by MaxAbsLog2FC)
1000

Plot options: Plot style
Heatmap

Plot option: gene label size
10

Plot options: Do you wish to include mock gene expression
Yes

Welcome to Lettuce Data Explorer!

This tool simplifies the exploration of our lettuce transcriptomic datasets. Quickly find lettuce genes using Arabidopsis symbols/IDs, GO terms, or protein domains, allowing you to focus on your genes of interest.

Step 1: Select a dataset to analyse

- Time-series expression:** Explore dynamic expression profiles ...
- Gene Regulatory Network Analysis:** Explore predicted transcriptional regulation ...
- Lesion Size Correlation:** Examine gene expression's correlation ...

Step 2: Select your genes of interest ...

- Lettuce GeneID:** Input Lettuce gene ID(s).
- Orthologues of Arabidopsis Genes:** Provide an Arabidopsis gene ID ...
- Genes with GO-term:** Enter a GO term ...
- Genes with Protein Domain:** Search genes ...

Step 3: Dataset-specific options

- Dataset-specific gene selection criteria ...
- Plot customisation options

Step 4: Click 'Generate Results'

Figure A: Homepage of the Lettuce Data Explorer shiny app

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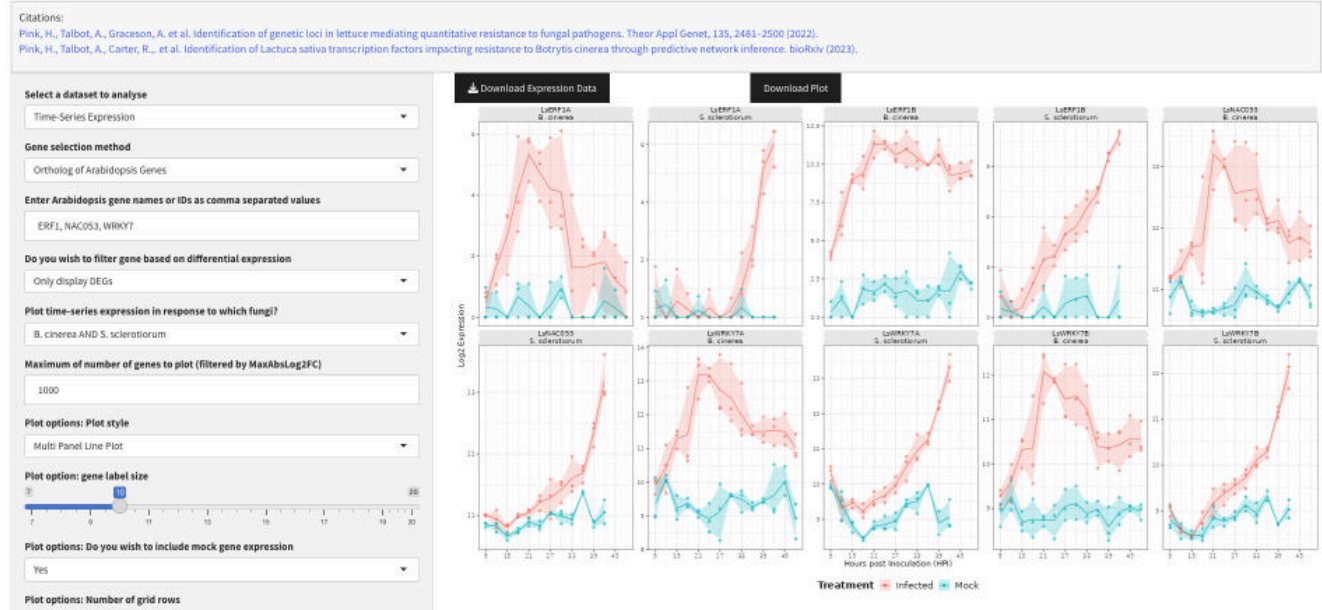


Figure B: An example output in the Shiny App, where a user has selected a “Multi Panel Line Plot” of Time-Series Expression for lettuce orthologues of “ERF1, NAC053, WRKY7” with filters applied to only display genes differentially expressed in the time-series. Download buttons above the plot enable the user to either download the plot or download the data used to produce the plot.

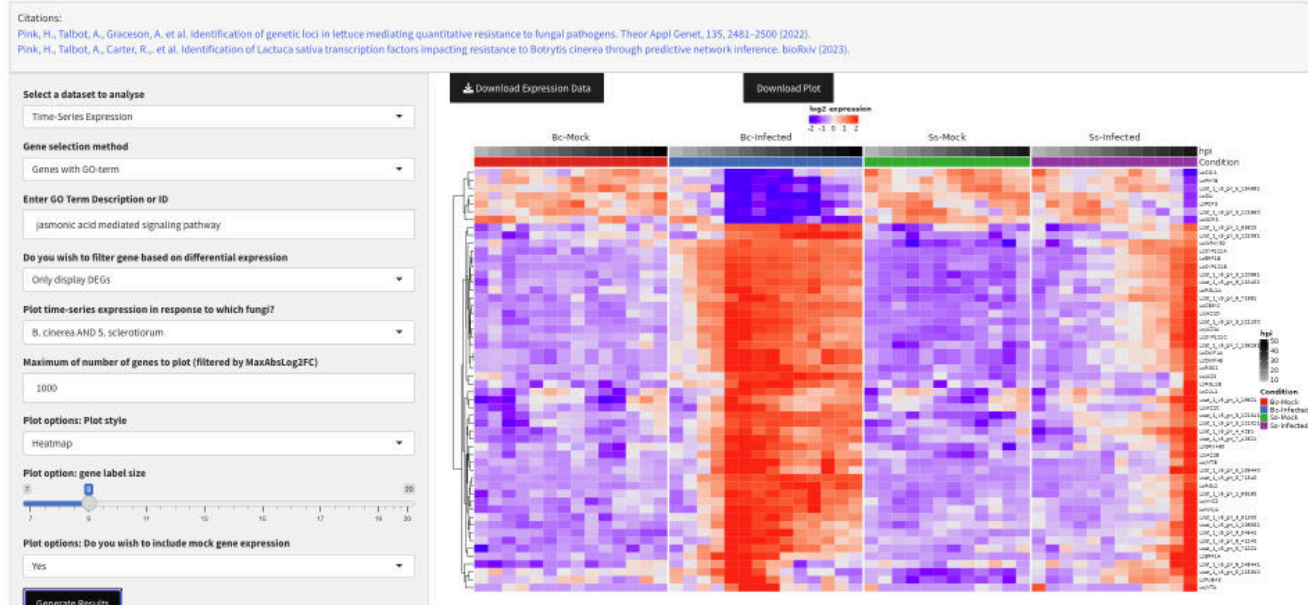


Figure C: An example output in the shiny app, where a user has selected a Heatmap displaying time-series expression for lettuce genes which are orthologues of Arabidopsis genes which are annotated with the GO-term “jasmonic acid mediated signalling pathway” (GO:0009867). The user has also selected to filter for just genes that are differentially expressed in the time-series. Download buttons above the plot enable the user to either download the plot or download the data used to produce the plot.

Lettuce Data Explorer

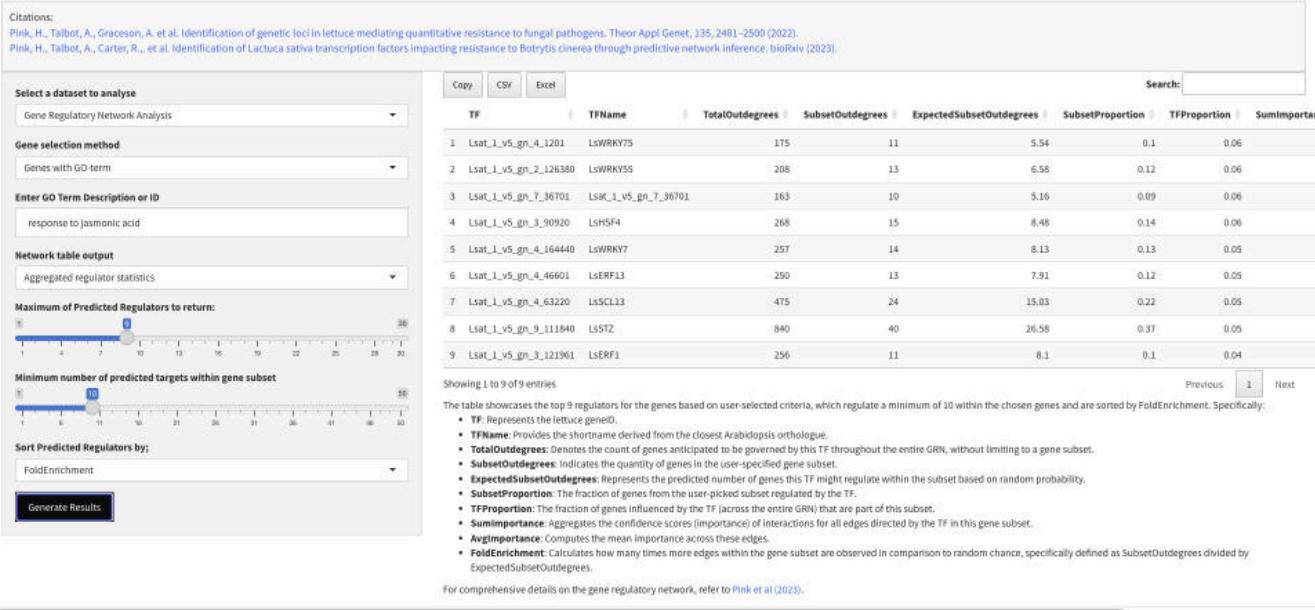


Figure D: An example output in the shiny app, identifying predicted gene regulatory network hubs for specific genes of interest. The user has selected to view “Aggregated regulator statistics” for the transcription factors which are predicted to regulate lettuce orthologues of genes annotated with the GO-term “response to jasmonic acid”. The user has applied an additional filter, requiring the transcription factor to have at least 10 predicted targets within the subset to be listed, and the results have been ordered by “FoldEnrichment”.

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