2022

Exploring genetic variability of *Pinus pinaster* in the molecular response pathways to the pinewood nematode as a source of markers for assisted selection

Inês Modesto

Dissertation presented to obtain the **Ph.D degree in Plant Biology**

PhD Program Plants for Life

Oeiras, August 2022

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER | UNIVERSIDADE NOVA DE LISBOA

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Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Dissertation presented to obtain the degree of Doctor of Science: Bioinformatics

Ghent University

Oeiras, August 2022

This work was performed at:

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This work was financed by Fundação para a Ciência e a Tecnologia, I.P. (FCT/MCTES), through the doctoral fellowship SFRH/BD/111687/2015, the grants GREEN-it (UID/Multi/04551/2013) and BioISI (UIDB/04046/2020 and UIDP/04046/2020), and through the project PTDC/BAA-MOL/28379/2017, LISBOA-01-0145-FEDER-028379 (FCT/MCTES and FEDER).

Acknowledgments

At the end of this journey, I would like to thank everyone that, in a way or another, contributed to it and made the finishing of this thesis possible.

To my supervisor Prof. Célia Miguel I would like to thank for all the support, guidance and availability during all these years. Your input and the discussions we had helped me be the scientist I am today.

To Prof. Yves Van de Peer for welcoming me in his lab, giving me all the necessary means to work on my project and always pointing me in the direction of the people that could help me.

To Dr. Isabel Carrasquinho for all the discussions about plant breeding and quantitative genetics, topics I did not know much about in the beginning of this project and that I now understand better much due to our conversations.

To Lieven Sterck and Polina Novikova for guiding me in the new world of bioinformatics.

To my thesis committee, Prof. Ana Cristina Figueiredo and Prof. Octávio Paulo, I would like to thank for the valuable input you gave me throughout the project.

To my colleagues from the ForGen Lab, Susana Lopes, Ana Alves, Bruno Costa, Vera Inácio, Ana Milhinhos, Inês Chaves, and all the people that came and went throughout these years, for their support, lab tips and for making the daily life at the lab joyful. To Vera Inácio for all the lab work and support at the end of the project and Bruno Costa for helping with servers and other bioinformatics issues. I would like to specially thank everyone that helped in the inoculation assays, working under intense heat in the greenhouses, including not only my group and my supervisors Prof. Célia Miguel and Dr. Isabel Carrasquinho, but also Ana Margarida Rodrigues from the Plant Metabolomics Lab, Hugo Matias from the ITQB

greenhouses, Lurdes Inácio and Maria João Barrento from INIAV, among others.

To all my colleagues and friends at VIB, for all the moments shared during lunch and afternoon breaks, the strange and yet fun conversations in the lounge room, and especially the sharing of chocolate, candy and cake.

To my friends in Gent, Cristina, François, Shubhada, Luca, Rodrigo, Carolina, Heike, Michiel, Sofia, Beste, André, Marc, Thomas, and all the people with whom I crossed my path, for making life in Gent interesting and fun, for all the Friday evenings out and other adventures. To Cristina, François, Shubhada and Luca, for welcoming me in Gent and making this city feel like a second home. To Cristina and François for keeping me company in the late evenings in the lab, and the fun returning home journeys. To Carolina, my neighbor that I had to go meet in another city, whom became my sister in Gent. And the Portuguese team, Carolina, Ricardo and André, for all the support and good moments during the last months of the PhD.

Aos meus amigos de Portugal, Yana, Diogo, Joaninha, Bin, Fernando, Simões, Pimpão, Mariana, por todas as sextas-feiras de convívio, jogos de tabuleiro, e parvoíce. À Inês, Luís, Andreia, Bernardo, Joana Castro, Filipa, por todos os bons momentos passados, quer seja à beira de uma piscina, em viagem, ou um simples café. À Joana Campos e ao Paulo, que sempre arranjam tempo para uma cerveja, seja em Lisboa, em Faro, em Londres, em Gent, ou online (e ainda falta irmos às ferratas!). Ao Miguel e ao Bruno por serem os meus companheiros de escalada, no rocódromo e na rocha. À Patrícia, que me apoiou sempre, mesmo do outro lado do oceano. À Carla, à Yana, à Cátia, à Joana Campos e à Carolina, por me ajudarem a manter a sanidade mental durante o lockdown, em pessoa e à distância. À Carla, por ser uma ótima colega de casa, especialmente em tempos de pandemia. A todos um obrigado pelo apoio e os momentos de descontração, sem os quais não seria possível chegar ao fim desta etapa.

To my Belgian family, Karien, Frank, Meme and Pepe, and my Spanish family, Stijn, Cristina, Alejandro and Dani, for welcoming me with open arms.

Aos meus pais e à minha irmã, pelo apoio incondicional que me deram sempre, e aos meus pais por todas as oportunidades que me proporcionaram e me permitiram chegar aqui.

To Thijs, for all the love and patience, and for fully supporting and encouraging me in this journey, especially in the hardest moments. I could not ask for someone more caring and understanding by my side.

Summary

Pine wilt disease (PWD) is caused by the endoparasitic nematode *Bursaphelenchus xylophilus*, or pinewood nematode (PWN). This disease is a serious environmental problem in Eastern Asia and Southwestern Europe. In Iberian Peninsula, it severely affects maritime pine (*Pinus pinaster* Ait.) forests. Despite the high susceptibility of *P. pinaster* to PWD, heritable resistance has been observed in selected half-sib families after PWN inoculation, showing that the implementation of breeding programs for PWD resistance is a viable and valuable strategy for managing this disease. Using a previously characterized half-sib family, we aimed at understanding the molecular basis of *P. pinaster* resistance to PWN inoculation. By comparing the transcriptional changes after inoculation in resistant and susceptible plants, pathways and genes involved in PWN resistance were highlighted. Jasmonic acid (JA) defence pathway, secondary metabolism, including terpene and lignin biosynthesis pathways, oxidative stress response genes, and resistance genes, seem to play key roles in achieving resistance, while the activation of the salicylic acid (SA) defence pathway was linked to susceptibility to PWN. Lignin quantification confirmed a significant increase of cell wall lignification in stem tissues of resistant plants, while higher SA levels were detected in the tissues of susceptible plants, supporting that cell wall reinforcement and hormone signalling mechanisms are essential in *P. pinaster* resistance to PWN. By sequencing the small non-coding RNAs (sRNAs) in the same samples, we were able to investigate their putative roles in the post-transcriptional regulation of *P. pinaster* immune response to PWN. MicroRNAs (miRNAs) differentially expressed after inoculation were predicted to target genes involved in JA defence pathway, response to oxidative stress and terpenoid biosynthesis, while miRNAs differentially expressed in resistant *versus* susceptible plants were predicted to target *RLKs* and *GDP-L-fucose synthase*, which may be involved in the initiation of PTI and ETI, in addition to JA pathway genes. These results emphasize the importance of such

pathways and genes in *P. pinaster* response and resistance to PWN. Based on the generated sRNA data, the possibility of bidirectional trans-kingdom RNA silencing was also explored. The identification of several putative interactions between *P. pinaster* genes and PWN miRNAs was further supported by degradome analysis. Putative targets of *P. pinaster* miRNAs were also identified in PWN, suggesting a role for trans-kingdom miRNA-mediated gene silencing in PWN parasitism as in *P. pinaster* resistance to PWD. Finally, the search for SNPs in the generated transcriptome data, allowed for the identification of 186,506 polymorphisms which may be relevant for future association studies aiming at finding molecular markers for PWD resistance to be used in *P. pinaster* breeding. Furthermore, a small set of these SNPs, highly divergent between resistant and susceptible samples, was genotyped for a larger sample size and weak associations with the phenotype after PWN inoculation were confirmed for two SNPs and one haplotype. In this way, candidate genes with functions possibly relevant for *P. pinaster* resistance to PWD were highlighted. Overall, important steps were taken into understanding the mechanisms involved in PWD resistance in *P. pinaster*.

Sumário

A doença da murchidão do pinheiro (DMP) é causada pelo nemátode da madeira do pinheiro (NMP, *Bursaphelenchus xylophilus*), que parasita o interior do tronco de várias espécies de árvores coníferas. Esta doença representa problema ambiental significativo na Ásia oriental e sudoeste da Europa. Na Península Ibérica, a DMP afeta severamente as florestas de pinheiro-bravo (*Pinus pinaster* Ait.). Apesar da grande suscetibilidade do pinheiro-bravo a esta doença, foram verificados níveis variáveis de resistência após inoculação com NMP em famílias de meios-irmãos selecionadas. Esta observação sugere que é possível implementar programas de melhoramento genético para a resistência à DMP, sendo esta uma estratégia importante na gestão da doença. Com o objetivo de compreender a base molecular da resistência do pinheiro-bravo ao NMP, foi selecionada uma família previamente caracterizada, com uma boa classificação relativamente à sobrevivência após inoculação. Ao comparar as alterações transcricionais após a inoculação em plantas resistentes e suscetíveis, foram destacados genes e vias metabólicas envolvidos na resistência ao NMP. A ativação da via de defesa do ácido jasmónico, as vias de síntese de metabolitos secundários, incluindo terpenos e lenhina, genes de resposta a stress oxidativo, e genes de resistência, parecem ter funções cruciais na resistência, enquanto a ativação da via de defesa do ácido salicílico foi associada à suscetibilidade ao NMP. A quantificação de lenhina confirmou um aumento deste composto nas paredes celulares de caules de plantas resistentes, enquanto níveis mais elevados de ácido salicílico foram encontrados em plantas suscetíveis, suportando a hipótese de que o reforço das paredes celulares e a resposta hormonal são essenciais na resistência do pinheiro-bravo ao NMP. A sequenciação de *small non-coding RNAs* (sRNAs) das mesmas amostras, permitiu investigar o possível papel destes na regulação pós-transcricional da resposta imune do pinheiro-bravo ao NMP. Os alvos previstos dos microRNAs (miRNAs) diferencialmente expressos após a inoculação

incluem genes envolvidos na via de defesa do ácido jasmónico, na resposta ao stress oxidativo e na síntese de terpenos. Por outro lado, os alvos previstos dos miRNAs possivelmente envolvidos na resposta de resistência incluem genes da via de defesa do ácido jasmónico, recetores transmembranares (*RLKs*) e *GDP-L-fucose synthase*, que codifica uma enzima possivelmente envolvida na ativação da resposta de defesa da planta. Estes resultados realçam a importância destes genes e vias metabólicas na resposta e resistência do pinheiro-bravo ao NMP. Com base nos dados de sRNAs gerados, a possibilidade de haver silenciamento cruzado e bidirecional de RNA, foi também explorada. A identificação de várias interações possíveis entre genes de pinheiro-bravo e miRNAs de NMP foi suportada pela análise de dados de degradoma. Também foram identificados genes de NMP como possíveis alvos dos miRNAs de pinheiro-bravo, sugerindo que o silenciamento de genes *trans-kingdom* através de miRNAs é importante tanto para o parasitismo do NMP como para a resistência do pinheiro-bravo a este nemátode. Por fim, a pesquisa de *single nucleotide polymorphisms* (SNPs) nos dados gerados de transcritoma permitiu identificar 186,506 polimorfismos. Estes SNPs poderão ser relevantes para estudos de associação com o objetivo de desenvolver marcadores moleculares de resistência à DMP, para utilização em programas de melhoramento como forma de distinguir precocemente plantas resistentes de suscetíveis. Além disso, foi genotipado um pequeno número de SNPs com elevada divergência entre plantas resistentes e suscetíveis, numa amostra maior de plantas. Foi encontrada uma associação fraca entre o fenótipo das plantas após inoculação com NMP e dois destes SNPs, assim como com um haplótipo. Desta forma, foi possível realçar genes candidatos com funções potencialmente relevantes para a resistência do pinheiro-bravo ao NMP. Em conclusão, este estudo permitiu dar passos importantes na compreensão dos mecanismos envolvidos na resistência do pinheiro-bravo à DMP.

Samenvatting

Pijnboomverwelkingsziekte (PVZ) wordt veroorzaakt door de endoparasitaire nematode *Bursaphelenchus xylophilus* of de pijnboomnematode (PBN). In Azië en het zuidwesten van Europa is PVZ uitgegroeid tot een belangrijk milieuprobleem. Op het Iberisch Schiereiland tast het *Pinus pinaster*-bossen ernstig aan. Ondanks de hoge gevoeligheid van *Pinus pinaster* voor PVZ zijn er bepaalde genetische varianten uit geselecteerde *half-sib*-families die overgeërfde resistentie vertonen na inoculatie met de nematode. Dit geeft aan dat de implementatie van kweekprogramma's voor resistentie mogelijk is en een waardevolle strategie kan zijn om PVZ te beheren en beheersen. Door gebruik te maken van eerder gekarakteriseerde *half-sib*-families hebben we getracht om de moleculaire basis van *P. pinaster* resistentie tegen PBN-inoculatie beter te begrijpen. Door het vergelijken van transcriptionele veranderingen na inoculatie in zowel resistente als gevoelige planten hebben we een aantal *pathways* en genen kunnen onderscheiden die geassocieerd worden met PVZ-resistentie. Onder meer de jasmijnzuur verdedigingspathway, secundaire metabolische *pathways*, inclusief terpenoïde en lignine biosynthese, oxidatieve stressresponsgenen, en resistentiegenen, blijken een hoofdrol te spelen in het ontstaan van resistentie. Anderzijds kan de activatie van de salicylzuur verdedigingspathway worden geassocieerd met gevoeligheid voor het ontwikkelen van PVZ. Kwantificatietechnieken bevestigen een significante verhoging van celwandlignificatie in de weefsels van de stam van resistente planten, terwijl hogere niveaus van salicylzuur gedetecteerd werden in de weefsels van gevoelige planten. Dit bevestigt dat een versterking van de celwand en mechanismen van hormoonsignalisatie essentieel zijn voor *P. pinaster* PVZ-resistentie. Door het sequeneren van *small non-coding RNAs* (sRNAs) van dezelfde stalen konden we ook hun vermoedelijke rol onderzoeken in de post-transcriptionele regulatie van de immuunrespons van *P. pinaster* tegen de nematode. MicroRNAs (miRNAs) die een differentiële expressie

vertoonden na inoculatie hadden genen als voorspeld doelwit die betrokken zijn bij de jasmijnzuur verdedigingspathway, en genen betrokken bij de reactie op oxidatieve stress en terpenoïde biosynthese. Dit terwijl miRNAs die mogelijks betrokken zijn bij resistentie als voorspeld doelwit vooral *RLKs* en *GDP-L-fucose synthase* hadden, die geassocieerd worden met de initiatie van PTI en ETI, maar ook genen van de jasmijnzuur verdedigingspathway. Deze resultaten benadrukken het belang van de vermelde *pathways* en genen voor de reactie van *P. pinaster* en resistentie voor PVZ. Met de sRNA data hebben we ook de mogelijkheid van een bi-directionele *trans-kingdom RNA silencing* onderzocht. Verschillende *P. pinaster* genen werden geïdentificeerd als mogelijke doelen voor de nematode miRNAs, resultaten die ook ondersteund werden door degradoomanalyse. Vermeende interactoren van *P. pinaster* miRNAs werden ook geïdentificeerd in PVZ, wat aangeeft dat *trans-kingdom* miRNA-gemedieerde interferentie een rol speelt in zowel parasitisme door de nematode als in de *P. pinaster* PVZ-resistentie. Ten slotte konden er, door het detecteren van SNPs in de transcriptoomdata die we eerder analyseerden, 185,506 polymorfismes worden geïdentificeerd die relevant zijn voor associatiestudies, met als doel het vinden van moleculaire merkers voor PVZ-resistentie die zouden kunnen gebruikt worden in *P. pinaster* kweekprogramma's. Een kleine set van deze SNPs die zeer verschillend waren tussen resistente en gevoelige stalen hebben we verder gegenotypeerd op een steekproef van grotere omvang. Hierbij werden zwakke associaties met het fenotype na inoculatie met de nematode bevestigd voor twee SNPs en één *haplotype*. Op deze manier werden kandidaatgenen benadrukt met functies die mogelijks relevant zijn voor *P. pinaster* PVZ-resistentie. Algemeen kunnen we stellen dat er belangrijke stappen werden ondernomen om de mechanismen van PVZ-resistentie in *P. pinaster* beter te begrijpen.

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List of abbreviations

4CL	4-coumarate-CoA ligase
ABA	abscisic acid
AOS	allene oxide synthases
AS	bifunctional abietadiene synthase
BAK1	brassinosteroid-insensitive 1-associated kinase 1
bHLH	basic helix-loop-helix
bp	base pairs
BP	biological process
CAD	cinnamyl-alcohol dehydrogenase
CC	cellular component
CCoAOMT	caffeoyl-CoA O-methyltransferase
CCR	cinnamoyl-CoA reductase
CDS	coding regions
CHS	chalcone synthase
CHS	chalcone synthase
COMT	caffeic acid 3-O-methyltransferase
CTAB	cetrimonium bromide
DAMP	damage-associated molecular pattern
DE	differential expression
DEG	differentially expressed gene
DMP	doença da murchidão do pinheiro
DNA	Deoxyribonucleic acid
dpi	days post-inoculation
DPME	dihydropinosylvin monomethyl ether
DXS	1-D-xylulose-5-phosphate synthase
EDS1	enhanced disease susceptibility 1
ERF	ethylene response factor
ET	ethylene
ETI	effector-triggered immunity
FDR	false discovery rate
FLS2	flagellin-sensitive 2
Gb	gigabase pairs
GERD	(-)-germacrene D synthase
GLOX	aldehyde oxidase
GO	gene ontology
half-sib	half-sibling

HDS	1-hydroxy-2-methyl- 2-(E)-butenyl-4-diphosphate synthase
HIPP41	heavy metal-associated isoprenylated plant protein 41
HMGCR	hydroxymethylglutaryl-CoA reductase
hpi	hours post-inoculation
HRPG	*hydroxyproline-rich glycoproteins extensins*
JA	jasmonic acid
JA-Ile	jasmonate-isoleucine
JAZ/Tify	jasmonate-ZIM domain
KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIN12	kinesin-like protein 12F
LAC	laccase
LDOX	leucoanthocyanidin dioxygenase
LOX	lipoxygenase
LPS	bifunctional levopimaradiene synthase
MAF	minor allele frequency
MAPK	mitogen-activated protein kinase
MEE12	maternal effect embryo arrest 12
MeJA	methyl jasmonate
MeSA	methyl salicylate
MF	molecular function
miRNA	micro RNA
MKS1	map kinase substrate 1
MQ	mapping quality
mRNA	messenger RNA
NAMPs	nematode-associated molecular pattern
NLR	nucleotide-binding/leucine-rich-repeat
NMP	nemátode da madeira do pinheiro
NonSyn	nonsynonymous
OPR	12-oxophytodienoic acid reductase
OPR3	12-oxophytodienoate reductase 3
PAD4	phytoalexin deficient 4
PAL	phenylalanine ammonia lyase
PAMP	pathogen-associated molecular pattern
PCMP-E91	pentatricopeptide repeat-containing protein E91
PCR	polymerase chain reaction
PER	peroxidase
PHR1	phosphate starvation response 1

PLA2G	phospholipase A2
PME	pinosylvin monomethyl ether
PP2C	protein phosphatase 2C
PR	pathogenesis-related
PTI	pattern-triggered immunity
PVZ	pijnboomverwelkingsziekte
PWD	pine wilt disease
PWN	pinewood nematode
QTL	quantitative trait loci
R	resistant
Res	resistant
RKN	root-knot nematode
RLK	receptor-like kinase
RLP	receptor-like protein
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	reactive oxygen species
RT-qPCR	real-time quantitative PCR
S	susceptible
SA	salicylic acid
SAG101	senescence-associated gene 101
SNP	single nucleotide polymorphism
sRNA	small RNA
SSR	simple-sequence repeat
STS	pinosylvin synthase
Sus	susceptible
Syn	synonymous
tasiRNA	trans-acting small interfering RNA
TPS	terpene synthase
UGT5	UDP-glycosyltransferase 5
VAP1	venom-allergen like protein 1
VAP2	venom-allergen like protein 2

Chapter 1

Introduction

Section 1.3. has been adapted from the following publication:

Modesto, I., Mendes, A., Carrasquinho, I. & Miguel, C. M. (2022) Molecular defence response of pine trees (*Pinus* spp.) to the parasitic nematode *Bursaphelenchus xylophilus*. *Cells*, 11, 3208. https://doi.org/10.3390/cells11203208

Inês Modesto wrote the original draft except for section 1.3.3., reviewed and edited section 1.3.3., and prepared all the figures and tables.

1. Introduction

1.1. *Pinus pinaster* and its importance in the European forest

Pinus pinaster Aiton (maritime pine) is a conifer tree native to the Western Mediterranean basin and widely distributed across Southwestern Europe, including Iberian Peninsula, Southern France, and Western Italy (Figure 1.1) (Grivet et al., 2011; Grivet et al., 2017). *P. pinaster* is found in a variety of ecosystems, occupying a broad range of altitudes, climate conditions and soils. Due to its high tolerance to different environments, ability to grow on poor sandy soils and fast growth characteristics, *P. pinaster* has been used for soil protection, reforestation and intensive plantations for commercial exploitation (Abad Viñas et al., 2016). *Pinus pinaster* has a high level of genetic variation, which has been structured both by natural events such as glaciations and by anthropogenic interference due to extensive plantation (Bucci et al., 2007; De-Lucas et al., 2009; Eckert et al., 2010). This resulted in highly differentiated populations and local adaption to distinct conditions (Grivet et al., 2011; Grivet et al., 2017). Growth traits, such as height, stem form and diameter at breast height (Correia et al., 2008; Correia et al., 2010; González-Martínez et al., 2002), as well as adaptive traits, such as survival, polycyclism, water-use efficiency, drought tolerance (Aranda et al., 2010; Correia et al., 2008; Correia et al., 2010; Gaspar et al., 2013) and resistance to pests or diseases (Burban et al., 1999; Elvira-Recuenco et al., 2014; Menéndez-Gutiérrez et al., 2017a), are highly variable in *P. pinaster* populations and differentiated across geographical regions. Significant genotype-environmental correlations have also been described using several types of molecular markers, revealing temperature as an important driver of local adaptation (Gómez et al., 2005; Grivet et al., 2011; Jaramillo-Correa et al., 2015).

Several studies focused on associating *P. pinaster* genetic variation to traits of interest. A number of loci were linked to growth, wood cellulose content (Lepoittevin et al., 2012), height (Bartholomé et al., 2016a; Cabezas et al.,

2015; Hurel et al., 2021), stem straightness (Bartholomé et al., 2016a), spring phenology, and susceptibility to fungal pathogens (Hurel et al., 2021). This information can be useful to implement in breeding programs, including in breeding strategies using genomic selection (Hurel et al., 2021; Sterck et al., 2022). However, to be able to successfully detect such associations in conifer species, which tend to present very fast decay in linkage disequilibrium, a high density of molecular markers is necessary to increase the likelihood of capturing markers close to causal variants (Sterck et al., 2022). With this in mind, efforts have been made to develop genotyping arrays with increasingly higher number of SNP markers (Bartholomé et al., 2016b; Chancerel et al., 2013; Isik et al., 2015; Plomion et al., 2016). Recently, a 50K array containing molecular markers of four tree species, including 13,000 *P. pinaster* SNPs, was developed in the scope of the project B4EST (Sterck et al., 2022).

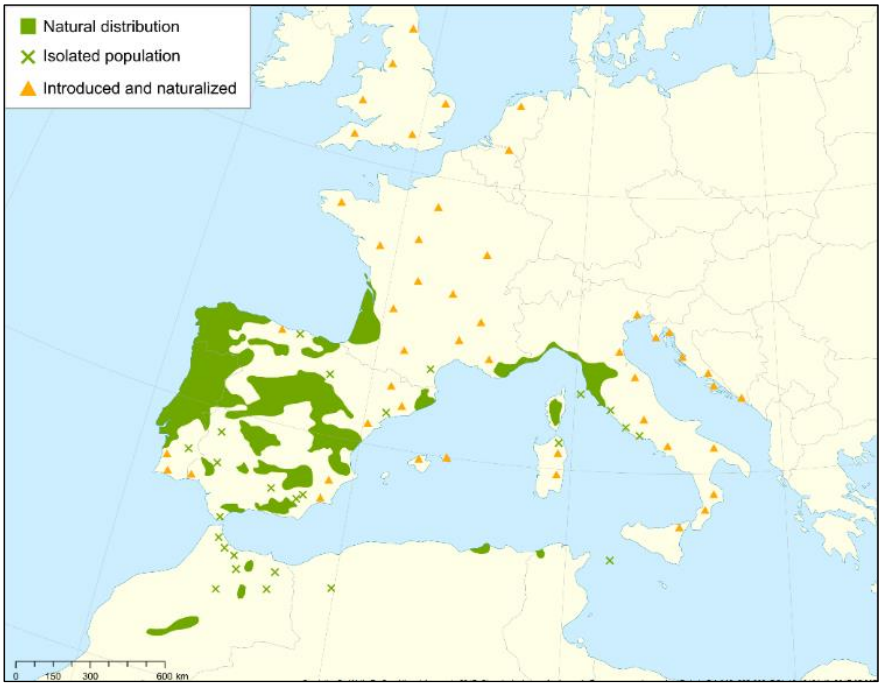

Figure 1.1. Distribution map of *Pinus pinaster*. Image obtained from Caudullo et al. (2017).

In Europe, coniferous trees compose 46% of the forests, from which 29% are pine species like *P. pinaster* and *Pinus sylvestris* (FOREST EUROPE, 2020). In several areas of Europe, *P. pinaster* corresponds to more than 70% of the forest species, namely in the West coast of Portugal and Galicia (Spain), the basin of the Douro river (Portugal), Castile and Leon (Spain), and in Landes (southwestern France) (Abad Viñas et al., 2016; Alonso-Esteban et al., 2022). Due to artificial plantation and naturalization, *P. pinaster* is now also present in Southeastern France, Greece, Adriatic countries, United Kingdom, Belgium and the Netherlands (Figure 1.1) (Abad Viñas et al., 2016).

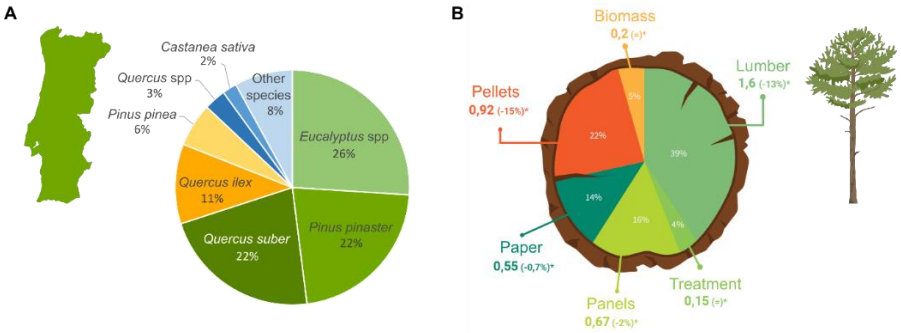

Figure 1.2. Main forest tree species (A) and main industrial uses of *Pinus pinaster* wood (B) in Portugal. Image created based on data obtained from ICNF (2019) (A). Image adapted from Centro PINUS (2021) (B).

In Portugal, *P. pinaster* is the second most abundant tree species (22%), together with cork oak, and the most abundant conifer species (ICNF, 2019) (Figure 1.2A). *Pinus pinaster* has a high economic relevance in the forest industry, representing 80% of the jobs in this sector. It is used as a source of wood for furniture, construction, or panels, as well as for energy production (pellets and biomass) and paper production (Figure 1.2B). *Pinus pinaster* is also used for resin extraction, which has several uses in the manufacturing of chemicals (Centro PINUS, 2021), and has been explored for the extraction of compounds with medicinal value (e.g., Malekahmadi et al., 2019; Simpson et al., 2019). Additionally, *P. pinaster* forests have a high

ecological relevance, playing a role as wildlife habitat, in soil protection and in climate, being the greatest carbon sink in the Portuguese forest (ICNF, 2019). Despite its significance, *P. pinaster* forest area has been declining in Portugal mainly due to wildfires, and the occurrence of diseases and pests (Centro PINUS, 2021). One of the diseases with higher impact in the last three decades has been pine wilt disease (Vicente et al., 2012).

1.1.1. *Pinus pinaster* genomic resources

Conifer species have very large genomes, ranging from 20 to 30 Gb, and with high complexity due to their repetitive nature (Mackay et al., 2012). *Pinus pinaster* has a highly heterozygous diploid genome of around 28 Gb distributed in 12 chromosomes (Sterck et al., 2022). These characteristics make sequencing and analysing such genomes a challenge. Despite the difficulties, the use of high throughput technologies have allowed for genomic sequences to be released for several conifer species in recent years (Birol et al., 2013; Grivet et al., 2017; Nystedt et al., 2013; Zimin et al., 2017). Efforts have also been made to make *P. pinaster* genome available, using a hybrid approach combining Illumina, 454 and long-read Nanopore sequencing of DNA from a single megagametophyte, an haploid tissue (Sterck et al., 2022). However, the assembling and processing of such data is complex and time consuming, and the genome assembly has not yet been publicly released.

A comprehensive reference transcriptome is, however, available for *P. pinaster* (Canales et al., 2014; Cañas et al., 2017; de María et al., 2020). The first reference transcriptome published included genes expressed in a variety of tissues from adult trees (cones, male and female strobili, buds, xylem and phloem), seedlings (needles, stems and roots), embryonic masses, as well as somatic and zygotic embryos in different developmental stages (Canales et al., 2014). In Cañas et al. (2017), laser capture microdissection followed by transcriptomic analysis allowed for the discovery of transcripts in specific cell types and tissues in *P. pinaster*

seedlings, such as the apical meristem, root cortex and root vascular tissues, among others. As a result, an improved reference transcriptome has been made available and an expression atlas was created, where gene expression level and localization can be accessed (ConGenIE.org). More recently, information of gene expression in different seedling tissues (needles, stems and roots) under drought conditions has been added to this reference transcriptome (de María et al., 2020). *Pinus pinaster* reference transcriptome has allowed for the identification of a large number of protein coding genes involved in a wide range of processes, representing a valuable resource for functional genomics research. *Pinus pinaster* transcriptomics, genomics and genetic information is available in several public databases, such as SustainPineDB (https://www.scbi.uma.es/sustainpinedb/), ConGenIE.org (http://v22.popgenie.org/microdisection) and Gymno PLAZA (https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/organism/view/Pinus+pinaster).

1.2. Pine wilt disease, a worldwide threat to conifer forests

Pine wilt disease (PWD) is one of the most serious threats to conifer forests worldwide, causing the loss of millions of pine trees annually (Kim et al., 2020). This disease is caused by pinewood nematode [PWN, *Bursaphelenchus xylophilus* (Steiner & Bührer, 1934; Nickle 1970)], which originated in North America (Figure 1.3). PWD was first reported in Japan in the beginning of the 20th century, where PWN was introduced. Since then, PWD has been spreading through other East Asian countries, such as China, Korea, and Taiwan. In Europe, it was first detected in Portugal in the late 1990's (Mota et al., 1999) and more recently in Spain (Abelleira et al., 2011). Since its introduction, PWD has caused an enormous loss of pine forest coverage in Japan. From 2004 to 2014, it was estimated that PWD caused an annual loss of 5000 hectares, resulting in large economic and ecological impacts. In Europe, PWD is predicted to spread to over 8-

34% of its geographical area by 2030, potentially leading to an estimated cumulative forestry stock loss of 22 billion euros (Kim et al., 2020).

Although PWN can infect a large range of conifer species, different species show distinct levels of susceptibility (Jones et al., 2008; Takeuchi, 2008). In North America, little damage is caused by PWD, as pine species are mostly resistant to it (e.g., *Pinus strobus* and *Pinus taeda*). Some species from other geographical regions have also demonstrated resistance, such as *Pinus pinea* and *Pinus nigra* in Europe (Franco et al., 2011). However, several species in Asia, such as *Pinus thunbergii*, *Pinus densiflora* and *Pinus massoniana*, as well as the European species *Pinus pinaster* (Figure 1.4) and *Pinus sylvestris* have shown high susceptibility to the disease (Takeuchi, 2008).

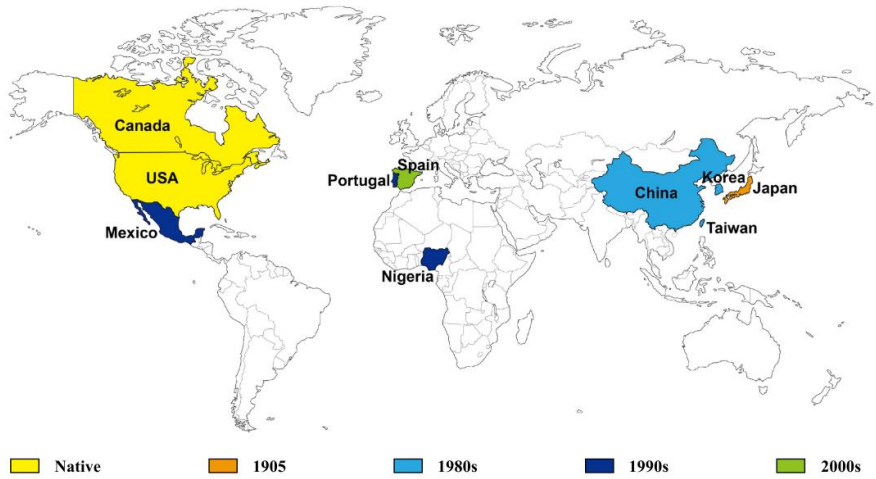

Figure 1.3. History of pinewood nematode invasion around the world. Image obtained from Kim et al. (2020).

The absence of symptoms after PWN infection may be due to resistance to PWD, in which the plant is able to limit the multiplication and spreading of the PWN, or tolerance, in which the infected tree remains healthy despite the multiplication of the PWN (Trudgill, 1991; Woodcock et al., 2018). In the case of *P. pinaster* (Menéndez-Gutiérrez et al., 2017b) and *P. thunbergii*

(Ichihara et al., 2000; Kusumoto et al., 2014; Son et al., 2015), plants without symptoms had lower amounts of PWNs when compared to symptomatic plants (Menéndez-Gutiérrez et al., 2017b), suggesting resistance to PWD. For the species *P. strobus and Pinus rigida*, similar results were observed (Son et al 2015). As it is not known if the multiplication or spreading of PWN is constrained in all the mentioned species throughout this work, the term "resistance" may include both true resistance and tolerance to PWD.

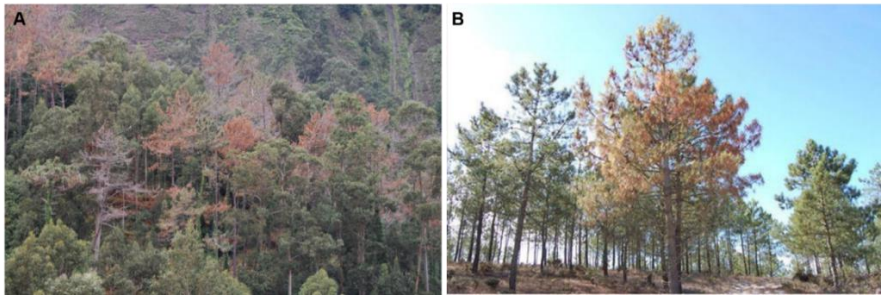

Figure 1.4. *Pinus pinaster* trees displaying symptoms of pine wilt disease. Images obtained from http://www.rephrame.eu/ptk-036.php (A) and Vicente et al. (2012) (B).

To limit the spreading of PWD, several phytosanitary measures were implemented throughout the years in the affected countries (Zhao, 2008). In Portugal, a phytosanitary strip of 3 km surrounding the area where PWN was detected, was established a few years after the first detection (Mota and Vieira, 2008). However, PWN still spread throughout most of the Portuguese territory, and a new phytosanitary buffer zone was established, surrounding the Portuguese frontier with Spain (ICNF, 2018). Presently, control measures consist mainly of detecting and eliminating symptomatic trees, followed by burning or fumigation of the wood, preventing movement of contaminated wood, and controlling the insect vector (Rodrigues, 2008; Zhao, 2008). The expansion of PWD in European forests is likely to be further aggravated by climate change, as PWD is associated with higher

temperatures and drought, conditions predicted to increase in frequency and intensity in the coming years (Gruffudd et al., 2019; Hirata et al., 2017).

1.2.1. Disease development and transmission

Pine wilt disease depends on a complex system involving the pinewood nematode (Figure 1.5A), an insect vector and a susceptible host (Futai, 2013; Jones et al., 2008). For the disease to spread, these three factors have to be present in combination with favourable temperature conditions (25°C to 30°C) (Yamaguchi et al., 2020). The insect vectors are longhorn beetles of the genus *Monochamus* spp. In Portugal, the only known vector is *Monochamus galloprovincialis* (Figure 1.5B) (Jones et al., 2008; Vicente et al., 2012).

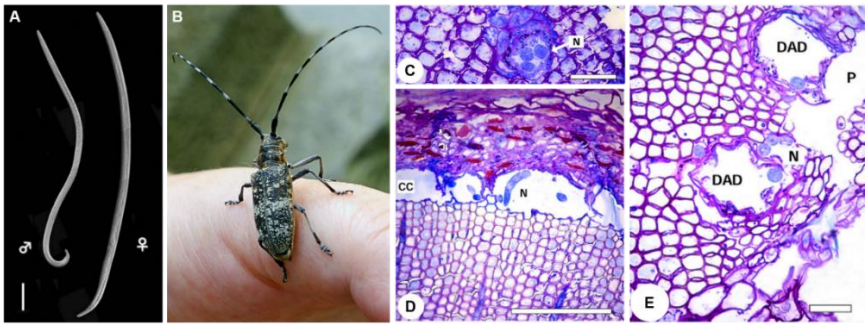

Figure 1.5. Pinewood nematode (A), the insect vector *Monochamus galloprovincialis* (B), and aspects of damage caused in the plant tissues by PWN (C-D). Pinewood nematode (N) migrates inside the plant stem through resin ducts **(C)**, and feeds on plant tissues causing damage to the cambial zone (CC) **(D)**, the destruction of epithelial cells of the cortical and axial (DAD) resin ducts, as well as the destruction of the pith tissue (P) **(E)**. Scale bars = 50 µm (A, C), 200 µm (D, E). Images obtained from Hasegawa and Miwa (2008) (A), https://commons.wikimedia.org/wiki/File:Monochamus_galloprovincialis_H26-42.jpg (B) and Rodrigues et al. (2021a) (C-E).

Pinewood nematode is an endoparasitic migratory nematode that infects the stem of susceptible trees (Jones et al., 2008; Kim et al., 2020; Vicente et al., 2012). These nematodes enter the plant tissues through wounds made by the insect vector while feeding on young tree branches. Once

inside the plant, PWNs move through resin canals, feed on epithelial and parenchymal cells, and reproduce, causing extensive damage (Figure 1.5C-E). The substances leaked by dead plant cells block water conductance in the xylem, causing cavitation and embolism of the tracheids (Futai, 2013). The tree starts to wilt and pine needles become chlorotic (Figure 1.6), leading to tree death. Before these visible symptoms, a reduction of oleoresin flow can be observed from artificial wounds in the stem (Jones et al., 2008).

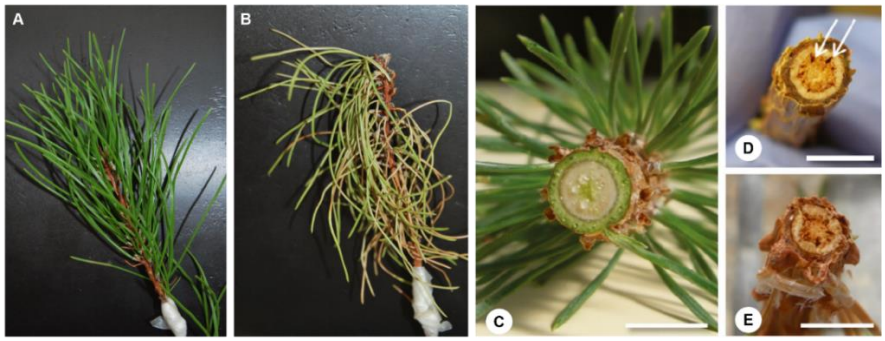

Figure 1.6. Symptoms of pine wilt disease in *Pinus* spp seedlings. Two-year old *Pinus pinaster* seedling without symptoms after inoculation with pinewood nematode **(A)**, and seedling displaying wilting symptoms and chlorotic needles **(B)**. Cross-section of healthy two-year old *Pinus sylvestris* seedling, showing resin exudation from the resin ducts **(C)**. Cross-section of *P. sylvestris* seedlings inoculated with pinewood nematode, showing desiccation of stem tissues, formation of cavities, and cessation of resin exudation **(D-E)**. Scale bars = 0.5 cm (C-E). Images obtained from Rodrigues et al. (2017) (C-E).

Inside the tree, PWNs develop through four stages of propagative juveniles, which moult into adult males and females (Figure 1.7). Under favourable conditions, PWNs complete their life cycle from egg to adult in 6 days (Vicente et al., 2012). As the tree starts to wilt, plant tissues are invaded by fungi and PWNs switch from phytophagous to a mycophagous phase. When the nematode population increases significantly and food becomes scarce, the nematodes enter the dispersal cycle, forming the specialized third-stage juvenile (DL3) around the pupal chambers of the insect vector.

They then moult into dauer juveniles (DL4), a non-feeding dispersive stage that enters the insect vector when they emerge. The insect vector transports the nematodes in its trachea and elytra to new healthy trees. PWNs can also be transmitted to dying trees or freshly cut logs during the insect vector's oviposition. Although this process does not lead to PWD, it contributes to the dispersal of the disease (Futai, 2013; Jones et al., 2008; Kim et al., 2020; Vicente et al., 2012).

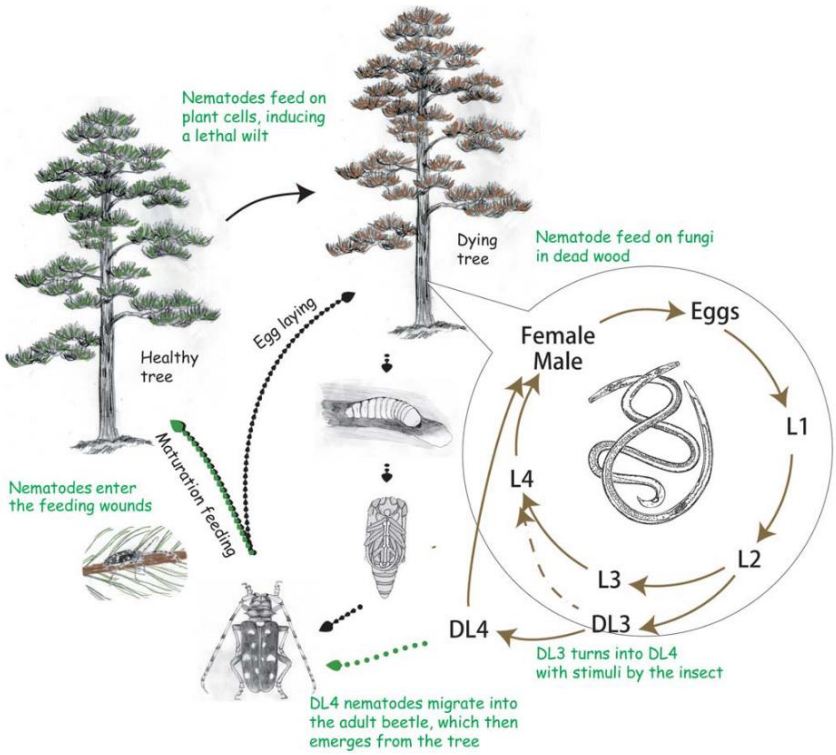

Figure 1.7. The life cycle of pinewood nematode. The nematode develops (brown arrows) through four larval stages (L1-L4) and moults into adult, which reproduces in the stem tissues while food is available. When food becomes scarce, PWN enters the dispersal stage by turning into a specialized larva (DL3). PWN subsequently moults into a dispersal larva (DL4) in the presence of the insect vector pupae and is carried to healthy trees by the adult insects when they emerge. Image obtained from Kikuchi et al. (2011).

Recent studies have pointed to a role of bacteria associated with PWN in disease development (Proença et al., 2017; Vicente et al., 2012). Different bacteria genera have been isolated from PWNs originating from different countries. Some of these bacteria secreted phytotoxins that may be involved in the pathogenesis of PWN. *In vitro* studies suggested that aseptic PWNs need to be inoculated in combination with the associated bacteria to cause PWD (Han et al., 2003; Zhao et al., 2003). However, other studies have shown that aseptic PWN can cause PWD, while bacteria isolates from PWNs did not cause disease (Jones et al., 2008; Zhu et al., 2012). A mutualistic symbiotic relationship between PWN and the associated bacteria has also been suggested (Zhao and Lin, 2005). Alternatively, bacteria carried by PWN may be endophytes with no direct effect on PWD, as these bacterial species are naturally present in pine trees and the environment (Proença et al., 2017). Therefore, the role of bacteria in PWN pathogenicity is still unclear and further studies are needed to understand the mechanisms involved (Proença et al., 2017; Vicente et al., 2012).

1.2.2. Breeding for resistance to PWD

Breeding for resistance can be an effective long-term approach to control the spreading of pests and diseases, as well as their associated damage (Naidoo et al., 2019; Sniezko and Koch, 2017). Genetic variability in survival to PWN inoculation has been observed in several pine species susceptible to PWD, opening the way for the implementation of breeding programs to PWD resistance (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017b; Nose and Shiraishi, 2008). Breeding programs have been successfully established for *P. thunbergii*, *P. densiflora* and *P. massoniana* in China and Japan (Nose and Shiraishi, 2008; Toda and Kurinobu, 2002; Xu et al., 2012). In Portugal and Spain, the first steps for the implementation of similar breeding programs for *P. pinaster* have also been taken (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017b; Ribeiro et al., 2012). From a reference population of 457 trees selected from a highly infested stand in Portugal (Ribeiro et al., 2012), 96 half-sib families were

evaluated for genetic variation of survival to PWN inoculation and significant genetic variability was detected (Carrasquinho et al., 2018). Predicted survival means at 157 days post-inoculation ranged from 6 to 23% and a moderate family heritability for survival (0.37) was obtained. The guidelines for a resistance improvement program were established and the 15 top-ranked families were selected for starting a clonal seed orchard (Carrasquinho et al., 2018). In Spain, similar inoculation assays were performed with 81 half-sib families selected from the Galician breeding program for growth and stem form (Menéndez-Gutiérrez et al., 2017b; Zas and Merlo, 2008). A moderate family heritability for mortality after PWN inoculation (0.59) was also obtained, but mortality was positively correlated with tree hight, suggesting that the current selection criteria for the Galician breeding program may increase the susceptibility of this population to PWD (Menéndez-Gutiérrez et al., 2017b).

1.3. Molecular defence response of pine trees (*Pinus* spp.) to the parasitic nematode *Bursaphelenchus xylophilus*

1.3.1. Immune signalling pathways

Although the molecular mechanisms involved in plant defence response to biotic stresses have been studied mainly in Arabidopsis and crop species, immune signalling pathways seem to be conserved across plant families (Gillet et al., 2017; Zipfel, 2014). The first level of plant defence is called pattern-triggered immunity (PTI) and initiates with the recognition by receptors localized on the cell surface (receptor-like kinases, RLKs, or receptor-like proteins, RLPs), of molecules known as pathogen-associated molecular patterns (PAMPs), conserved in large groups of pathogens, parasites, or pests, or of host derived molecules resulting from plant cell damage, known as damage-associated molecular patterns (DAMPs) (Couto and Zipfel, 2016; Zipfel, 2014). In the case of nematodes, defence is triggered by nematode-associated PAMPs (NAMPs), such as the pheromones ascarosides, chitin present in nematode eggshells and

pharynx, and likely other molecules present on their surface coat (Holbein et al., 2016). The recognition of a foreign organism triggers a series of signalling events in the plant cell, including bursts of calcium and reactive oxygen species (ROS), as well as the activation of mitogen-activated and calcium-dependent protein kinases (MAPKs and CDPKs) (Bigeard et al., 2015). Adapted pathogens can, however, suppress the plant's immunity though the release of effectors. In turn, if these effectors are recognised by resistance genes, often intracellular nucleotide-binding/leucine-rich-repeat (NLR) receptors, the more robust defence response known as effector triggered immunity (ETI) is initiated (Cui et al., 2015). Although ETI is typically seen in response to biotrophic pathogens, relevant roles have been described for resistance genes, including NLR receptors, in achieving resistance to parasitic nematodes (Sato et al., 2019; Zheng et al., 2021) and herbivorous insects (Erb and Reymond, 2019; Hogenhout and Bos, 2011). Both the activation of PTI and ETI lead to a transcriptional reprogramming and the expression of defence response genes, including the activation of phytohormones pathways, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Dodds and Rathjen, 2010).

1.3.2. Defence mechanisms highlighted by the transcriptional response to PWN

In recent years, several studies on gene expression after PWN inoculation have been published in a variety of susceptible pine species and in the resistant *P. pinea* and *P. yunnanensis* (Table 1.1). For *P. thunbergii* (Hirao et al., 2012; Nose and Shiraishi, 2011) and *P. massoniana* (Liu et al., 2017) comparative analyses of differential gene expression were made between susceptible and selected resistant varieties or individuals. The experimental conditions varied among these studies, such as the age of the plants used (2 years-old to adult trees), the use of seedlings or grafts, growth conditions (greenhouse, growth chamber or field), the PWN isolates used (possibly with different levels of virulence), the amount of PWNs inoculated (1000-30000 PWNs) and sampling timepoints (Table 1.1). These differences may

affect the plants' response to PWN inoculation, as well as disease progression, and consequently gene expression, protein, and metabolite profiles. Nevertheless, comparing the transcriptional response to PWN inoculation in several pine species allowed for the identification of several common molecular mechanisms involved both in the susceptible and resistant responses.

1.3.2.1. Phytohormone signalling

Phytohormones are signalling molecules with vital roles in plant development and response to stress, including response to biotic stresses. The recognition of a pathogen or pest and subsequent trigger of PTI or ETI by the plant cell leads to the accumulation of hormones, such as SA, JA, and ET (Pieterse et al., 2011). These three hormones are well described as having major roles in initiating downstream immune responses. SA is generally associated with response to biotrophic and hemi-biotrophic pathogens, while JA and ET have been associated with response to herbivory and necrotrophic pathogens, and the activation of these pathways is considered mutually exclusive (Caarls et al., 2015; Pieterse et al., 2011). However, this dichotomy is not always observed in species other than Arabidopsis, and JA has been implicated in the response to a wider range of pathogens and pests in monocots and gymnosperms (Campos et al., 2014). Other hormones such as auxins, gibberellins, cytokinins, brassinosteroids or abscisic acid (ABA), which are usually linked to plant development or response to abiotic stresses, can also play roles in plant defence response against biotic stresses (De Vleesschauwer et al., 2014). Despite the importance of phytohormones in plant immune response, little emphasis has been given to their role in pine response to PWN inoculation except in a hormone quantification study with *P. pinaster* (Rodrigues et al., 2021b).

Table 1.1. Details of the gene expression studies with pine trees infected with pinewood nematode (PWN).

Host species	Type of study	Susceptible/ Resistant	Type of analysis	Time points	Plant material	Inoculated PWNs	Experiment conditions	References
P. thunbergii	Gene expression (LongSAGE)	Susceptible species, resistant varieties	Susceptible *vs* resistant	72 hpi	2-3 yo seedlings	5000	field	Nose and Shiraishi, 2011
P. thunbergii	Gene expression (SSH)	Susceptible species, resistant varieties	Susceptible *vs* resistant	24 hpi, 72 hpi, 7 dpi, 14 dpi	2 yo grafts	10000	field	Hirao et al., 2012
P. thunbergii, P. massoniana	Gene expression (RNA-seq)	Susceptible species	Susceptible response	24 hpi, 48 hpi, 72 hpi, 96 hpi, 5 dpi, 6 dpi	3 yo seedlings	10000	greenhouse	Chen et al., 2021a
P. massoniana	Gene expression (SSH)	Susceptible species	Susceptible response	24 hpi, 72 hpi	3 yo seedlings	1500	growth chamber	Xu et al., 2013
P. massoniana	Gene expression (RNA-seq)	Susceptible species, resistant varieties	Susceptible *vs* resistant	24 hpi, 15 dpi, 30 dpi	4 yo ramets	10000	nursery	Liu et al., 2017
P. massoniana	Gene expression (RNA-seq)	Susceptible species	Susceptible response	24 hpi, 48 hpi, 72 hpi	2 yo seedlings	2000	greenhouse	Xie et al., 2020
P. densiflora	Gene expression (ACP, SSH)	Susceptible species	Susceptible response	21 hpi, 24 hpi, 7 dpi	4 yo and 8 yo seedlings	6000 to 30000	filed and nursery	Shin et al., 2009
P. densiflora	Gene expression (RNA-seq)	Susceptible species	Pathogenic *vs* non-pathogenic nematode	28 dpi	adult trees (11-13 meters)	60000	field	Lee et al., 2019
P. pinaster	Gene expression (RNA-seq)	Susceptible species	Susceptible response	6 hpi, 24 hpi, 48 hpi, 7 dpi	3 yo seedlings	2000	field	Gaspar et al., 2017
P. pinaster, P. pinea	Gene expression (pyrosequencing)	Susceptible and resistant species	Susceptible *vs* resistant	24 hpi	2 yo seedlings	1000	growth chamber	Santos et al., 2012
P. pinaster, P. yunnanensis	Gene expression (RNA-seq)	Susceptible and resistant species	Susceptible *vs* resistant	6 hpi, 24 hpi, 48 hpi, 7 dpi	3 yo seedlings	2000	field	Gaspar et al., 2020

hpi – hours post-inoculation; dpi – days post-inoculation; yo – years old

Rodrigues et al. (2021b) reported higher levels of methyl jasmonate (MeJA) and SA in susceptible *P. pinaster* plants when compared to resistant ones, suggesting these hormones have a role in susceptibility to PWN. The differential expression of genes encoding JA biosynthesis enzymes was observed in susceptible *P. pinaster* (Gaspar et al., 2017). However, the differential expression of other JA responsive genes or SA signalling pathway genes were not reported for this species. In *P. massoniana*, JA biosynthesis genes, a transcription factor that represses the JA response (*JAZ/Tify*), *PR-3*, *PR-4,* and *PR-5* were reported as upregulated (Xie et al., 2020; Xu et al., 2012). In susceptible *P. densiflora* (Shin et al., 2009), as well as in susceptible and resistant *P. thunbergii* (Hirao et al., 2012; Nose and Shiraishi, 2011), *PR-2*, *PR-3*, *PR-4,* and *PR-5* were also upregulated. SA responsive genes, such as *WRKY* transcription factors, namely *WRKY6* and *WRKY51*, were reported as upregulated in susceptible *P. densiflora* trees (Lee et al., 2019), while *PR-1* genes were more expressed in susceptible *P. thunbergii* plants when compared to resistant ones (Hirao et al., 2012; Nose and Shiraishi, 2011). These results suggest an important role for SA and JA pathways in susceptibility to PWN, although the information available is still scarce. Overall, the role of phytohormones in PWN response and resistance needs further clarification in the pine species of interest. Quantification of hormones in several timepoints after PWN inoculation, as well as investigating the effect of hormone application in the plant response, might help in understanding their relevance in PWD.

1.3.2.2. Secondary metabolism

The importance of secondary metabolites in plant defence response, and in particular in conifers defence response, has been well described for the interaction with several pests and pathogens (Ahuja et al., 2012; Eyles et al., 2010; Keeling and Bohlmann, 2006). The expression of genes encoding secondary metabolites biosynthetic enzymes in response to PWN inoculation has also been reported for a number of pine species. Quantification of these metabolites in PWD susceptible and resistant

species has been described, as well as the effect of some of these compounds in PWN mobility and survival (Table 1.2). Secondary metabolites include terpenes and phenylpropanoids, among other compounds.

Table 1.2. Secondary metabolites with a toxic effect on pinewood nematode (PWN).

Secondary metabolite	Type of compost	Species of origin	Effect on PWN	References
α-humulene	Sesquiterpene	*Pma*	repellent	Suga et al., 1993
Calarene	Sesquiterpene	*Pma*	repellent	Suga et al., 1993
β-bisabolene	Sesquiterpene	*Pma*	repellent	Suga et al., 1993
Dihydroabietane	Diterpene	*Pma*	repellent	Suga et al., 1993
α-pinene	Monoterpene	*Pma*	repellent	Suga et al., 1993
		Pma	nematicide	Liu et al., 2020
β-pinene	Monoterpene	*Pma*	nematicide	Liu et al., 2020
β-myrcene	Monoterpene	*Pma*	nematicide	Liu et al., 2020
D-limonene	Monoterpene	*Pma*	nematicide	Liu et al., 2020
Longifolene	Sesquiterpene	*Pma*	nematicide	Liu et al., 2020
Pinosylvin monomethyl ether (PME)	Stilbene	*Pma*, *Pst*, *Ppa*	nematicide	Suga et al., 1993
		Pst	nematicide	Hwang et al., 2021
Dihydropinosylvin monomethyl ether (DPME)	Stilbene	-	nematicide	Suga et al., 1993
		Pst	nematicide	Hwang et al., 2021
		Pst	nematicide	Hanawa et al., 2001
Pinosylvin	Stilbene	-	nematicide	Suga et al., 1993
Methyl ferulate	Phenolic compound	*Pma*	nematicide	Suga et al., 1993
Ferulic acid	Phenolic acid	-	nematicide	Suga et al., 1993
(-)-nortrachelogenin	Lignan	*Pma*	nematicide	Suga et al., 1993
(+)-pinoresinol	Lignan	*Pma*	nematicide	Suga et al., 1993

Pma – Pinus massoniana; Pst – Pinus strobus; Ppa – Pinus palustris; PWN – pinewood nematode

Biosynthesis of terpene compounds

The upregulation of genes involved in terpene backbone biosynthesis pathway after PWN inoculation has been reported for *P. massoniana* (Liu et al., 2017), *P. pinaster* (Gaspar et al., 2017; Gaspar et al., 2020; Santos

et al., 2012), *P. pinea* (Santos et al., 2012) and *P. yunnanensis* (Gaspar et al., 2020). For instance, *hydroxymethylglutaryl-CoA reductase* (HMGCR) was upregulated both in *P. massoniana* (Liu et al., 2017) and *P. pinaster* (Gaspar et al., 2017), while *1-D-xylulose-5-phosphate synthase* (*DXS*) was upregulated in *P. massoniana* (Liu et al., 2017), *P. pinaster* (Gaspar et al., 2017; Gaspar et al., 2020; Santos et al., 2012) and *P. yunnanensis* (Gaspar et al., 2020), and *1-hydroxy-2-methyl- 2-(E)-butenyl-4-diphosphate synthase* (*HDS*) was upregulated in *P. massoniana* (Liu et al., 2017) and *P. pinea* (Santos et al., 2012).

Several terpene synthases genes were more expressed in resistant *P. massoniana* plants than in susceptible ones after PWN inoculation, including *(-)-limonene synthase*, *(-)*-β-*pinene synthase*, *(+)-α-pinene synthase* and *longifolene synthase* (Liu et al., 2020; Liu et al., 2017). Two of these terpene synthases, namely α-pinene (*PmTPS4*) and longifolene (*PmTPS21*) synthases, were further characterized (Liu et al., 2020). The enzyme α-pinene synthase produced the monoterpenes α-pinene, β-pinene, β-myrcene and D-limonene, while longifolene synthase produced the sesquiterpene longifolene and the monoterpene α-pinene. All of these compounds had an inhibitory effect on PWN survival when applied separately to PWN *in vitro* cultures, and a stronger effect when applied in combination. Therefore, the higher expression levels of terpene synthase genes probably result in the increased synthesis of terpene compounds with nematicidal effect, leading to resistance in *P. massoniana*. Other terpene compounds extracted from *P. massoniana* were shown to have a repellent effect on PWN (Table 1.2) (Suga et al., 1993). However, no information about the concentration of such terpene compounds in resistant or susceptible *P. massoniana* plants is yet available. In other pine species, the upregulation of terpene synthases genes after PWN inoculation was also observed. Shin et al. (2009) reported the upregulation of *limonene cyclase* in susceptible *P. densiflora* plants, while *α-pinene synthase* was upregulated in the resistant species *P. pinea* (Trindade et al., 2016).

Despite these reports of differential gene expression in inoculated pine plants, connecting it to increased levels of terpene compounds has proven difficult. For susceptible *P. pinaster* and *P. sylvestris* plants, or for the resistant *P. pinea*, *P. halepensis* or *P. radiata*, no alterations were found in the concentration of volatile and non-volatile terpenes after inoculation with PWN (Nunes da Silva et al., 2015; Rodrigues et al., 2017) in any of the studied timepoints (3 hpi to 2 months post-inoculation). However, an increase in diterpenes and sesquiterpenes was observed in *P. pinea* and *P. halepensis* after mechanical wounding, mimicking the insect vector feeding (Rodrigues et al., 2017), while a significant increase in *P. pinea* limonene concentration was observed after feeding by *M. galloprovincialis* (Gonçalves et al., 2020). In *P. pinaster*, feeding by *M. galloprovincialis* caused an increase in several terpene compounds in susceptible plants, mainly in β-pinene, α-pinene, β-caryophyllene, and germacrene D, while a slight increase in β-myrcene and limonene was observed (Gonçalves et al., 2020). As PWNs enter the tree stem through wounds made during *M. galloprovincialis* feeding, the amount of terpene compounds produced and their relative proportions after wounding may impact the success of PWN infestation and consequently the resistance/susceptibility phenotypic outcome after infection. Studies to link gene expression and the synthesis of terpene compounds are still missing. Moreover, characterizing the enzymes with terpene synthase functions encoded by genes upregulated after wounding or PWN inoculation, similar to what was described for two *P. massoniana* enzymes (Liu et al., 2020), would elucidate their relevance and the role of their products in resistance to PWN.

Phenylpropanoids biosynthesis

Phenylpropanoids are long recognized for their roles in plant response to abiotic and biotic stresses, being key elements in resistance to pests and pathogens (Ahuja et al., 2012; Chin et al., 2018; Vogt, 2010). The phenylpropanoid biosynthesis pathway branches out into several pathways, such as flavonoid and stilbenoid biosynthesis. Therefore, this

class of secondary metabolites includes a vast variety of compounds, such as flavonoids, isoflavonoids, anthocyanidins, stilbenes, tannins, suberin, lignans and lignin (Vogt, 2010). The synthesis of these compounds is frequently induced by pathogens or pests in a large variety of plants. In PWD, several phenylpropanoids have been quantified in PWN susceptible and resistant species (Hwang et al., 2021; Nunes da Silva et al., 2015; Pimentel et al., 2016; Trindade et al., 2022) and the effect of some of these compounds on PWN survival has been studied (Table 1.2) (Hanawa et al., 2001; Hwang et al., 2021; Suga et al., 1993). Furthermore, a large number of genes involved in phenylpropanoids biosynthesis were upregulated after PWN inoculation in various pine species, revealing its importance in pine response to PWN (Table 1.3).

The constitutive levels of total phenolic content have been measured in several resistant and susceptible pine species in an attempt to associate these levels with the phenotypic outcome after PWN inoculation (Nunes da Silva et al., 2015; Pimentel et al., 2016; Trindade et al., 2022). However, high levels of phenolics have been found both in resistant species, such as *P. pinea*, *P. halepensis* and *P. radiata*, and in the susceptible species *P. pinaster* (Trindade et al., 2022). Furthermore, results were not in accordance across studies, possibly due to the use of plants with different ages [2-3 y.o. in Nunes da Silva et al. (2015); 12 y.o. in Trindade et al. (2022)] and the collection of data in different timepoints [24-72 hpi in Pimentel et al. (2016) and Trindade et al. (2022); 2 months post-inoculation in Nunes da Silva et al. (2015)]. Therefore, measurements of total phenolics seem of little value to discriminate between PWN resistant and susceptible phenotypes. Instead, the synthesis of specific phenolic compounds may be linked to resistance (Hwang et al., 2021; Suga et al., 1993; Trindade et al., 2022).

Table 1.3. Expression of genes related to secondary metabolism pathways in several pine species after inoculation with pinewood nematode.

Pathway	Genes	*P. densiflora*	*P. massoniana*	*P. thunbergii*	*P. pinaster*	*P. pinea*	*P. yunnanensis*	*P. strobus*
Phenylpropanoid biosynthesis	*phenylalanine ammonia-lyase (PAL)*	up			up			up
	4-coumarate-CoA ligase (4CL)	up						up
	caffeoyl-CoA O-methyltransferase (CCoAOMT)	up	up					
	caffeic acid O-methyltransferase (COMT)		up					
Flavonoid biosynthesis	*chalcone synthase (CHS)*	up	up	up	up	up		
	chalcone isomerase	up			up			
	flavonoid hydroxilase	up						
	leucoanthocyanidin dioxygenase (LDOX)	up		up; S>R				
Stilbenoid biosynthesis	*pinosylvin synthase (STS)*	up			up		up	up
	pinosylvin O-methyltransferase (PMT)							up
Lignans biosynthesis	*phenylcoumaran benzylic ether reductase*		up					
Lignin biosynthesis	*cinnamoyl-CoA reductase (CCR)*	up			down		up	
	cinnamyl-alcohool dehydrogenase (CAD)		up; down in S					
	peroxidase (PER)	up	up; S<R	up; S<R	up		up	
	laccase (LAC)				up			
Transcription factors	*bHLH*	up						up
	MYB	up					up	up
	WRKY	up				up		up
References		Shin et al., 2009; Lee et al., 2019	Xu et al., 2013; Liu et al., 2017; Xie et al., 2020; Chen et al., 2021a	Chen et al., 2021a; Nose & Shiraishi 2011; Hirao et al., 2012	Gaspar et al., 2017; Gaspar et al., 2020	Santos et al., 2012	Gaspar et al., 2020	Hwang et al., 2021

up – upregulated; S – susceptible; R – resistant

The first genes in the phenylpropanoid biosynthesis pathway, such as *phenylalanine ammonia-lyase* (*PAL*), *4-coumarate-CoA ligase* (*4CL*), *caffeoyl-CoA O-methyltransferase* (*CCoAOMT*) or *caffeic acid O-methyltransferase* (*COMT*), were upregulated in several susceptible pine species after PWN inoculation, namely in *P. densiflora*, *P. massoniana* and *P. pinaster* (Table 1.3) (Gaspar et al., 2017; Shin et al., 2009; Xu et al., 2013). *PAL* and *4CL* were also upregulated in the resistant species *P. strobus* (Hwang et al., 2021), suggesting that the activation of the phenylpropanoid biosynthesis pathway is a common response to PWN and may be important in resistance.

Several genes of the flavonoid biosynthesis pathway, such as *chalcone synthases* (*CHS*), were also induced by PWN inoculation in the susceptible species *P. densiflora*, *P. massoniana*, *P. thunbergii* and *P. pinaster*, as well as in the resistant *P. pinea* (Chen et al., 2021a; Gaspar et al., 2017; Nose and Shiraishi, 2011; Santos et al., 2012; Shin et al., 2009; Xu et al., 2013). In *P. densiflora*, a higher expression of flavonoid biosynthesis pathway genes was observed in resistant varieties (Kuroda et al., 2011), suggesting that the synthesis of flavonoids may have a role in PWN resistance. Accordingly, high constitutive and PWN induced levels of the flavonoids taxifolin and rutin were detected in the resistant species *P. halepensis*, while the susceptible *P. pinaster* and *P. sylvestris* had lower levels of these compounds (Trindade et al., 2022). Furthermore, levels of total flavonoids decreased in susceptible *P. massoniana* plants after PWN inoculation (Xie et al., 2020). In many plant-nematode interactions, the activation of the flavonoid biosynthesis pathway has been associated with resistance to nematodes (Chin et al., 2018). Products of this pathway have been shown to be toxic to several nematode species. For instance, naringenin, the product of CHS, caused a reduction in burrowing nematode's (*Radopholus similis*) egg hatching, while kaempferol and quercetin repelled both root-knot nematodes (*Meloidogyne incognita*) and burrowing nematodes (Wuyts et al., 2006). These compounds, and others with roles in plant resistance to parasitic nematodes (see Chin et al., 2018), may also affect PWN.

Therefore, the levels of flavonoids should be further investigated in resistant pine species and varieties, as well as the toxicity and repellent effect of such compounds in PWNs.

Pinosylvin synthase (*STS*), which encodes a stilbene biosynthesis enzyme, was upregulated in *P. densiflora* and *P. pinaster* inoculated plants (Gaspar et al., 2017; Shin et al., 2009), and in the resistant species *P. yunnanensis* and *P. strobus* (Gaspar et al., 2020; Hwang et al., 2021). In *P. strobus*, an increase in the pinosylvin derivates dihydropinosylvin monomethyl ether (DPME) and pinosylvin monomethyl ether (PME) was observed together with this gene upregulation, while in the susceptible species *P. koraiensis* and *P. densiflora* PME was not detectable and DPME was present only in trace amounts in *P. koraiensis* (Hwang et al., 2021). These compounds were shown to be toxic to PWN in *in vitro* assays, affecting the nematode mobility and survival (Hwang et al., 2021; Suga et al., 1993). Interestingly, PME was more toxic to adult PWNs, while DPME was more toxic to juveniles (Hwang et al., 2021). High constitutive levels of another stilbene, resveratrol, were observed in *P. pinea* and *P. halepensis* (Trindade et al., 2022). Levels of this compound also increased after PWN inoculation in *P. halepensis*. Therefore, the synthesis of stilbenoid compounds seems to be relevant in achieving resistance in some pine species.

Genes involved in the synthesis of lignans were upregulated in *P. massoniana* (Xu et al 2013), while the synthesis of (+)-seoisolariciresinol was induced by PWN inoculation in *P. halepensis* (Hwang et al., 2021). Furthermore, the lignans (-)-nortrachelogenin and (+)-pinoresinol have been shown to be nematicidal for PWN (Table 1.2) (Suga et al., 1993). However, the role of lignan compounds in PWN response has not been much explored, both in resistant and susceptible pine species or varieties.

Genes encoding transcription factors likely to be involved in the regulation of the phenylpropanoid, flavonoid and anthocyanin pathways, such as *bHLH*, *MYB* or *WRKY* (Vogt, 2010), were upregulated after PWN inoculation in *P. densiflora*, *P. pinaster*, *P. pinea*, *P. yunnanensis* and *P.*

strobus (Table 1.3) (Gaspar et al., 2020; Hwang et al., 2021; Lee et al., 2019), supporting the importance of these pathways in pine response to PWN. However, most of the phenylpropanoid compounds reported were associated with resistance in one or few pine species. It is possible that each species may depend on a different combination of phenylpropanoids to achieve resistance. On the other hand, considering the existing variety of such compounds in plants, the lack of overlap between species may be simply due to the lack of extensive data. Further research focusing on the quantification of the several classes of phenylpropanoids before and after PWN inoculation, guided by the transcriptomics studies available, might provide new insights into the conserved and unique resistance mechanisms in the different pine species and varieties.

Lignin biosynthesis and cell wall reinforcement

Lignin is another product of the phenylpropanoid biosynthesis pathway. Several genes encoding enzymes specific to lignin biosynthesis were upregulated in *P. densiflora*, *P. massoniana*, *P. thunbergii*, *P. pinaster* and *P. yunnanensis* in the initial response after PWN inoculation (24-72 hpi) (Table 1.3) (Gaspar et al., 2017; Gaspar et al., 2020; Hirao et al., 2012; Liu et al., 2017; Shin et al., 2009). Moreover, higher expression of genes involved in this pathway seem to be associated with the resistance phenotype in susceptible pine species. For instance, *peroxidase* genes, encoding an enzyme involved in the last step of lignin synthesis, were more expressed in resistant plants of *P. massoniana* and *P. thunbergii* (Hirao et al., 2012; Liu et al., 2017). Higher levels of lignin in cell walls around the inoculation zone were in fact associated with resistance in *P. thunbergii* (Kusumoto et al., 2014). Furthermore, this lignin accumulation has been linked to a limitation in PWN migration in *P. thunbergii* stem tissues (Kusumoto et al., 2014). Therefore, increased lignification around the inoculation zone seems to be a conserved defence mechanism in resistant varieties within susceptible pine species. This can interfere with PWN migration as observed in *P. thunbergii*, but possibly also with PWN ability

to digest plant cell walls and feed on their content (Holbein et al., 2016). It remains to be clarified if constitutive levels of lignin in the stem also vary within pine species and if these levels can also influence the plant's phenotype after PWN inoculation. Cell wall reinforcement may also result from the cross-linking of the hydroxyproline-rich glycoproteins extensins (HRPG), catalysed by peroxidases, which has also been associated with increased resistance to pathogens. Cross-linking of structural cell wall proteins such as extensins is the first histochemical modification observed in cell walls damaged by PWN, followed by lignification (Kusumoto et al., 2014; Nairn et al., 2008).

1.3.2.3. Oxidative stress response

During nematode infection, plants are typically under intense oxidative stress, where the reactive oxygen species (ROS) can be generated as part of the plant defence mechanisms, by dying plant cells, or by the nematodes themselves (Holbein et al., 2016). After nematode recognition, ROS produced by the plant act as signalling molecules in the activation of the defence response, having a role in the strengthening of plant cell walls via cross-linking and may have a toxic effect on nematodes (Couto and Zipfel, 2016a; Holbein et al., 2016). However, ROS are toxic to plant cells and may lead to their death if not transformed into innocuous molecules. Accordingly, during PWN infection, genes involved in ROS detoxification were differentially expressed after PWN inoculation in *P. densiflora*, *P. massoniana*, *P. thunbergii*, *P. pinaster* and *P. yunnanensis* (Table 1.4) (Gaspar et al., 2017; Gaspar et al., 2020; Hirao et al., 2012; Liu et al., 2017; Nose and Shiraishi, 2011; Shin et al., 2009; Xu et al., 2013). Furthermore, a few genes were reported to be more expressed in resistant than in susceptible plants, such as *peroxidases* (Hirao et al., 2012; Liu et al., 2017), *catalases* (Liu et al., 2017; Nose and Shiraishi, 2011), *superoxide dismutase* and *glutathione reductase* (Liu et al., 2017).

Table 1.4. Expression of oxidative stress response genes that encode enzymes involved in detoxification of reactive oxygen species (ROS) after pinewood nematode inoculation in several pine species.

Genes	*P. densiflora*	*P. massoniana*	*P. thunbergii*	*P. pinaster*	*P. yunnanensis*
superoxide dismutase	up	down, S<R		up	up
glutathione reductase		down, S<R			
glutathione peroxidase (GPx)		up		up	
L-ascorbate peroxidase	up	down			
catalase (CAT)		down, S<R	S<R		
catalase isozyme		up			
peroxidase (PER)	up	S<R	S<R		up
glutathione S-transferase	up			up	up
peroxiredoxin	up				
thioredoxin	up				
References	Shin et al 2009	Liu et al 2017; Xu et al 2013	Hirao et al 2012; Nose & Shiraishi 2011	Gaspar et al 2017	Gaspar et al 2020

up – upregulated; S – susceptible; R – resistant

Measurements of ROS in *P. massoniana* revealed that, after PWN inoculation, levels of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) increased in an early stage of the infection (24 hpi) for both resistant and susceptible plants, and gradually decreased at 15 to 30 days post-inoculation (dpi) only in resistant plants (Liu et al., 2017). At 24 hpi, the levels of H_2O_2 were higher in resistant plants when compared with susceptible ones, which was inverted at 15 and 30 dpi, when a steep increase was observed in susceptible plants. Thus, there seems to be a more efficient ROS detoxification in resistant plants, especially in more advanced phases of the disease, probably due to the action of the enzymes encoded by the genes more expressed in resistant *P. massoniana* plants.

Considering the importance that ROS detoxification seems to have in achieving resistance in *P. massoniana*, it would be interesting to measure ROS at several timepoints after PWN inoculation in other pine species of

interest, comparing resistant and susceptible varieties. Quantifying the enzymes encoded by the differentially expressed genes reported and correlating them with ROS concentration should highlight which enzymes are important for pine response and resistance to PWN.

1.3.2.4. Plant defence response genes

Pathogenesis-related proteins are induced by pathogens or pests as part of the host plant defence. They comprise a variety of proteins with different properties and functions, such as chitinases (PR-3, PR-4, PR-8, and PR-11), thaumatin-like proteins (PR-5) or proteinase inhibitors (PR-6). Although some of these proteins have been associated with resistance to specific pathogens, PR proteins are thought to be part of a generalized plant defence response to a broad range of pathogens and pests, even though not always effective (Van Loon, 1999). During PWN infection, the expression of several *PR* genes is induced in pines trees, including *PR-1* (unknown function; *P. thunbergii*, *P. massoniana*), *PR-2* (beta-1,3-glucanase-like protein; *P. thunbergii*, *P. densiflora*), *PR-3* (chitinases; *P. thunbergii*, *P. densiflora*, *P. massoniana*), *PR-4* (chitinases or chitin-binding proteins; *P. thunbergii*, *P. densiflora*, *P. massoniana*), *PR-5* (thaumatin-like protein; *P. thunbergii*, *P. densiflora*, *P. massoniana*, *P. pinaster*, *P. pinea*), *PR-6* (proteinase inhibitors; *P. thunbergii*), *PR-10* (ribonuclease-like protein; *P. thunbergii*, *P. densiflora*, *P. massoniana, P. pinaster*) and *PR-14* (lipid-transfer protein; *P. massoniana*, *P. pinea*) (Hirao et al., 2012; Nose and Shiraishi, 2011; Santos et al., 2012; Shin et al., 2009; Xu et al., 2013; Zhou et al., 2017). Although the role of these proteins in PWN resistance in unknown, some of these genes were more expressed in resistant pine varieties.

The chitinases *PR-3* and chitin-binding *PR-4* were more expressed in resistant *P. thunbergii* (Nose and Shiraishi, 2011) when compared to susceptible plants. Chitin is a main component of nematode eggshell (Fukushige and Futai, 1985; Holbein et al., 2016) and possibly the

pharyngeal lumen walls (Sato et al., 2019), suggesting that chitinases may compromise egg integrity and embryo development, as well as PWN feeding. Treatment with chitinase plant extracts caused premature egg hatching and increased juvenile mortality in the root-knot nematode *Meloidogyne hapla* (Mercer et al., 1992). Assessing the effects of chitinase extracts from pine trees, especially extracts from resistant varieties, in the several life stages of PWN would elucidate their role in PWD resistance. *PR-10* were also more expressed in resistant *P. thunbergii* (Hirao et al., 2012) plants. It is unknown if these proteins have an impact on nematode growth, multiplication or spread. Evaluating the effects of pine extracts of these proteins, similarly to what is here suggested for chitinases, would clarify this topic.

Other genes previously associated with plant defence response, such as *mannose/glucose-specific lectin* or *ricin B-related lectin*, were also upregulated early after inoculation (24-72 hpi in *P. pinaster*, *P. pinea* and *P. massoniana*) (Gaspar et al., 2017; Santos et al., 2012; Xu et al., 2013). Plant lectins have been shown to interact with mono- or oligosaccharides from several pests and pathogens and some were reported as toxic (Vandenborre et al., 2011). Interestingly, some lectins are involved in *A. thaliana* defence response against the root-knot nematode *Meloidogyne incognita* (Ripoll et al., 2003).

1.3.2.5. Resistance genes

Plant resistance genes are receptors that detect effectors released by a pathogen, parasite, or pest, starting a highly specific defence response that is effective in stopping the spread and multiplication of the invading organism. Resistance genes include the membrane receptors RLKs or RLPs, which recognize apoplastic effectors, and more often the intracellular receptors NLRs, which identify cytoplasmic effectors. These receptors may interact with the effector directly or indirectly by monitoring alterations caused by the effector in a plant co-factor (Cui et al., 2015; Zheng et al.,

2021). Several studies have shown important roles of resistance genes in plant interactions with parasitic nematodes and herbivorous insects (Erb and Reymond, 2019; Hogenhout and Bos, 2011; Sato et al., 2019; Zheng et al., 2021). For instance, the RLP Cf-2 recognizes the potato cyst nematode (*Globodera rostochiensis*) apoplastic effector venom allergen-like protein 1 (VAP1), indirectly, while the NLR receptor Gpa2 recognises the potato cyst nematode *Globodera pallida* cytoplasmic effector GpRBP-1 (Zheng et al., 2021). *VAP1* gene was also identified in PWN genome and the knockdown of this gene resulted in significantly lower PWN migration in the stem of pine seedlings when compared to wildtype PWNs (Kang et al., 2012). This suggests that VAP1 also acts as an effector in PWN-pine interactions by supressing pine defence response. Another apoplastic effector, BxSapB1, has also been recently described (Hu et al., 2019), without which PWN has lower virulence than wildtype PWNs. Furthermore, a NAMP has been characterized, namely BxCDP1, which leads to the initiation of PTI in a brassinosteroid-insensitive 1-associated kinase 1 (BAK1)-dependent manner (Hu et al., 2020).

The receptors involved in the recognition of these NAMP and effectors, and subsequent activation of pine defence response, are unknown. It is possible that different receptors are involved in the activation of the immune response in resistant and susceptible plants, or that resistance genes detect PWN effectors and activate the more robust ETI in resistant plants. This is supported by the downregulation of *NLRs* and resistance genes in susceptible *P. massoniana* (Xie et al., 2020). The activation of the ETI usually takes place in plants adapted to the pathogen or pest, implying a coevolution of the two organisms and often an arms race (Cui et al., 2015). As PWN is an invasive parasite, susceptible pine species have not evolved in the presence of PWN. Therefore, genetic resistance to PWD occurring in natural stands has likely evolved due to the selective pressures of another pathogen or pest. In fact, one gene may confer resistance to more than one type of organism, as in the case of *Mi-1.2*, which confers resistance to root-knot nematodes (*Meloidogyne* spp.) (Milligan et al., 1998), the potato aphid

(*Macrosiphum euphorbiae*) (Rossi et al., 1998), the white fly (*Bemisia tabaci*) (Nombela et al., 2003), and the tomato psyllid (*Bactericera cockerelli*) (Casteel et al., 2006). To better understand pine response and resistance to PWN, it would be interesting to investigate if ETI is activated in resistant plants, what effectors may be recognized by resistance genes and what receptors are involved in these responses.

1.3.3. Post-transcriptional regulation mediated by small RNAs in pine response to PWN

Small RNAs (sRNAs) are a class of non-coding RNAs, with 20 to 35 nucleotides, that are key players in post-transcriptional and transcriptional gene silencing (Tang et al., 2022). MicroRNAs (miRNAs), one of the best studied sRNA classes, are mostly involved in post-transcriptional gene silencing by guiding the cleavage or translation inhibition of complementary target transcripts (Brant and Budak, 2018b) with roles in a variety of processes, including plant development, as well as response to abiotic and biotic stresses (Dong et al., 2022; Khraiwesh et al., 2012). In most cases, mutant plants for components of the sRNAs biogenesis and function pathways exhibit higher susceptibility to pathogens, suggesting an important role of sRNAs in the regulation of plant defence mechanisms (Weiberg and Jin, 2015). During plant defence response, sRNAs are known to be involved in the regulation of plant hormone synthesis and signalling, callose deposition, expression of NLR receptors and of other resistance proteins, ROS detoxification and secondary metabolites synthesis (Huang et al., 2016a), being essential players in PTI and ETI immune responses. After nematode infection, several host sRNAs have also been associated with resistance traits. Transcription factors and hormone signalling genes are examples of sRNA targets associated with plant defence mechanisms against parasitic nematodes (Khanna et al., 2021; Kulshrestha et al., 2020). The great majority of studies on sRNAs involved in plant response to nematode diseases focus on sedentary endoparasitic nematodes and their involvement in feeding sites development (Dutta et al., 2021; Hewezi, 2020;

Jaubert-Possamai et al., 2019; Khanna et al., 2021). Nevertheless, the first steps have been taken to understand the role of miRNAs in PWD response.

1.3.3.1. MicroRNA expression in response to PWN

The differential expression of miRNAs in response to PWD has been reported for *P. massoniana* (Xie et al., 2017). These authors showed that in *P. massoniana* inoculated with PWN, several miRNAs were differentially expressed in the needles during the first 3 dpi. The predicted targets for these miRNAs were associated with plant hormone signalling (e.g., zeatin synthesis and ethylene signalling), RNA transport, splicing, and fatty acid metabolism, among other processes. The expression of hormone signalling predicted targets showed a significant increase at 2 dpi followed by a decrease at 3 dpi and negatively correlated with the expression of their corresponding miRNAs. The impact of the infection on hormone synthesis and accumulation was confirmed by the quantification of indole-3-acetic acid (IAA) and zeatin contents in *P. massoniana* needles, which were shown to initially decrease (3 dpi), then increase (9 dpi) and finally decrease significantly at 14 dpi (Xie et al., 2017). The suppression of growth-related hormone signalling and synthesis at 14 dpi was suggested by the authors to be a consequence of the damage caused by PWN. On the other hand, the initial decrease in hormone content (3 dpi) likely results from growth-defence trade-offs, suggesting that the tree has relocated its energy for the defence response in detriment of growth and development (He et al., 2022). However, the expression of miRNAs involved in the regulation of plant immune response was not detected in this study, probably because only the pine needles were sampled, while PWN infects stem tissues. Therefore, the role of post-transcriptional regulation in pine response to PWN is still largely unknown.

1.3.4. Defence response induced by the application of phytohormones and secondary metabolites

In recent years, the application of elicitors to pine trees in order to induce plant immunity prior to PWN infection has been investigated as a PWD control method (Jeon et al., 2022; Mannaa et al., 2020; Nunes da Silva et al., 2014; Nunes da Silva et al., 2021). Elicitors can be a variety of substances, such as plant hormones, purified molecules derived from pathogens or pests, or synthetic molecules, which induce plant defence response (Bektas and Eulgem, 2015). These substances have the potential to be used as bio-control agents, as they are more economical and eco-friendly approaches than the traditional use of insecticides against the insect vector or trunk injection of nematicides. A few elicitors have been evaluated for PWD with positive results in reducing disease progression or PWN multiplication, namely methyl salicylate (MeSA) and chitosan (Jeon et al., 2022; Mannaa et al., 2020; Nunes da Silva et al., 2014; Nunes da Silva et al., 2021).

The application of MeSA in *P. densiflora* and *P. thunbergii* seedling leaves in the form of spray, one and two weeks before PWN inoculation, significantly decreased disease progression when compared to seedlings without treatment (Jeon et al., 2022; Mannaa et al., 2020; Park et al., 2020). Treatment of *P. densiflora* plants with MeSA and subsequent inoculation with PWN seem to induce genes and pathways similar to those previously associated with PWN resistance, such as *peroxidases*, *extensins*, flavonoid biosynthesis genes and genes involved in ROS detoxification (Jeon et al., 2022; Park et al., 2020). However, the expression levels of these genes were much higher in plants treated with MeSA than in non-treated plants, reinforcing the importance of such pathways in reaching resistance to PWD.

Similarly, the application of chitosan, a compound derived from chitin, in the soil increased *P. pinaster* resistance to PWN, as shown by the significantly lower number of PWNs in treated plants in several timepoints after

inoculation when compared to untreated ones (Nunes da Silva et al., 2014; Nunes da Silva et al., 2021). Chitosan application induced catalase activity, an enzyme involved in ROS detoxification, as well as the production of phenolic compounds, anthocyanins (flavonoids), carotenoids (terpenes) and lignin (Nunes da Silva et al., 2021). Therefore, application of chitosan and MeSA induced similar pathways that seem to be crucial for PWD resistance in pine trees.

On the other hand, the trunk injection of MeJA had a small effect on improving *P. densiflora* resistance to PWN (Park et al., 2020), while spraying *P. massoniana* seedlings with MeJA seems to induce the production of diterpenes and deter the insect vector *Monochamus alternatus* from feeding on the stem of elicited plants (Chen et al., 2021b). These observations show that MeJA has also the potential to be used as a control compound at the level of the host-insect vector interaction, but further studies are needed to confirm that MeJA application has the same effect on other pine trees and insects of the genus *Monochamus.*

1.4. Thesis aims and outline

As reviewed, pine trees undergo a significant transcriptional change after PWN infection that may be followed by changes in the synthesis of proteins and metabolites (e.g., Hwang et al., 2021; B., Liu et al., 2020; Rodrigues et al., 2021a). The presented overview of the defence response pathways induced in several pine species by PWN inoculation reveals that at least part of this defence seems to be conserved. The analysis of resistant pine varieties and families highlighted resistance mechanisms, some of which shared among the studied pine species. ROS detoxification, limiting PWN migration through cell-wall reinforcement, and production of secondary metabolites that affect PWN mobility or survival, seem to contribute to achieve resistance to PWN. However, the resistance response has been studied mainly in *P. thunbergii* and *P. massoniana*, and has not been reported for *P. densiflora* or *P. pinaster*. Furthermore, although miRNAs were implicated in the regulation pine growth in *P. massoniana*, their role in regulating pine immune response to PWN has not been described.

Although most *P. pinaster* individuals show high susceptibility to PWD, genetic variation in the response to PWN inoculation has been observed, with variable degrees of resistance being reported for different half-sib families and populations (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017a; Menéndez-Gutiérrez et al., 2017b). However, the mechanisms involved in achieving resistance in *P. pinaster* are unknown.

In this project, we took advantage of plant material previously characterized by Carrasquinho et al. (2018), in which 96 half-sib families were evaluated for the genetic effects on survival after PWN inoculation and ranked according to these effects. For the present work, we chose one of the 15 top-ranked families, the half-sib family 440. This family presented a predicted survival mean at 157 days after inoculation of 15% (in a range of 6% to 23%).

The main goals of this work were to understand the molecular mechanisms relevant for *P. pinaster* resistance to PWN, and to develop molecular markers associated with PWD resistance. To achieve these goals, three specific aims were established:

1) To uncover the genes and pathways involved in the response to PWN infection in susceptible and resistant *P. pinaster* plants.

2) To address the role of post-transcriptional regulation in *P. pinaster* response and resistance to PWN infection.

3) To identify SNPs in candidate genes that might be associated with *P. pinaster* phenotype after PWN inoculation.

After the introduction (Chapter 1), the analysis of transcriptomic data is presented in Chapter 2, in which differential gene expression after PWN inoculation is compared between susceptible and resistant *P. pinaster* plants. This analysis was followed by hormone and lignin quantification in plants with contrasting response to PWN inoculation to support some of the key findings from the transcriptomics analysis. In Chapter 3, the expression of small RNAs was assessed to identify miRNAs involved in the response to PWN inoculation and in PWN resistance. The integration of these results with the transcriptomics data presented in Chapter 2 was used to predict the target genes and associated pathways under post-transcriptional regulation by the identified miRNAs. In Chapter 4, the RNA-seq data presented in Chapter 2 was used to identify SNPs potentially interesting for association analysis. Association tests of a subgroup of the identified SNPs in a larger sample were performed for the discovery of molecular markers relevant for future association studies. Finally, the significance of the results here obtained and future research perspectives are discussed in Chapter 5.

1.5. References

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Chapter 2

Insights Into the Mechanisms Implicated in *Pinus pinaster* Resistance to Pinewood Nematode

This chapter has been published in *Frontiers in Plant Science*:

Modesto, I., Sterck, L., Arbona, V., Gómez-Cadenas, A., Carrasquinho, I., Van de Peer, Y. & Miguel, C. M. (2021) Insights Into the Mechanisms Implicated in *Pinus pinaster* Resistance to Pinewood Nematode. *Frontiers in Plant Science*, 12, 1–18. https://doi.org/10.3389/fpls.2021.690857.

Inês Modesto participated in the conceptualization of the experiments, performed the inoculation and RNA experiments, analysed and interpreted the data, prepared the figures and tables, and wrote the original draft of the manuscript.

2. Insights Into the Mechanisms Implicated in *Pinus pinaster* Resistance to Pinewood Nematode

2.1. Abstract

Pine wilt disease (PWD), caused by the plant-parasitic nematode *Bursaphelenchus xylophilus*, has become a severe environmental problem in the Iberian Peninsula with devastating effects in *Pinus pinaster* forests. Despite the high levels of this species' susceptibility, previous studies reported heritable resistance in *P. pinaster* trees. Understanding the basis of this resistance can be of extreme relevance for future programs aiming at reducing the disease impact on *P. pinaster* forests. In this study, we highlighted the mechanisms possibly involved in *P. pinaster* resistance to PWD, by comparing the transcriptional changes between resistant and susceptible plants after infection. Our analysis revealed a higher number of differentially expressed genes in resistant plants (1916) when compared with susceptible plants (1226). Resistance to PWN is mediated by the induction of the jasmonic acid defense pathway, secondary metabolism pathways, lignin synthesis, oxidative stress response genes and resistance genes. Quantification of the acetyl bromide-soluble lignin confirmed a significant increase of cell wall lignification of stem tissues around the inoculation zone in resistant plants. In addition to less lignified cell walls, susceptibility to the pinewood nematode seems associated with the activation of the salicylic acid (SA) defense pathway at 72 hpi, as revealed by the higher SA levels in the tissues of susceptible plants. Cell wall reinforcement and hormone signaling mechanisms seem therefore essential for a resistance response.

2.2. Introduction

Pine wilt disease (PWD) is caused by *Bursaphelenchus xylophilus*, or pinewood nematode (PWN), which is transmitted by the insect vector *Monochamus* spp. while feeding on healthy trees. Upon entering the tree

stem, PWN spreads through the resin canals, feeds on plant cells or fungi that populate the decaying tree, and breeds (Evans et al., 1996; Kim et al., 2020; Vicente et al., 2012).

During the last century, PWD has become a worldwide threat to conifer forests (Webster and Mota, 2008), being particularly damaging for trees of the genus *Pinus*. In the Iberian Peninsula, it was first detected in the late 1990's (Mota et al., 1999), spreading rapidly through Portugal and reaching Spain. In this region, it infects mostly *Pinus pinaster* trees, which are highly susceptible (Evans et al., 1996). Given the high economic and ecological value of *P. pinaster* in southwestern Europe due to its use in paper, wood and resin production, its importance for soil protection and as wildlife habitat, PWD has a huge impact on the local economy and environment (Vicente et al., 2012; Webster and Mota, 2008).

Remarkably, varieties with high resistance levels have been described in susceptible pine species (Toda and Kurinobu, 2002; Xu et al., 2012). In *P. pinaster*, different levels of resistance were also observed in plants after artificially inoculated with PWN (Menéndez-Gutiérrez et al., 2017a, 2017b; Carrasquinho et al., 2018). Since control measures implemented so far have failed in stopping PWD spreading, breeding resistant varieties may be a highly effective control strategy. Breeding programs have been successfully implemented for *Pinus thunbergii*, *Pinus densiflora* and *Pinus massoniana* (Toda and Kurinobu, 2002; Xu et al., 2012). For *P. pinaster*, genetic variation in susceptibility to PWN inoculation was observed in two independent studies (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017a) and a moderate family heritability for survival (0.37; Carrasquinho et al., 2018) and mortality (0.59; Menéndez-Gutiérrez et al., 2017a) after inoculation was detected, suggesting that implementation of breeding programs can also be valuable. Furthermore, plants without symptoms had very few PWNs when compared to symptomatic plants (Menéndez-Gutiérrez et al., 2017a), suggesting asymptomatic plants were able to

control the multiplication of PWN, showing, therefore, true resistance to the parasite (Trudgill, 1991; Woodcock et al., 2018).

The identification of the mechanisms involved in resistance to PWN may inform on effective strategies to fight the disease. In general, plant defense response initiates upon recognition of the pathogen at the cellular level (Couto and Zipfel, 2016). Cell membrane receptor-like kinases (RLKs) or receptor-like proteins (RLPs) recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), initiating a series of signaling events that culminate in transcriptional reprogramming and expression of defense response genes. This pattern-triggered immunity (PTI) represents the first level of plant defense against pathogens (Couto and Zipfel, 2016; Dodds and Rathjen, 2010). However, adapted pathogens release effectors to suppress host immunity. In turn, these effectors may be recognized by intracellular nucleotide-binding/leucine-rich-repeat (NLR) receptors, inducing a more robust defense response, the effector-triggered immunity (ETI) (Cui et al., 2015). Several RLK/RLP and NLR receptors have been implicated in resistance to plant-parasitic nematodes (Sato et al., 2019; Zheng et al., 2021). However, these studies focus on sedentary and biotrophic species, and intracellular NLR receptors may not have a relevant role in resistance to migratory non-biotrophic nematodes such as PWN. The activation of PTI and ETI triggers hormone-dependent plant immune responses, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways (Buscaill and Rivas, 2014; Tsuda and Katagiri, 2010). Other hormones like gibberellins, auxins, cytokinins and abscisic acid (ABA), although usually associated with development or response to abiotic stresses, have also been shown to play an important role in plant-microbe interactions (De Vleesschauwer et al., 2014).

Although limited knowledge is available about the possible mechanisms involved in resistance to PWD in individuals within susceptible pine species, a few studies have focused on the comparison of transcriptional responses

between PWD resistant and susceptible plants. For *P. thunbergii* (Hirao et al., 2012; Nose and Shiraishi, 2011) and *P. massoniana* (Liu et al., 2017) resistance was associated with higher expression levels of genes related to the synthesis of secondary metabolites, namely flavonoids (Kuroda et al., 2011) and terpenes (Liu et al., 2017), cell wall reinforcement, including genes related to plant cell wall lignification (Hirao et al., 2012; Liu et al., 2017), and ROS detoxification. Furthermore, higher lignification seemed to limit PWN migration in resistant *P. thunbergii* plants (Kusumoto et al., 2014). Recently, susceptibility in *P. pinaster* was associated with the activation of salicylic acid and jasmonic acid pathways, as part of an inefficient trigger of the hypersensitive response (Rodrigues et al., 2021).

The first transcriptomic analysis addressing the PWN response in *P. pinaster* was based on the comparison to *P. pinea*, described as less susceptible than *P. pinaster* (Santos et al., 2012), while more recent reports in *Pinus pinaster* described the transcriptional changes after PWN infection during a susceptible interaction (Gaspar et al., 2017; Gaspar et al., 2020). However, despite the identification of *P. pinaster* genotypes considered resistant (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017a; Menéndez-Gutiérrez et al., 2017b), the transcriptional response associated with resistance has not been previously analyzed.

Our aim was to identify the molecular mechanisms involved in *P. pinaster* resistance to PWD. In the absence of available *P. pinaster* clones showing either susceptibility or resistance towards the PWN, we took advantage of within family variation (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017a) and used half-siblings from a single family in the transcriptomic analysis. In this way, differences in gene expression resulting from genetic variation in traits other than response to PWN were minimized. While it would be interesting to extend this analysis to other families, the strategy used here contributes to highlight the most relevant genes for the PWN response by exploring the behavior of one of the top-ranking half-sib families regarding genetic effects on survival to PWN infection, previously

characterized by Carrasquinho *et al.* (2018). We hypothesize that differences in survival to PWN infection may be related to different transcriptional responses in the first days after inoculation, as it was observed in other *Pinus* spp (Hirao et al., 2012; Liu et al., 2017; Nose and Shiraishi, 2011; Xu et al., 2012). In order to test this, we inoculated several plants within the selected family and analyzed the differential expression in susceptible and resistant plants at 72 hours post-inoculation (72hpi) (Hirao et al., 2012; Nose and Shiraishi, 2011; Xu et al., 2012). Through a comparative transcriptomic analysis of PWN resistant and susceptible plants, complemented with the investigation of cell wall lignification and hormone signaling, we obtained the first insights into the resistance mechanisms possibly involved and detected candidate resistance genes that can be a valuable resource for future studies.

2.3. Materials and methods

2.3.1. PWN inoculum

B. xylophilus isolate Bx013.003 (Carrasquinho et al., 2018; Rodrigues et al., 2021) was obtained from an infested field tree exhibiting wilting symptoms in central Portugal (39°43'33.8''N, 9°01'55.7''W) and was included in the collection of INIAV's Nematology Laboratory, Oeiras, Portugal. The sequence of the ITS region is available at GenBank (NCBI) under the accession number MF611984.1. Nematodes were kept in pure culture at 25±1°C on a non-sporulating Botrytis cinerea strain grown on autoclaved barley grains. Prior to inoculation, nematodes were allowed to grow on sterilized wood. Nematodes were separated from the culture media using the "tray" method (Whitehead and Hemming, 1965) and suspended in water in a concentration of 1000 PWN/mL.

2.3.2. Plant inoculation, sampling and symptoms evaluation

Twenty-three potted 4-year-old *P. pinaster* plants from the half-sib family 440 were maintained in a greenhouse and placed according to a completely

randomized experimental design. The plants were derived from seeds obtained from the mother tree 440, which is included in the reference population for PWD resistance from "Herdade da Comporta" (38°21'28.52''N, 8°45'49.89''W) in southern Portugal (Ribeiro et al., 2012), resulting from a mass selection program initiated in 2009. Within a half-sib family, part of the individuals may prove resistant while the majority are susceptible. Family 440 was previously characterized by Carrasquinho et al. (2018) as one of the 15 top-ranked half-sib families (among 96 evaluated families) regarding the genetic effects on survival after PWN inoculation. Predicted survival means at 157 days after inoculation ranged from 6% to 23% using 2-year-old plants, having family 440 shown a predicted survival mean of 15% (Carrasquinho et al., 2018). The plants were inoculated in September 2016, following the method of Futai and Furuno (1979). A suspension aliquot with 500 nematodes was pipetted into a small longitudinal wound made in the main stem with a sterile scalpel below the apical shoot region (Figure 2.1A,B). Inoculated wounds were covered with parafilm to prevent drying of the inoculum. Eighteen plants were inoculated with PWN and five controls were inoculated with sterile water. Stem samples of approximately 5cm, including the inoculation zone (Figure 2.1A), were collected 72 hpi (Hirao et al., 2012; Nose and Shiraishi, 2011; Xu et al., 2013) and immediately frozen in liquid nitrogen. After removal of the inoculation zone (and apical stem), the remaining part of each plant was kept in the greenhouse and observed for symptoms weekly for a period of 210 days (Figure 2.1C-E). Plants were classified according to a scale from 0 (no visible symptoms) to 4 (more than 75% of needles brown/wilted) (Figure 2.1E). The first symptoms were visible 14 days post-inoculation (dpi) and evolved progressively until de end of the experiment. Plants presenting symptoms (1-4 in the symptoms scale) were considered susceptible, while plants without any symptoms (0) were classified as resistant. As this classification is based on external symptoms and not on nematode counting, plants here considered resistant may in fact be tolerant, maintaining a healthy phenotype despite PWN multiplication

(Trudgill, 1991; Woodcock et al., 2018), although true resistance, in which plants were able to inhibit PWN multiplication, was observed in other *P. pinaster* families (Menéndez-Gutiérrez et al. 2017a). It should be noted that at 72 hpi PWNs are expected to have spread through plant tissues several centimeters away from the inoculation zone (Ichihara et al., 2000; Kusumoto et al., 2014; Son et al., 2015).

Height and diameter at the base of the stem were measured before inoculation. A two-sample unpaired *t*-test was performed using R v3.5.1 (https://www.r-project.org) to evaluate significant differences in these parameters between resistant and susceptible plants.

2.3.3. Total RNA extraction and transcriptome sequencing

Total RNA was extracted from each stem sample, after debarking, using the method described in Le Provost et al. (2007). RNA concentrations were measured using Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA USA) with the RNA BR Assay Kit and integrity was verified with LabChip GX (PerkinElmer, Hopkinton, MA USA). Four susceptible and five resistant plants with the most contrasting phenotypes, i.e., plants that died faster (symptoms scale level 4) and plants without symptoms (symptoms scale level 0) during the entire observation period, were selected for library preparation, as well as four control samples. Libraries were prepared with the Illumina TruSeq Stranded mRNA Kit and sequenced on Illumina HiSeq 2500 (Fasteris, Switzerland), providing 125 bp single-end reads. Each sample was run in two independent lanes.

2.3.4. Quality control, transcriptome assembly and read mapping

The quality of the RNA-seq data was evaluated with FastQC v0.11.2 (Andrews, 2010). Adapter and quality trimming were performed using clc_adapter_trim and clc_quality_trim, respectively, from CLC Assembly Cell v7.0.4 (Quiagen, Hilden, Germany), with default parameters.

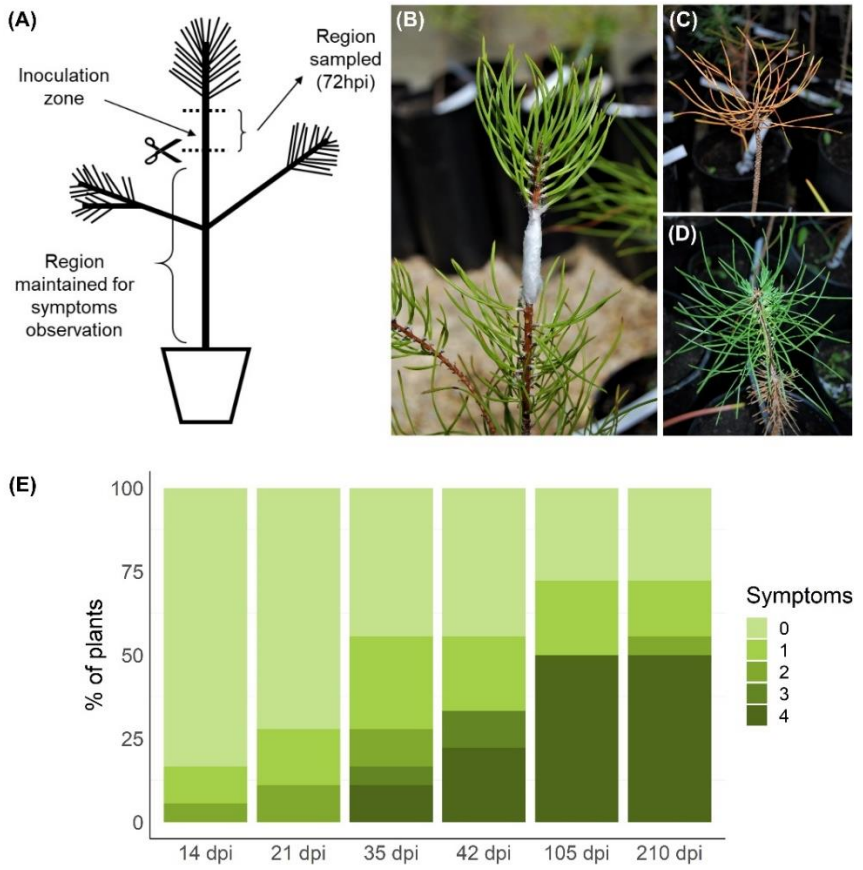

Figure 2.1. Inoculation, sampling, and symptoms observation. Plants were inoculated in the stem, below the apical region **(A,B)**. Samples of the stem, including the inoculation zone, were collected 72 hours post-inoculation (hpi). After debarking, these samples were homogenized and total RNA was extracted. The remaining part of the plant, below the cutting region, was maintained for symptoms observations for 210 days post-inoculation (dpi). Symptoms were evaluated weekly and registered according to a five-level scale based on percentage of brown/wilted needles: 0, 0% **(D)**; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% **(C)**. Symptom progression in selected timepoints is represented in **(E)**. Plants without any visible symptom at the end of the experiment were considered resistant.

At the moment of our analysis, a reference transcriptome was available for *P. pinaster* (Cañas et al., 2017). However, this transcriptome did not include samples submitted to any kind of biotic stress. Therefore, in order to include transcripts that may be specific to PWN infection response, we performed

a *de novo* assembly with reads from all inoculated samples using Trinity v2.6.6 (Grabherr et al., 2013) with default parameters. The resulting contigs were compared with the previously available *P. pinaster* transcriptome and PWN genome (Kikuchi et al., 2011) using BLASTn (DeCypher Tera-BLASTn, TimeLogic, California, USA) and highly similar sequences ($e \leq 10^{-5}$) were filtered out. To further exclude contigs originating from PWN, a BLASTx (DeCypher Tera-BLASTx) was performed with the National Center for Biotechnology Information (NCBI) Protein database (accessed January 2019) and all the sequences with blast hits to a nematode species were excluded. In this way, 34,737 new transcripts were added to the 206,574 from the previous *P. pinaster* reference transcriptome (Table S2.1). For these 34,737 transcripts, Transdecoder v2.1.0 (Haas, 2019) was used to predict protein coding regions.

Reads were mapped to the *P. pinaster* transcripts containing predicted coding regions (CDS), including both the newly predicted and the ones available in Gymno PLAZA 1.0 database (http://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza) (70,870 transcripts). The nematode reference transcriptome (17,704 sequences) (Kikuchi et al., 2011) was obtained from WormBase ParaSite (http://parasite.wormbase.org) and used to filter out the reads corresponding to the pathogen. Reads were mapped using the BWA alignment software v0.7.5a (BWA-MEM) (Li, 2013) with default parameters. The mapping results were filtered and only uniquely mapped reads where kept for read counting using SAMtools v1.3 (Li et al., 2009). *P. pinaster* and PWN transcripts and respective counts were separated in two files, and only *P. pinaster* data was used for differential expression analysis.

2.3.5. Functional annotation

Protein sequences were obtained from Gymno PLAZA 1.0 for the available transcriptome and Transdecoder predictions were generated for the newly discovered transcripts. To functionally annotate the *P. pinaster*

transcriptome, a similarity search was performed using BLASTp (DeCypher Tera-BLASTp) alignments and the NCBI RefSeq Plant database (accessed February 2019). InterProScan was used to identify protein domains, assign gene ontology (GO) terms, and assign Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. KEGG annotation was further improved by using KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007). In the set of differentially expressed genes (DEGs), transcription factors were identified and classified using iTAK (Zheng et al., 2016). Genes potentially involved in disease resistance were identified with DRAGO 2 available from the Plant Resistance Genes database (Osuna-Cruz et al., 2017).

2.3.6. Differential expression and enrichment analyses

The differential expression (DE) analysis was done using DESeq2 (Love et al., 2014) with a 0.05 false discovery rate (FDR) threshold. Results were filtered for genes with Log2(fold change) ≥|2|. Venn diagrams were drawn (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Gene set enrichment analysis was performed using BiNGO plugin (Maere et al., 2005) for Cytoscape (Shannon et al., 2003). The hypergeometric statistical test was used, and Benjamini & Hochberg FDR was applied for multi testing correction, with a significance level ≤ 0.05. GO redundancy was decreased by using Revigo tool (Supek et al., 2011) with a soft trim threshold of 40%. Pathway enrichment analysis was performed using the hypergeometric statistical test implemented in BiNGO with the same parameters.

2.3.7. Quantitative RT-qPCR validation

To validate DE results, 10 genes with different expression patterns in susceptible and resistant plants were selected for quantitative RT-qPCR. Primers were designed using PerlPrimer (Marshall, 2004) (Table S2.2). cDNA synthesis was performed from total RNA samples of 3 resistant, 3

susceptible and 3 control plants using SuperScript™ IV First-Strand Synthesis System (Invitrogen, USA) and oligo($dT)_{20}$ primer. RT-qPCR was run in a LightCycler 480 Instrument II (Roche, Switzerland) using SYBR Green I Master (Roche) and the following conditions: 5min at 95°C, 40 cycles of 95°C for 10s, 58-63°C for 15s (Table S2.2), and 72 °C for 12s. Primer specificity was monitored by analyzing the melting curves. Three technical replicates were performed for each biological replicate. Transcript profiles were normalized using the reference genes *actin*, *40S rRNA* (Pascual et al., 2015) and *histone H3* (de Vega-Bartol et al., 2013). Relative expression levels of candidate genes were calculated with the Pfaffl (2001) method.

2.3.8. Lignin content

Powdered stem samples were freeze-dried and 1mg was used for determining lignin content. Acetyl bromide-soluble lignin was determined according to Foster *et al.* (2010) and a standard curve was generated with alkali lignin (Sigma-Aldrich, 370959). Five susceptible, five resistant and two control samples were used for this analysis and three technical replicates were made for each biological replicate. A two-sample unpaired *t*-test was performed to evaluate significant differences between control and susceptible or resistant plants (R v3.5.1).

2.3.9. Hormone analysis

Hormone quantification was performed for five susceptible, five resistant and four control samples. Before extraction, freeze dried powdered stem samples were weighed in 2 mL-microtubes and spiked with 25 µl of an internal standard mixture (containing ABA-d_6, DHJA and C^{13}-SA concentration of 1 mg L^{-1}) to correct for analyte loses. Extraction was carried out in 1 mL ultrapure water for 10 min in a ball mill at room temperature using 2 mm glass beads. After extraction, homogenates were centrifuged at 10,000 rpm for 10 min at 4°C and supernatants recovered. The resulting solutions were partitioned twice against an equal volume of

di-ethyl ether after adjusting pH to 3.0 with a 30% acetic acid solution. The combined organic layers were evaporated under vacuum in a centrifuge concentrator (Jouan, Sant Germaine Cedex, France) and the dry residues reconstituted in 0.5 mL of a 10% aqueous methanol solution. Prior to injection, extracts were filtered through 0.20 µm PTFE syringe membrane filters and filtrates recovered in chromatography amber glass vials. Samples were analyzed by tandem LC/MS in an Acquity SDS UPLC system (Waters Corp., USA) coupled to a TQS triple quadrupole mass spectrometer (Micromass Ltd., UK) through an electrospray ionization source. Separations were carried out on a C18 column (Luna Omega Polar C18, 50×2.1 mm, 1.6 µm particle size, Phenomenex, USA) using a linear gradient of ultrapure acetonitrile and water, both supplemented with formic acid to a 0.1% (v/v) concentration, at a constant flow rate of 0.3 mL min^{-1}. During analyses, column temperature was maintained at 40°C and samples at 10°C to slow down degradation. Plant hormones were detected in negative electrospray mode following their specific precursor-to-product ion transitions (ABA, 263>153; JA, 209>59; JA-Ile, 322>130 and SA, 137>93) and quantified using an external calibration curve with standards of known amount. To evaluate for significant differences between control and susceptible or control and resistant plants, a two-sample unpaired *t*-test was performed (R v3.5.1).

2.4. Results

To identify genes that may be involved in resistance to pine wilt disease, an artificial PWN inoculation assay was performed with plants from a previously characterized half-sib family (Carrasquinho et al., 2018). After sampling the stem of inoculated plants at 72 hpi, plants were observed and evaluated weekly for PWD symptoms for 210 dpi. In each timepoint, plants were classified on a scale from 0 (absence of symptoms) to 4 (more than 75% of brown/wilted needles) (Figure 2.1E). The first symptoms were visible at 14 dpi and at 35 dpi the first plants died (level 4). After 210 dpi, 28% of the plants continued showing no symptoms (level 0) and were

considered resistant. The remaining plants were considered susceptible. From the susceptible plants, 69% had died (level 4) by the end of the experiment. The first four plants reaching level 4 in the symptoms scale were selected as the susceptible plants to be sequenced by RNA-seq. Resistant and susceptible plants showed no significant differences in height and diameter at the stem base (Figure S2.1).

2.4.1. *De novo* transcriptome assembly, functional annotation, and mapping

RNA-seq data from samples of stem tissue from 4 susceptible, 5 resistant and 4 control plants yielded 17-20 million reads per sample, with sizes ranging between 70-125 bp and an average quality score of 36. The *de novo* transcriptome assembly produced 250,339 transcripts (Table S2.1), from which 215,602 were highly similar to the *P. pinaster* transcriptome previously available (Cañas et al., 2017b), the PWN genome (Kikuchi et al., 2011) or sequences available from other nematode species. From the remaining 34,737 transcripts, 1,445 had a predicted protein coding sequence (CDS) (Data S1). In combination with the transcripts retrieved from the Gymno PLAZA 1.0 database, a reference transcriptome of 70,870 transcripts with predicted proteins was obtained. From these transcripts, 46,625 were functionally annotated with BLASTp (DeCypher Tera-BLASTx) similarity search. Using InterProScan, at least one protein domain was identified for 44,839 transcripts, of which 31,192 had GO annotations assigned. By joining InterProScan and KAAS annotations, 17,059 transcripts were associated with at least one KEGG pathway.

Read sequences were mapped to the *P. pinaster* and PWN transcriptomes. On average, a mapping ratio of 93% was obtained, of which 69% were uniquely mapped. The percentage of reads derived from PWN in infected plants varied between samples, from 0.2% to 0.7%. PWN reads mapped to genes previously described as important for pathogenicity (Table S2.3) (Espada et al., 2016; Kikuchi et al., 2011; Shinya et al., 2013a), such as

genes encoding enzymes involved in plant cell wall degradation (e.g. *endo-β-1,4-glucanase*, *pectate lyase*, *expansin*), peptidases (e.g. *cysteine proteinase*, *aspartic protease*), anti-oxidant proteins (e.g. *peroxiredoxin*, *glutathione S-transferase*, *thioredoxin*, *superoxide dismutase*) (Shinya et al., 2013a) and effector protein genes, such as *venom-allergen like protein 1* (*VAP1*) and *VAP2*, which may cause the suppression of the plant immune response (Lozano-Torres et al., 2014). The expression of several pathogenicity genes during infection found in our dataset is consistent to what was described for other plant-parasitic nematodes (Goverse and Smant, 2014; Haegeman et al., 2012). For the differential expression analysis, only the reads uniquely mapped to *P. pinaster* transcriptome were retained.

2.4.2. Differential expression analysis highlighted specific enriched functions and pathways in resistant plants

Differential expression analysis was performed by comparing control plants to either resistant or susceptible ones. From the 40,391 transcripts with mapped reads, 1916 and 1226 were differentially expressed in resistant and in susceptible plants, respectively [Log2(fold change) >= 2, adjusted p-value ≤ 0.05; Tables S2.4 and S2.5]. In resistant plants, 1182 genes were upregulated and 734 downregulated, while in susceptible plants 720 were upregulated and 506 downregulated. Part of the DEGs was shared (44.6%), while 11.8 % were unique to susceptible and 43.6% were unique to resistant plants (Figure 2.2A). Analysis by RT-qPCR of 10 randomly selected genes show the same expression trends as the RNA-seq results (Figure 2.3A) with a positive correlation coefficient ($R^2 = 0.91$, Figure 2.3B).

After redundancy reduction, 38 and 53 GO terms were enriched for upregulated genes in susceptible (Table S2.6) and resistant plants (Table S2.7), respectively. Several GO terms that are related with biotic stress response, such as DNA-binding transcription factor activity, response to oxidative stress or defense response to bacterium, were enriched both in

susceptible and resistant plants (Figure 2.2B-D). GO terms as the MFs chitinase activity and terpene synthase activity (Figure 2.2B), the BP reactive oxygen species metabolic process (Figure 2.2C), or the CCs cell wall and exocyst (Figure 2.2D), were enriched only in resistant plants.

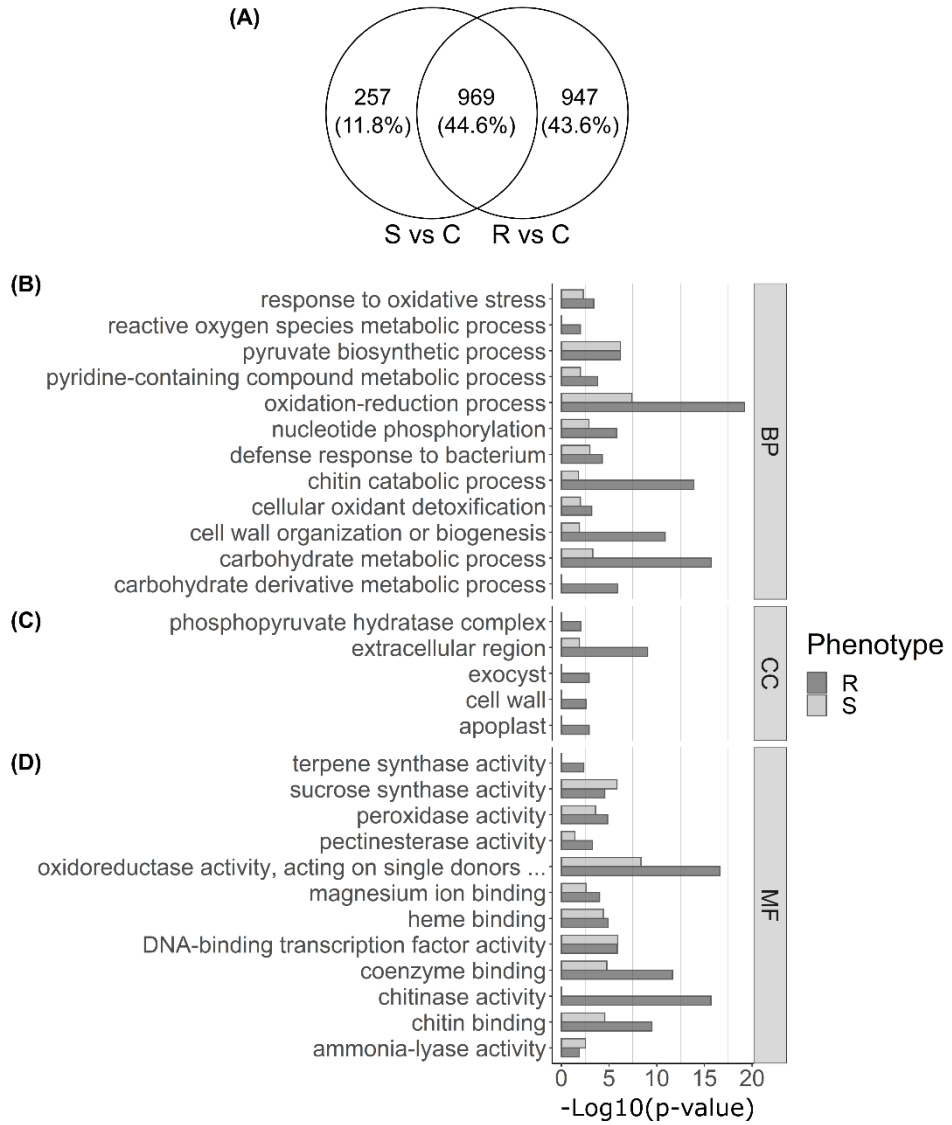

Figure 2.2. Venn diagram showing overlap of differentially expressed genes in susceptible (S) and resistant (R) samples (A) and gene set enrichment analysis (B–D). (A) Differential expression was calculated by comparing

susceptible (S) or resistant (R) samples with controls (C). **(B–D)** GO terms overrepresented in the upregulated genes in resistant (dark gray) and susceptible (light gray) samples are displayed, separated by **(B)** biological process (BP), **(C)** cellular component (CC), and **(D)** molecular function (MF). The x-axis represents the significance of GO enrichment (-log10 of corrected *p*-values).

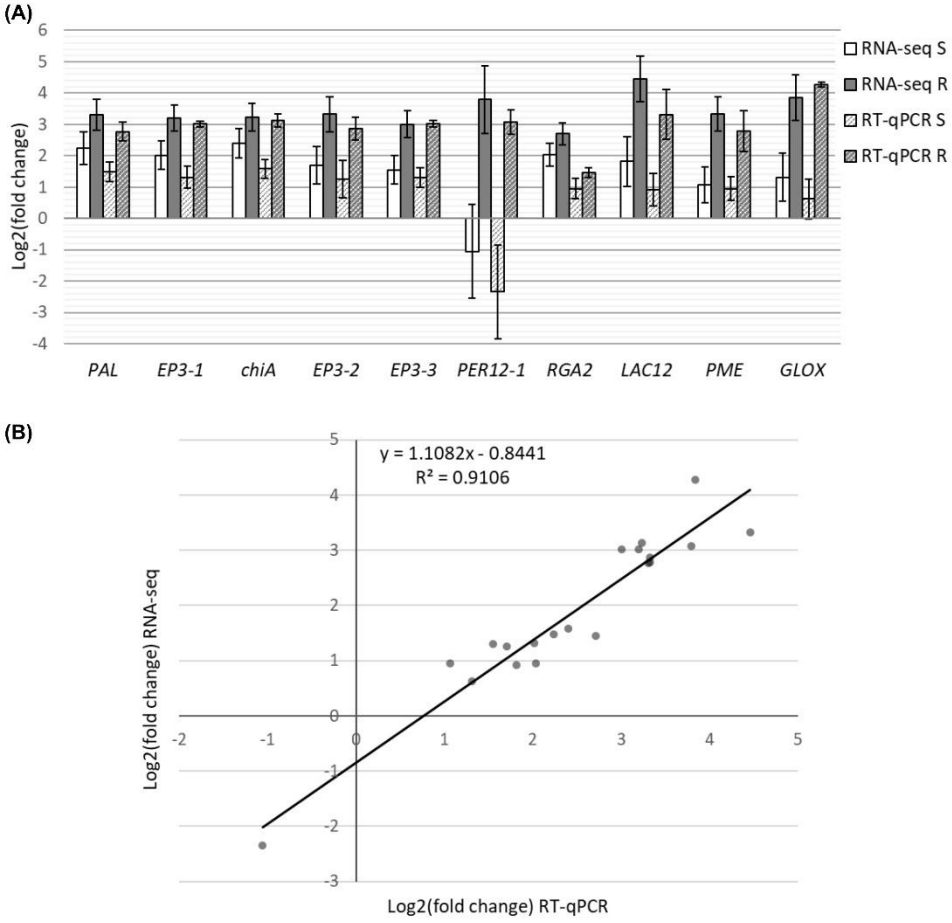

Figure 2.3. RT-qPCR analysis of 10 DEGs from the RNA-seq results. (A) Bars represent differential expression levels, in Log2(fold change), of susceptible (white) and resistant (gray) plants in comparison with controls. Results from both the RNA-seq analysis (filled colors) and the RT-qPCR analysis (stripes) are displayed. Error bars represent the standard error of the biological replicates used for RNA-seq (4–5) and RT-qPCR (3). **(B)** Correlation of expression levels between RNA-Seq and RT-qPCR.

For the upregulated genes, 13 pathways were enriched in resistant plants and 9 in susceptible plants (Figure 2.4). Pathways commonly associated with biotic stress response were enriched in both resistant and susceptible plants, including alpha-Linolenic acid metabolism, which leads to the synthesis of jasmonic acid (JA), phenylpropanoid biosynthesis, which leads to the synthesis of several compounds including lignin, plant hormone signal transduction and flavonoid biosynthesis. Pathways enriched only in resistant plants include amino sugar and nucleotide sugar metabolism and MAPK signaling pathway, while plant-pathogen interaction was enriched only in susceptible plants (Figure 2.4).

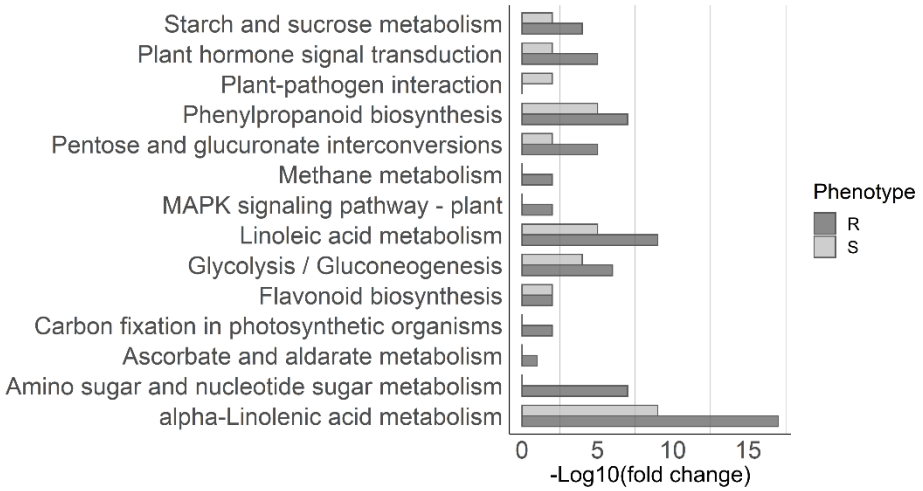

Figure 2.4. Pathway enrichment analysis. KEGG pathways overrepresented in the upregulated genes in resistant (dark gray) and susceptible (light gray) samples are depicted in the graph. The x-axis represents the significance of KEGG enrichment (-Log10 of corrected *p*-values).

2.4.3. Induction of secondary metabolism pathways and lignin accumulation was higher in resistant plants

Secondary metabolites play an important role in conifers defense response and have been associated with resistance to insects and pathogens (Ahuja et al., 2012; Eyles et al., 2010; Keeling and Bohlmann, 2006). Although

several genes involved in secondary metabolism pathways were differentially expressed after inoculation, a few genes related to the biosynthesis of terpenoids, such as *AS* (*bifunctional abietadiene synthase*, unigene128167), *LPS* (*bifunctional levopimaradiene synthase*, unigene10412) or *GERD* (*(-)-germacrene D synthase*, unigene144607 and unigene8510) had higher expression levels in resistant plants (Figure S2.2). Likewise, a few genes from the flavonoid biosynthesis pathway were more expressed in resistant plants (Figure S2.2), such as *CHS4* (*chalcone synthase 4*, isotig47436), *CHS2* (unigene147178) and *LDOX* (*leucoanthocyanidin dioxygenase*, unigene210255).

In contrast, a high number of genes in the phenylpropanoid biosynthesis pathway had different expression levels in resistant and susceptible plants (Figure 2.5). Several genes involved in lignin synthesis, including *peroxidase* (*PER*, Figure 2.5C) and *laccase* (*LAC*, Figure 2.5D) genes (Vogt, 2010; Xie et al., 2018), were more expressed in resistant plants. In addition, genes encoding for aldehyde oxidase (GLOX) enzymes, which produce hydrogen peroxide, a molecule necessary for lignin polymerization by peroxidases, had considerably higher expression levels in resistant plants (Figure S2.3). These results, suggesting the induction of lignin biosynthesis, were supported by experimental determination of the lignin content in stem tissues, which detected a significantly higher amount of lignin in resistant plants when compared to controls, while susceptible plants were not significantly different from controls (Figure 2.5E). Hydrogen peroxide may also play an important role in the activation of the plant defense response and is toxic for pests and pathogens (Holbein et al., 2016). The higher production of ROS was reflected by the high number of oxidative stress response genes upregulated (Figure S2.3).

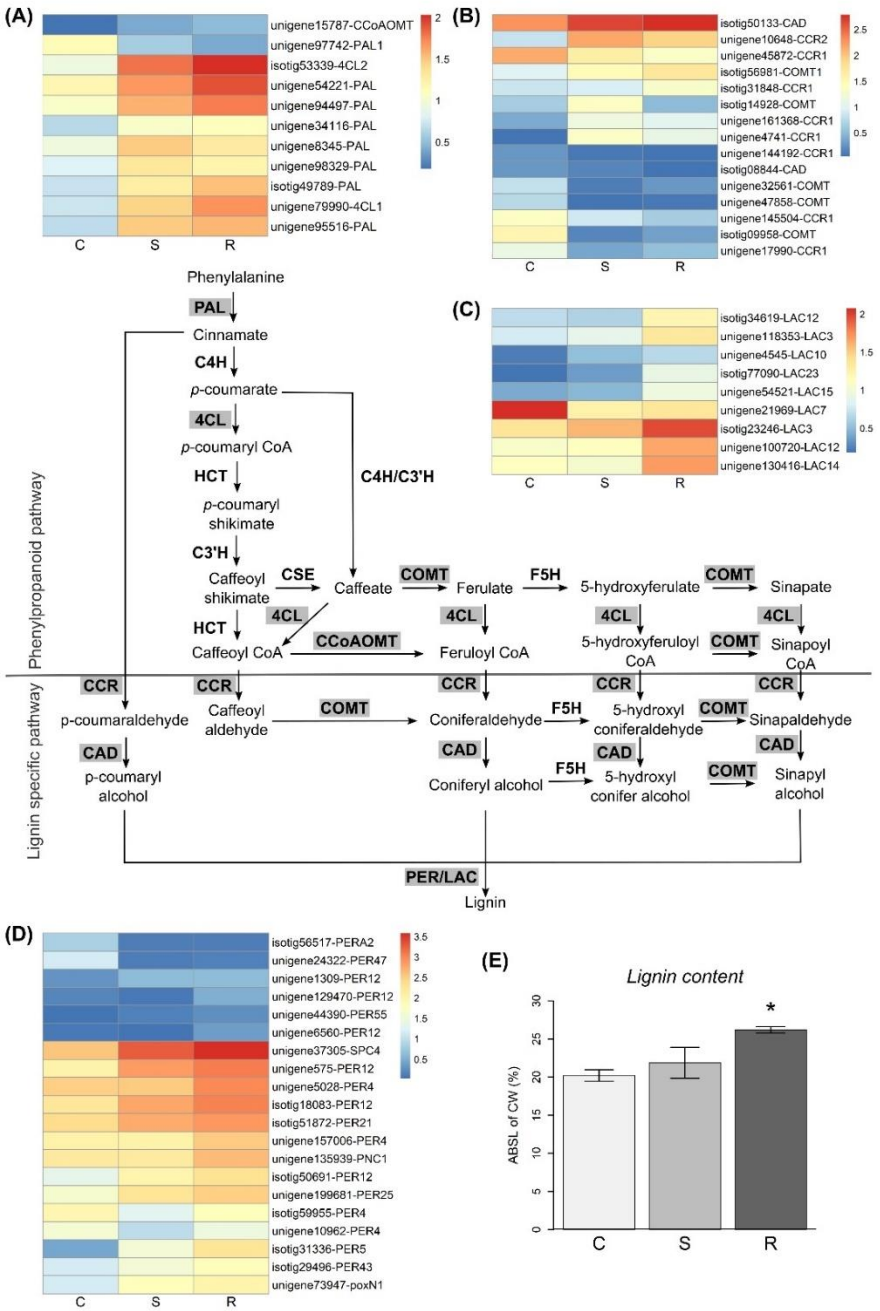

Figure 2.5. Lignin biosynthesis pathway. Lignin biosynthesis pathway is represented (adapted from Xie et al., 2018), with the differential expressed genes highlighted in gray. Heatmaps represent Log10(TPM) values of differentially expressed genes in the general phenylpropanoid pathway **(A)** and the lignin specific

pathway **(B–D)**. The final steps of lignin synthesis are carried out by laccases (LAC, **C**) and peroxidases (PER, **D**). The percentage of acetyl bromide soluble lignin of cell wall (ABSL of CW) measured in control (C), susceptible (S), and resistant (R) plants is represented in **(E)**. Error bars represent the standard error of the mean. Significant differences between control and inoculated plants, using Student's *t*-test, are indicated by an asterisk (**p*-value < 0.05).

2.4.4. Jasmonate response was induced in inoculated plants

Several genes involved in the JA biosynthesis pathway were upregulated in inoculated plants, with *Lipoxygenase* (*LOX*), *phospholipase A2* (*PLA2G*) and *12-oxophytodienoic acid reductase* (*OPR*) genes showing higher expression levels in resistant plants (Figure 2.6E). Analysis of hormone levels in the several sample types detected similar JA-Ile levels in resistant, susceptible and control plants (Figure 2.6A), while the JA levels were higher only in inoculated plants (Figure 2.6C), although with no significant differences between resistant and susceptible plants.

Consistent with these data, JA induced transcription factors, such as *ethylene response factors* (*ERF*), *MYC2* and the negative regulators *JAZ*/*Tify* were upregulated in all inoculated plants (Figure S2.4). However, 25 *chitinase*, 3 *PR-4* and 16 *PR-5* genes, usually associated with JA response (Davis et al., 2002; Piggott et al., 2004), were more strongly upregulated in resistant plants (Figure 2.7A-C).

ABA may act synergistically with JA in the activation of the MYC branch of JA response (Pieterse et al., 2011). In turn, JA induces the expression of *PYL4*, which encodes for an ABA receptor (Lackman et al., 2011). In our results, we observed the upregulation of *PYL4* concomitantly with *PP2C* (Merlot et al., 2001), an ABA signaling repressor gene (Figure 2.6E), only in resistant plants. Five transcription factors of the NAC family, implicated in ABA-JA interactions (Pieterse et al., 2011), were upregulated in all inoculated plants, although with higher intensity in resistant ones (Figure S2.4). Despite these differences in gene expression, the amount of ABA

measured in the samples was similar between control and inoculated plants, with resistant plants tending to have a smaller amount (Figure 2.6C).

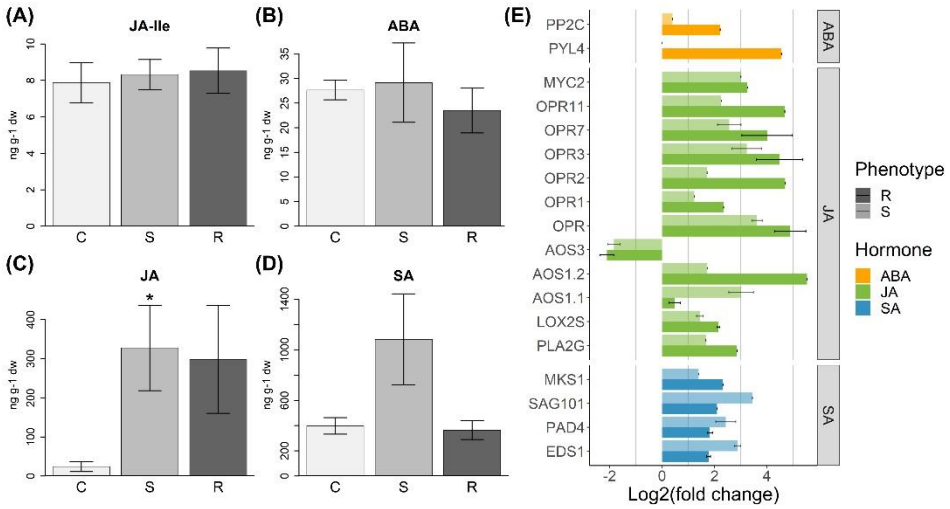

Figure 2.6. Hormone response to PWN inoculation. (A) Levels of jasmonate-Isoleucine (JA-Ile), **(B)** abscisic acid (ABA), **(C)** jasmonic acid (JA), and **(D)** salicylic acid (SA) (ng per 1g of plant dry weight) measured in control (C), susceptible (S), and resistant (R) plants. Error bars represent the standard error of the mean. Significant differences between control and inoculated plants, using Student's t-test, are indicated by an asterisk (*p-value < 0.05). **(E)** Differential expression of hormone responsive genes in resistant (R) and susceptible (S) plants, compared to controls. For each gene annotation, the average of the Log2(fold change) is represented. Error bars represent the standard error of the mean. For more details about the genes used and respective functional annotations see Supplementary Table S2.9.

2.4.5. Salicylic acid response is induced in susceptible plants

Genes encoding for proteins that induce the synthesis and accumulation of SA, namely EDS1, PAD4 and SAG101 (Caarls et al., 2015; Wiermer et al., 2005), were more expressed in susceptible plants (Figure 2.6E). On the other hand, a gene encoding for the SA signaling suppressor MKS1 (Andreasson et al., 2005; Pieterse et al., 2011) was more upregulated in resistant plants (Figure 2.6E). In accordance with these results, the induction of the SA response in susceptible plants was validated by

quantifying SA levels, which was higher in these plants than in controls and resistant plants (Figure 2.6D).

Several WRKY transcription factors, involved in the SA response pathway (Caarls et al., 2015), were upregulated in both susceptible and resistant plants, with a few showing higher expression in the susceptible ones (e.g. unigene36207-WRKY23, isotig49008-WRKY50) (Figure S2.4). In addition, the SA responsive pathogenesis-related protein 1 (PR-1) genes were also upregulated in all inoculated plants (Figure 2.7D). Although not significantly different, there seems to be a tendency for higher expression of PR-1 genes in susceptible plants.

2.4.6. Putative resistance genes showed different expression patterns in resistant and susceptible plants

The analysis with DRAGO 2 identified a set of genes that encode for the characteristic domains of proteins described in the literature as having a role in resistance to pathogens (Osuna-Cruz et al., 2017), including receptor-like kinases (RLK), receptor-like proteins (RLP), protein kinases and nucleotide-binding domain leucine-rich repeat (NLR) proteins (Table 2.1, Table S2.8). The RLP differ from RLK by the presence of a kinase domain and genes in which this domain was not detected were classified as RLPs. Several RLPs and RLKs were more expressed in resistant plants (e.g., unigene148155-IRL6, DN63749_c0_g2_i1-RLK, unigene73543-PIRL3) (Table 2.1, Table S2.8), while others were more expressed in susceptible plants (e.g., isotig67777-FLS2, unigene102513-RLP30, isotig84710-PXC2). Most genes encoding intracellular receptors NLRs had higher expression levels in resistant plants (Table 2.1).

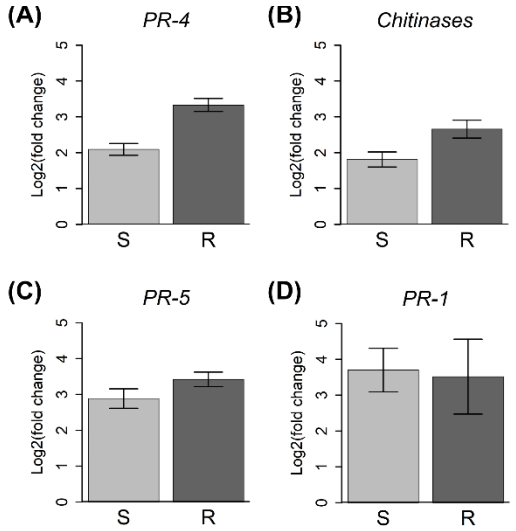

Figure 2.7. Differential expression of PR and chitinase genes. For each gene family, the average of the Log2(fold change) is represented for resistant (R) and susceptible (S) plants, compared to controls. Error bars represent the standard error of the mean. **(A)** *PR-4*, 3 genes; **(B)** *chitinases*, 25 genes; **(C)** *PR-5*, 16 genes; **(D)** *PR-1*, 4 genes. For more details about the genes used and respective functional annotations see Supplementary Table S2.9.

2.5. Discussion

A significant reprogramming of gene expression was observed in *P. pinaster* plants after inoculation with the PWN. This observation is not surprising, and it is in accordance with previous studies on *P. pinaster* inoculated with PWN (Gaspar et al., 2017; Gaspar et al., 2020; Santos and Vasconcelos, 2012) where susceptible plants have been analyzed. However, by focusing on both resistant and susceptible interactions in *P. pinaster* plants, we show here that although part of the transcriptional response to PWN was shared between both resistant and susceptible groups, significant qualitative and quantitative differences exist in gene expression. Importantly, some of these differences were confirmed to translate into relevant functional outcomes.

Table 2.1. Putative resistance genes detected by DRAGO tool (selected).

Transcript	Drago ann.	Blastp annotation	Gene	Log2FC	
				Sus	Res
isotig36950	RLP	PREDICTED: probable disease resistance protein At4g33300 [Nelumbo nucifera]	PDR	2.73	**4.12**
DN63749_c0_g2_i1	RLP	receptor-like serine/threonine-protein kinase At1g78530 isoform X1 [Physcomitrella patens]	RLK	0.00	**3.86**
unigene73543	RLP	plant intracellular Ras-group-related LRR protein 3 [Cynara cardunculus var. scolymus]	PIRL3	0.49	**3.40**
DN45869_c0_g1_i1	RLP	probable leucine-rich repeat receptor-like protein kinase At1g35710 [Medicago truncatula]	RLK	1.70	**3.11**
unigene148155	RLP	PREDICTED: plant intracellular Ras-group-related LRR protein 6 [Erythranthe guttata]	IRL6	1.14	**3.03**
unigene104083	RLP	PREDICTED: plant intracellular Ras-group-related LRR protein 6 [Erythranthe guttata]	IRL6	0.75	**2.91**
isotig19381	RLP	probable leucine-rich repeat receptor-like protein kinase At1g35710 [Durio zibethinus]	RLK	1.24	**2.90**
isotig55894	RLP	LOW QUALITY PROTEIN: probable leucine-rich repeat receptor-like protein kinase At1g35710 [Durio zibethinus]	RLK	0.51	**2.04**
unigene10412	RLP	putative disease resistance protein RGA3 [Aegilops tauschii subsp. tauschii]	RGA3	-21.84	**0.05**
isotig82402	RLP	PREDICTED: protein SUPPRESSOR OF npr1-1, CONSTITUTIVE 1-like [Gossypium hirsutum]	SNC1	-22.04	**-0.07**
unigene75605	RLP	leucine-rich repeat receptor-like protein kinase TDR [Glycine max]	TDR	**3.87**	2.30
DN44984_c1_g1_i1	RLP	receptor-like protein EIX2 isoform X1 [Glycine max]	EIX2	**3.90**	2.19
DN44458_c0_g1_i2	RLP	PREDICTED: receptor-like protein kinase HSL1 [Elaeis guineensis]	HSL1	**4.63**	2.15
isotig67777	RLP	LRR receptor-like serine/threonine-protein kinase FLS2 [Quercus suber]	FLS2	**2.54**	0.07
unigene102513	RLP	PREDICTED: receptor like protein 30-like [Vitis vinifera]	RLP30	**1.81**	-2.52
isotig56462	RLP	receptor-like protein kinase HSL1 [Papaver somniferum]	HSL1	**-0.55**	-2.17
isotig38664	RLP	receptor-like protein 12 [Durio zibethinus]	RLP12	**0.54**	-2.19
unigene120230	RLP	putative leucine-rich repeat receptor-like serine/threonine-protein kinase At2g24130 [Setaria italica]	RLK	**3.26**	1.28
unigene98132	RLK	PREDICTED: receptor kinase-like protein Xa21 isoform X1 [Juglans regia]	Xa21	**1.30**	-3.54
isotig36058	RLK	putative receptor-like protein kinase At3g47110 [Populus trichocarpa]	RLK	**0.14**	-2.02
unigene37276	NLR	PREDICTED: TMV resistance protein N-like [Malus domestica]	N	-23.22	**-0.01**
unigene49085	NLR	TMV resistance protein N-like [Arachis hypogaea]	N	0.89	**2.13**
isotig52629	NLR	TMV resistance protein N [Vigna radiata var. radiata]	N	2.13	**3.37**
unigene104666	NLR	disease resistance-like protein DSC1 [Citrus clementina]	DSC1	0.00	**4.23**
isotig43179	NLR	PREDICTED: TMV resistance protein N-like isoform X2 [Eucalyptus grandis]	N	1.58	**2.84**

Higher differential expression levels [Log2(fold change)], in comparison to control samples, are highlighted in bold. Drago ann. – Drago annotation; RLP – receptor-like protein; RLK – receptor-like kinase; NLR – nucleotide-binding/leucine-rich-repeat receptor; Log2FC – Log2(fold change), Sus – susceptible; Res – resistant.

Several possible mechanisms involved in PWN resistance in *P. pinaster* are here described. Some clear differences in *P. pinaster* resistant and susceptible transcriptional responses were visible at 72 hpi, highlighting the activation of different phytohormone pathways, contrasting expression of resistance genes, lignin biosynthesis and, possibly, different levels of synthesis of other secondary metabolites. The induction of JA or SA in resistant and susceptible plants, respectively, can be pivotal to determine if the plant defense response is effective against PWN.

2.5.1. Activation of SA and JA pathways

The synthesis and accumulation of SA is induced by EDS1 and its interacting proteins, PAD4 and SAG101, which have also a role in repressing the JA pathway (Pieterse et al., 2011; Wiermer et al., 2005; Zhang and Li, 2019). Genes encoding for these proteins were more upregulated in susceptible plants, suggesting an activation of SA pathway at 72hpi. At the same time, *MKS1*, which encodes for a protein that can repress SA signaling (Andreasson et al., 2005), was more expressed in resistant plants. This indicates that the activation of SA immune response at the studied time point may be characteristic of a susceptible response. In fact, levels of SA were higher in susceptible plants compared to resistant and controls, supporting this hypothesis.

SA and JA immune responses are often antagonistic, with SA pathway being mostly associated with response to biotrophic pathogens and JA pathway with response to necrotrophic pathogens and herbivory (Dar et al., 2015). Although JA and JA-Ile levels were similar in resistant and susceptible plants, SA may still inhibit JA response in susceptible plants, independently of JA biosynthesis (Caarls et al., 2015). The repression of JA pathway in susceptible plants, or activation in resistant plants, is supported by a higher expression of JA responsive genes in the latter. These include *chitinase*, *PR-4*, *PR-5*, *JAZ/Tify* transcription factors and JA biosynthesis genes (Wasternack and Hause, 2013). Therefore, SA/JA

antagonism may play a role in the outcome to PWN inoculation in *P. pinaster*, with the activation of SA pathway in susceptible plants and JA pathway in resistant ones, at 72hpi. In a recent study where hormone levels were analyzed for another *P. pinaster* family described in Carrasquinho et al. (2018), high levels of SA and jasmonic acid methyl ester (JA-ME) were detected in susceptible plants at 72hpi (Rodrigues et al., 2021), supporting an important role for SA in PWN susceptibility. In *P. thurnbergii*, the induction of JA and SA responsive genes was also observed in susceptible plants (Hirao et al., 2012). Therefore, this hormonal response seems to be shared not only among *P. pinaster* families, but also susceptible pine species.

The role of ABA in plant immunity seems to be an ambivalent one (Ton et al., 2009). In some interactions, ABA can inhibit SA and JA/ET response (Adie et al., 2007; Hillwig et al., 2016; Lorenzo et al., 2004; Nahar et al., 2012), while in others it enhances JA response against fungi or herbivory (Bodenhausen and Reymond, 2007; Liu et al., 2020a; Ton et al., 2009; Ton and Mauch-Mani, 2004), activating the MYC branch of JA pathway (Pieterse et al., 2011; Vos et al., 2019). In this work, the similar levels of ABA seen in both inoculated and control plants suggests it does not play a part in defense response to PWN, as it was concluded for *P. pinaster* response to *Fusarium circinatum* (Hernandez-Escribano et al., 2020). However, the overexpression of both a positive regulator of ABA response, *PYL4*, and a repressor of ABA signaling, *PP2C* (Lackman et al., 2011), in resistant plants seems to indicate some role for the ABA pathway in the early stages of the infection. PYL4 is a receptor that recognizes ABA, activating ABA signaling pathway, and has been implicated in the crosstalk between ABA and JA during stress response (Lackman et al 2011). Furthermore, PYL4 induces the expression of both ABA signaling pathway genes, such as *PP2C*, and JA signaling pathway genes, such as *MYC2* or *JAZ* TFs (Liu et al 2020a). In this way, the upregulation of *PYL4* may lead to the activation of ABA pathway independently of ABA accumulation.

2.5.2. Involvement of pathogenesis-related and resistance genes

The expression of *PR* and *chitinase* genes is commonly induced by defense response phytohormones (Van Loon et al., 2006; Pieterse et al., 2011). In this work, a higher expression of several chitin-binding PR-4 and chitinase encoding genes was observed in resistant plants. As chitin is a component of nematode eggshell (Fukushige and Futai, 1985; Holbein et al., 2016), these chitinases may compromise egg integrity and embryo development. In the RKN *Meloidogyne hapla*, treatments with chitinase plant extracts caused premature egg hatching and increased juvenile mortality (Mercer et al., 1992). It would be interesting to see if chitinase extracts from resistant *P. pinaster* plants have similar effects in PWM.

Several differentially expressed genes were identified as putative resistance genes. Interestingly, for many of these, different patterns of expression were detected in resistant and susceptible plants, emphasizing the differences between resistant and susceptible immune responses. For instance, the upregulation of a *FLS2* and a *RLP30* only in susceptible plants seem to reflect the activation of the SA pathway in these plants, since these genes have been described as SA responsive (Zhang and Li, 2019). On the other hand, the higher expression of NLR receptors in resistant plants may lead to the recognition of PWN effectors. Several studies have previously shown relevant roles for NRL receptors in plant resistance to parasitic nematodes (Sato et al., 2019; Zheng et al., 2021) and herbivorous insects (Erb and Reymond, 2019; Hogenhout and Bos, 2011). For instance, the NLR receptor encoded by gene *Mi-1.2* confers resistance to tomato root-knot nematodes (*Meloidogyne* spp) (Milligan et al., 1998), the potato aphid (*Macrosiphum euphorbiae*) (Rossi et al., 1998), the white fly (*Bemisia tabaci*) (Nombela et al., 2003) and the tomato psyllid (*Bactericerca cockerelli*) (Casteel et al., 2006). In the same way, it is plausible that PWN delivers effectors to the plant cell cytoplasm while feeding through the stylet. The recognition of these effectors by NLR receptors could induce a stronger defense response in resistant plants.

2.5.3. Induction of secondary metabolism pathways

Secondary metabolites can be induced both by SA or JA, and their importance in plant defense response is well established (Dar et al., 2015), particularly in conifer trees (e.g. Martin et al., 2002; Moreira et al., 2009; Zas et al., 2014; Zeneli et al., 2006; Zhao et al., 2004; Zulak et al., 2009). The overexpression of genes involved in the synthesis of secondary metabolites was induced by PWN inoculation in several pine species (Gaspar et al., 2017; Gaspar et al., 2020; Shin et al., 2009; Xu et al., 2013), particularly in resistant varieties (Hirao et al., 2012; Kuroda et al., 2011; Liu et al., 2017). In this work, similar results were obtained, with the induction of several genes involved in the phenylpropanoid biosynthesis pathway, including flavonoid or lignin biosynthesis, and the induction of a few genes involved in terpenoid biosynthesis.

The synthesis of terpene compounds has been implicated in resistance to several pests in pine species (Keeling and Bohlmann, 2006) and seems to be induced by JAs, including in *P. pinaster* (Moreira et al., 2009; Sampedro et al., 2011; Zas et al., 2014). For instance, specific diterpenes produced by AS and LPS, encoded by two genes that were here more expressed in *P. pinaster* resistant plants after PWN inoculation, were associated with *Pinus resinosa* resistance to bark beetle (Mason et al., 2017). An increased expression of terpene synthase genes was also observed in resistant *P. massoniana* plants in response to PWN (Liu et al., 2017), and the products of two of these enzymes, α-pinene and longifolene, directly inhibited the survival rate of PWN *in vitro* (Liu et al., 2020b). Other terpenoid compounds found in the resistant species *Pinus strobus* and *Pinus palustris* had nematicidal or repelling effect on PWN (Suga et al., 1993). In *P. pinaster*, plants can be grouped into several chemotypes according to the constitutive content in terpenoid compounds (Gonçalves et al., 2020; Rodrigues et al., 2017). Furthermore, feeding by the PWN insect vector *M. galloprovincialis* induced an increased production of these compounds in different patterns for the studied chemotypes, including α-pinene and

longifolene (Gonçalves et al., 2020). The impact of these chemotypes on PWN resistance is, however, unknown and it would be relevant to investigate it. As synthesis of terpenes seems to be an effective and conserved response to herbivory, and more precisely to PWN, in several conifer species, distinct levels of specific compounds may have a significant impact on nematode survival in resistant *P. pinaster* plants.

Higher induction of the flavonoid biosynthesis pathway has consistently been found in nematode resistant varieties of several plants species (Chin et al., 2018). For instance, chalcone synthase (CHS) encoding genes were frequently more expressed in resistant plants (Chin et al., 2018) and the product of these enzymes, naringenin, caused reduced egg hatching in the burrowing nematode (Wuyts et al., 2006). In turn, LDOX is involved in the synthesis of another compound with a similar effect, kaempferol, and quercetin, which repelled root-knot nematode and burrowing nematode juveniles (Wuyts et al., 2006). As these flavonoids can affect nematode egg development, nematode mobility and survival (Chin et al., 2018), the higher expression of genes encoding for CHS and LDOX enzymes in resistant *P. pinaster* plants may impact PWN and contribute to the observed phenotype. In *P. densiflora*, a higher expression of flavonoid biosynthesis pathway genes was also observed in resistant varieties (Kuroda et al., 2011).

The phenylpropanoid pathway was the secondary metabolism pathway more highly induced by PWN inoculation in *P. pinaster*, with special emphasis in the lignin biosynthesis pathway. Several genes specific to lignin synthesis, such as *PER* and *laccase* genes, had high expression levels in resistant plants and we were able to show that the higher gene expression translated into a significant increase in cell wall lignin content. The upregulation of *PER* genes and genes involved in cell wall strengthening was also associated with resistance in *P. thunbergii* (Hirao et al., 2012) and *P. massoniana* (Liu et al., 2017). Furthermore, higher lignification in regions surrounding plant tissue damaged by PWN has been observed in resistant *P. thunbergii* plants and associated with limited PWN

migration (Kusumoto et al., 2014). Our results support that lignification seems to be an efficient strategy to reduce the spread of PWN, consequent plant tissue damage and likely to interfere with nematode feeding on plant cells (Holbein et al., 2016; Naoumkina et al., 2010).

In our data, it was possible to detect PWN gene expression during the infection process important for the successful infestation of plant tissues. Among these, were genes encoding for antioxidant proteins, which protect PWN from ROS produced by the plant during defense response (Espada et al., 2016). Resistance to oxidative stress has been positively correlated with PWN virulence (Vicente et al., 2015), suggesting that detoxification is essential for successful infection. As above mentioned, the expression of genes encoding the hydrogen peroxide producing enzymes GLOX, possibly involved in increased lignification, was higher in resistant plants. The production of higher amounts of this toxic compound in resistant plants may surpass the PWN capacity for detoxification and negatively influence the nematode performance. In inoculated *P. massoniana* plants, levels of hydrogen peroxide were slightly higher in resistant plants at 24 hpi (Liu et al., 2017), but no data was collected at 72 hpi. At 15 dpi, levels were reversed, being significantly lower in resistant plants, which was associated with a higher expression of oxidative stress response genes (Liu et al., 2017). In *P. pinaster*, as well as in *P. thunbergii* (Hirao et al., 2012), oxidative stress response genes were also more expressed in resistant plants, indicating that a better protection from oxidative damage is important for PWN resistance in several *Pinus* ssp.

2.6. Concluding remarks

Investigation of *P. pinaster* defense response to PWN inoculation in resistant plants has not been previously reported. Combining differential gene expression analysis with hormone and lignin quantification, we identified pathways and mechanisms potentially involved in PWN resistance. The induction of different hormone pathways, namely the SA

pathway in susceptible plants versus the JA pathway in resistant plants, and the higher lignification of plant tissues around the inoculation zone in resistant plants seem to be of great relevance for the phenotypic outcome after inoculation. Secondary metabolism pathways, resistance genes, and oxidative stress response genes also seem to play an important role in PWN resistance. The high expression of these groups of genes in resistant plants may interfere with nematode feeding, survival, mobility, and reproduction. This study provides the foundation to understand PWN resistance in *P. pinaster*, highlighting a set of candidate genes greatly relevant for future functional characterization studies. The use of compounds here associated with resistance, such as JA and secondary metabolites, for pest-management strategies has been previously suggested (e.g., Erbilgin et al., 2006; Goławska et al., 2014; Scalerandi et al., 2018; Zas et al., 2014) and should be explored.

Overall, the implication of several distinct pathways in the resistance of *P. pinaster* to PWN is in accordance with the quantitative nature of the resistance trait and the observation of intermediate symptoms from susceptibility to complete resistance in this study and in previous reports (Carrasquinho et al. 2018, Menéndez-Gutiérrez et al. 2017a). The search for genetic variation in the candidate genes here identified using high-throughput genotyping technologies, further supported by their possible co-location with quantitative trait loci (QTLs) currently under investigation in a larger number of families, may provide relevant molecular markers for identification of resistant genotypes. These approaches will greatly aid selection of individuals from the most tolerant families to be used in current breeding or vegetative propagation programs.

2.7. Data Availability Statement

The sequence data for this study has been submitted to the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB26836 (https://www.ebi.ac.uk/ena/browser/view/PRJEB26836).

2.8. Author Contributions

IM, IC and CMM designed the experiment and performed the inoculation assay. IM performed the RNA experiments. VA and ACG performed the hormone quantification experiment. IM performed data analysis, guided and supervised by LS and YVP. IM and CMM interpreted data and prepared the manuscript. All authors discussed the results and reviewed the manuscript.

2.9. Funding

This work was supported by Fundação para a Ciência e a Tecnologia (FCT), through grants GREEN-it (UID/Multi/04551/2013), BioISI (UIDB/04046/2020 and UIDP/04046/2020), IF/01168/2013 and the doctoral fellowship SFRH/BD/111687/2015 (to IM). Support was also provided by project PTDC/BAA-MOL/28379/2017 - LISBOA-01-0145-FEDER-028379.

2.10. Conflict of interest

The authors declare that they have no conflict of interest.

2.11. Acknowledgments

We thank Dr. Lurdes Inácio (INIAV) for providing the nematode cultures, Hugo Matias (ITQB NOVA) for all the technical support in the greenhouse, and the “Biopolymer Analytical Platform” at Swedish University of Agricultural Sciences, Sweden for the lignin quantification analysis.

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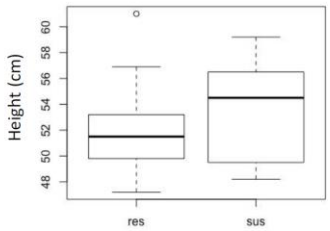
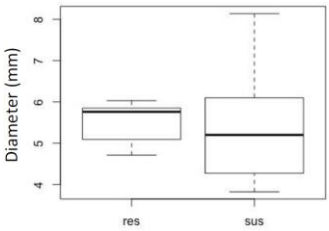
2.13. Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.690857/full#supplementary-material.

a)



b)



c)

	N	Mean (cm)	*SD*	*t*	*p*-value
Res	7	52.3	4.0	-0.47	0.64
Sus	11	53.2	3.8		

d)

	N	Mean (mm)	*SD*	*t*	*p*-value
Res	7	5.6	0.4	0.49	0.63
Sus	11	5.3	1.3		

Figure S2.1. Boxplots of the height and diameter at the base of the stem of inoculated plants (half-sib family 440) and t-test results for the comparison of these parameters' means between resistant (res) and susceptible (sus) plants. (a) Boxplot of height (cm) measurements. **(b)** boxplot of diameter at the base of the stem (mm) measurements. Both measurements were made before inoculations. **(c)** t-test results for heights comparison. **(d)** t-test results for diameter comparison. *N*, number of samples. *SD*, standard deviation.

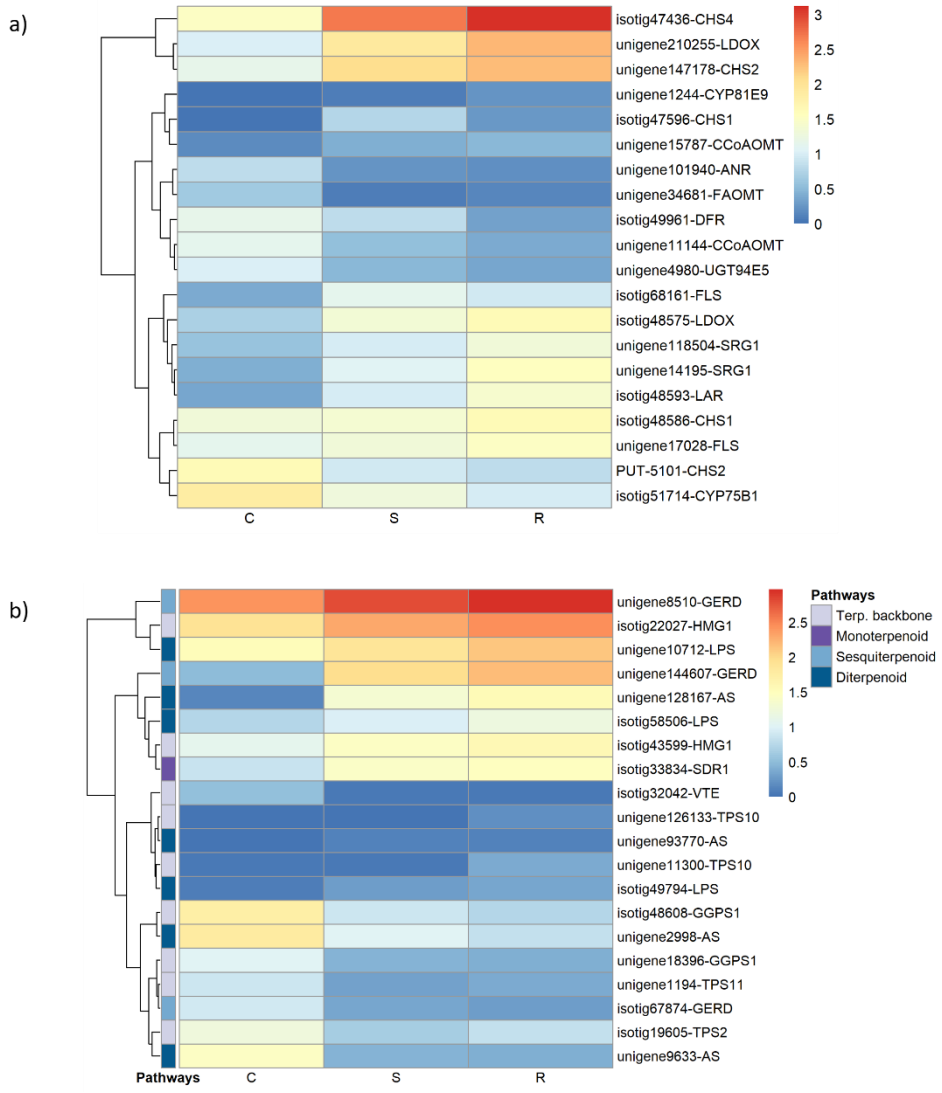


Figure S2.2. Heatmaps representing the expression patterns of genes involved in secondary metabolism. (a) Flavonoid biosynthesis pathway. **(b)** Terpenoid biosynthesis pathways, including terpenoid backbone biosynthesis (Terp. Backbone), monoterpenoid biosynthesis, sesquiterpenoid biosynthesis and diterpenoid biosynthesis pathways. The colour gradient represents mean expression levels (logTPM) of each gene for control (C), susceptible (S) and resistant (R) samples.

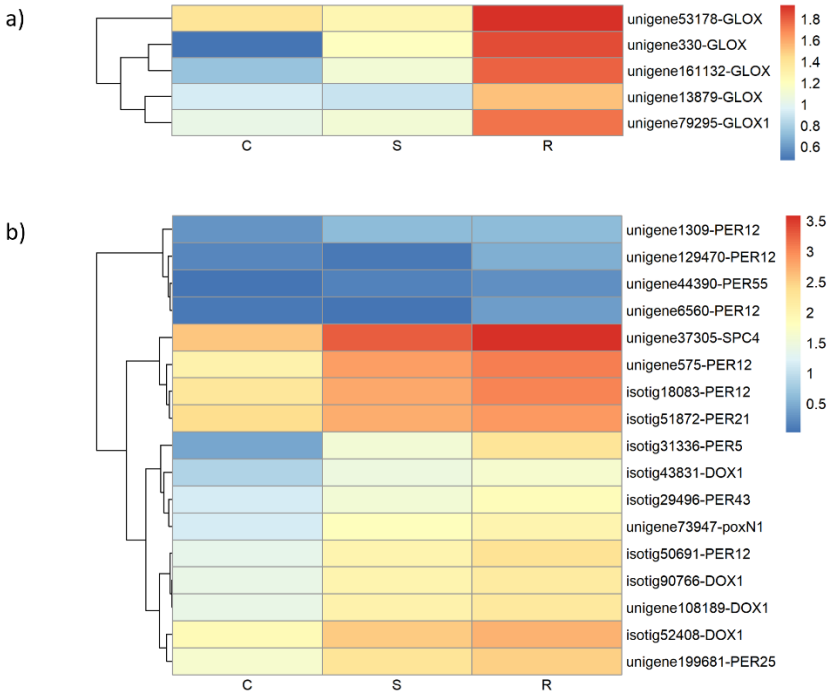


Figure S2.3. Heatmaps representing the expression patterns of genes involved in the synthesis of hydrogen peroxide (a) and response to oxidative stress (b). The colour gradient represents mean expression levels (logTPM) of each gene for control (C), susceptible (S) and resistant (R) samples.

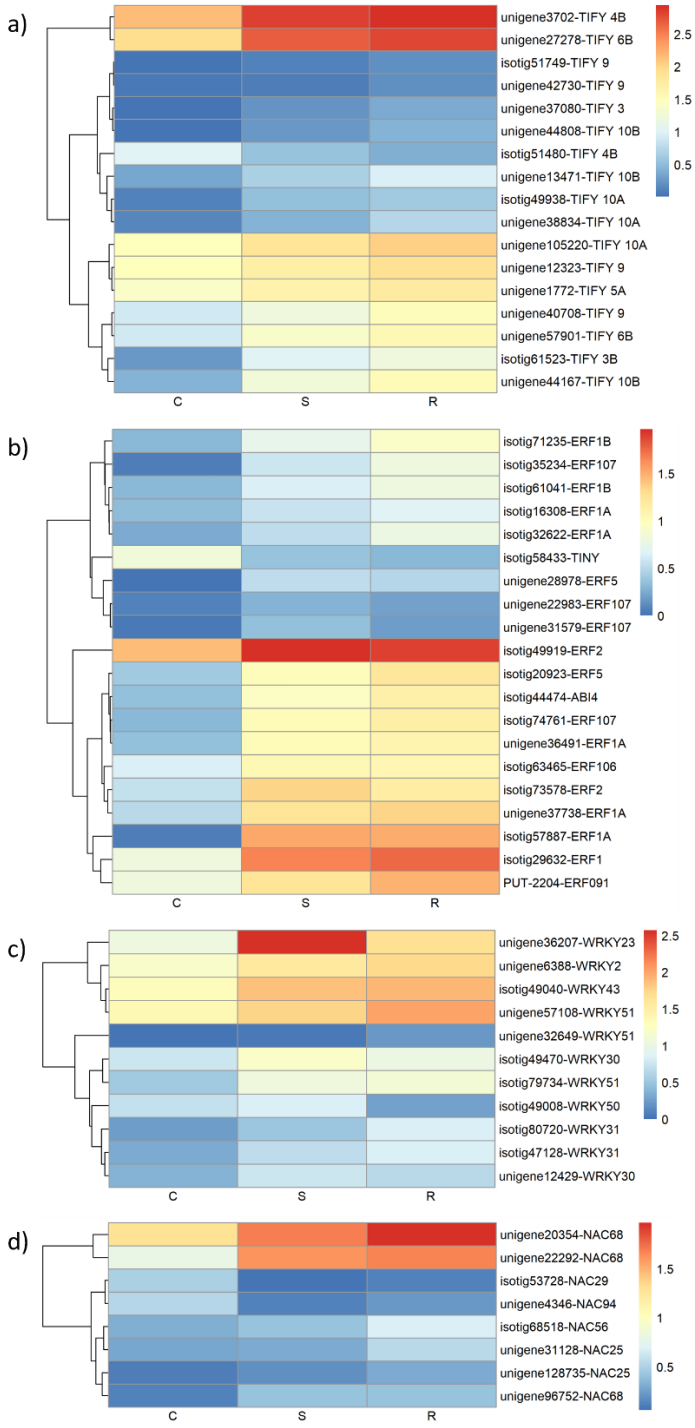

a)
unigene3702-TIFY 4B
unigene27278-TIFY 6B
isotig51749-TIFY 9
unigene42730-TIFY 9
unigene37080-TIFY 3
unigene44808-TIFY 10B
isotig51480-TIFY 4B
unigene13471-TIFY 10B
isotig49938-TIFY 10A
unigene38834-TIFY 10A
unigene105220-TIFY 10A
unigene12323-TIFY 9
unigene1772-TIFY 5A
unigene40708-TIFY 9
unigene57901-TIFY 6B
isotig61523-TIFY 3B
unigene44167-TIFY 10B
C
S
R
2.5
2
1.5
1
0.5
b)
isotig71235-ERF1B
isotig35234-ERF107
isotig61041-ERF1B
isotig16308-ERF1A
isotig32622-ERF1A
isotig58433-TINY
unigene28978-ERF5
unigene22983-ERF107
unigene31579-ERF107
isotig49919-ERF2
isotig20923-ERF5
isotig44474-ABI4
isotig74761-ERF107
unigene36491-ERF1A
isotig63465-ERF106
isotig73578-ERF2
unigene37738-ERF1A
isotig57887-ERF1A
isotig29632-ERF1
PUT-2204-ERF091
C
S
R
1.5
1
0.5
0
c)
unigene36207-WRKY23
unigene6388-WRKY2
isotig49040-WRKY43
unigene57108-WRKY51
unigene32649-WRKY51
isotig49470-WRKY30
isotig79734-WRKY51
isotig49008-WRKY50
isotig80720-WRKY31
isotig47128-WRKY31
unigene12429-WRKY30
C
S
R
2.5
2
1.5
1
0.5
0
d)
unigene20354-NAC68
unigene22292-NAC68
isotig53728-NAC29
unigene4346-NAC94
isotig68518-NAC56
unigene31128-NAC25
unigene128735-NAC25
unigene96752-NAC68
C
S
R
1.5
1
0.5

Figure S2.4. Heatmaps representing the expression patterns of hormone responsive transcription factors (TFs). Jasmonate responsive TFs JAZ/Tify **(a)** and ERF **(b)**, salicylic acid responsive TFs WRKY **(c)** and abscisic acid responsive TFs NAC **(d)**. The colour gradient represents mean expression levels (logTPM) of each gene for control (C), susceptible (S) and resistant (R) samples.

Table S2.1. De novo assembly and *P. pinaster* reference transcriptome statistics.

	New assembly	**Gymno PLAZA (1)**	**Transcriptome used**
Contigs	250,339	206,574	**241,311**
Filtering			
Contigs with blastn hits with Gymno PLAZA(1) *P. pinaster* trasnscriptome (e ≤ 10E-5)	195,983	-	-
Contigs with blastn hits with Gymno PLAZA(1) *P. pinaster* trasnscriptome and *B. xylophilus* genome (2) (e ≤ 10E-5)	161,183	-	-
Contigs with blastx hits with a nematode species different than *B. xylophilus*	63	-	-
Remaining contigs after filtering	**34,737**	-	-
Contigs (after filtering) with CDS	**1,445**	**69,425**	**70,870**

(1) Cañas et al. (2017), *Plant Journal*; (2) Kikushi et al. (2011), *PLoS Pathogens*

Table S2.2. Genes selected for quantitative RT-PCR, respective primer sequences, amplicon size and annealing temperatures used.

Transcript ID	Annotation	Gene abbreviation	Primer Fwd	Primer Rev	Amplicon size (bp)	Ta(ºC)
unigene6560	*peroxidase 12-like [Chenopodium quinoa]*	*PER12-1*	ATAACATCACCACAGGAGACAC	TGAGATCCAAGTTTGTTGCGT	119	59
isotig77090	*laccase-12 [Amborella trichopoda]*	*LAC12*	ATGCGTAGAAGATTAGTTTCCC	ATCCCTTTACCTTTACCAGACC	225	60
unigene161132	*aldehyde oxidase GLOX [Amborella trichopoda]*	*GLOX*	ACATTGGTTACGTCTTCTCCG	GCATGAGTTGTGAAGGATGG	228	59
isotig34276	*PREDICTED: disease resistance protein RGA2-like [Cucumis sativus]*	*RGA2*	GCAAACACCATGTTACCGTC	CAGAGTCAGGGAAAGCATAAAGG	102	59
isotig49789	*Phenylalanine ammonia-lyase [Morus notabilis]*	*PAL*	GCAGGACATATCCCATTTACAG	AAGCGTCCAGACATTTGAG	158	58
isotig52008	*PREDICTED: endochitinase EP3 [Theobroma cacao]*	*EP3-1*	ATCCACACCCAACTGGCT	GACAAGACTCCACCATTTCGT	201	60
isotig58212	*PREDICTED: endochitinase EP3-like [Pyrus x bretschneideri]*	*EP3-2*	TATCTCCTCCACATAGCACAG	GACCTTCTATACATACAGCGAC	153	59
unigene210324	*PREDICTED: endochitinase EP3-like [Gossypium hirsutum]*	*EP3-3*	TTATGCACGAAACTGGAGGG	GTCTTGAACGAAATGGTGGAG	205	63
unigene56017	*class IV endochitinase precursor [Vitis vinifera]*	*chi4*	TCTGTGAGTGGAGTCTTGAG	CCCTGAACTGGAACATGGA	218	58
isotig43946	*PREDICTED: pectinesterase-like [Musa acuminata subsp. malaccensis]*	*PME*	GTCCCATTCCAACCAACC	TGAGCCAACAACAGAACAC	226	63

Table S2.3. Genes expressed by *Bursaphelenchus xylophilus* in inoculated samples. Each column represents one sample (1-4, susceptible plants; 6-10, resistant plants), and the read counts per sample for each gene are displayed. (available online)

Table S2.4. Differential expressed genes in susceptible plants when compared to controls [Log2(fold change) ≥ |2|, FDR corrected p-value (padj) ≤ 0.05]. InterPro, KEGG and blastx annotations are presented. (available online)

Table S2.5. Differential expressed genes in resistant plants when compared to controls [Log2(fold change) ≥ |2|, FDR corrected p-value (padj) ≤ 0.05]. InterPro, KEGG and blastx annotations are presented. (available online)

Table S2.6. GO terms enriched in the upregulated genes in susceptible samples when compared with controls, after trimming for redundancy. Gene set enrichment analysis was performed using the hypergeometric statistical test and results were filtered for an adjusted p-value ≤ 0.05 (FDR).

GO Category	GO Accession No.	Description	corr p-value	No. Different Genes
Biological Process	GO:0055114	oxidation-reduction process	4.42E-08	72
Biological Process	GO:0005984	disaccharide metabolic process	3.31E-04	7
Biological Process	GO:0005975	carbohydrate metabolic process	4.55E-04	35
Biological Process	GO:0017144	drug metabolic process	1.02E-03	19
Biological Process	GO:0042866	pyruvate biosynthetic process	1.02E-03	8
Biological Process	GO:0046939	nucleotide phosphorylation	1.33E-03	8
Biological Process	GO:0006979	response to oxidative stress	4.53E-03	11
Biological Process	GO:0006733	oxidoreduction coenzyme metabolic process	5.91E-03	8
Biological Process	GO:0072521	purine-containing compound metabolic process	8.52E-03	10
Biological Process	GO:0072524	pyridine-containing compound metabolic process	9.33E-03	8
Biological Process	GO:0098869	cellular oxidant detoxification	9.92E-03	11
Biological Process	GO:0071554	cell wall organization or biogenesis	1.17E-02	10
Biological Process	GO:0016998	cell wall macromolecule catabolic process	1.57E-02	4
Biological Process	GO:0006032	chitin catabolic process	1.57E-02	4
Biological Process	GO:0051186	cofactor metabolic process	2.06E-02	14
Biological Process	GO:0009056	catabolic process	4.71E-02	18
Biological Process	GO:0042742	defense response to bacterium	1.02E-03	4
Molecular Fuction	GO:0003700	DNA-binding transcription factor activity	1.27E-06	23
Molecular Fuction	GO:0003824	catalytic activity	5.38E-13	186
Molecular Fuction	GO:0050662	coenzyme binding	0.00001706	22
Molecular Fuction	GO:0016157	sucrose synthase activity	1.45E-06	6

Molecular Fuction	GO:0016841	ammonia-lyase activity	2.92E-03	3
Molecular Fuction	GO:0016491	oxidoreductase activity	2.55E-14	70
Molecular Fuction	GO:0030955	potassium ion binding	1.57E-03	4
Molecular Fuction	GO:0008061	chitin binding	2.73E-05	5
Molecular Fuction	GO:0019842	vitamin binding	2.66E-03	8
Molecular Fuction	GO:0020037	heme binding	0.000039456	22
Molecular Fuction	GO:0004743	pyruvate kinase activity	1.57E-03	4
Molecular Fuction	GO:0043169	cation binding	2.73E-03	54
Molecular Fuction	GO:0030599	pectinesterase activity	3.79E-02	4
Molecular Fuction	GO:0019139	cytokinin dehydrogenase activity	4.31E-02	2
Molecular Fuction	GO:0016798	hydrolase activity, acting on glycosyl bonds	0.010664	17
Molecular Fuction	GO:0000287	magnesium ion binding	2.58E-03	9
Molecular Fuction	GO:0004618	phosphoglycerate kinase activity	3.47E-02	2
Molecular Fuction	GO:0016701	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	4.5105E-09	13
Molecular Fuction	GO:0004601	peroxidase activity	2.58E-04	11
Molecular Fuction	GO:0005506	iron ion binding	2.31E-02	13
Celular Component	GO:0005576	extracellular region	1.34E-02	5

Table S2.7. GO terms enriched in the upregulated genes in resistant samples when compared with controls, after trimming for redundancy. Gene set enrichment analysis was performed using the hypergeometric statistical test and results were filtered for an adjusted p-value ≤ 0.05 (FDR).

GO Category	GO Accession No.	Description	corr p-value	No. Different Genes
Biological Process	GO:0042742	defense response to bacterium	4.79E-05	5
Biological Process	GO:0051952	regulation of amine transport	1.89E-03	8
Biological Process	GO:0071554	cell wall organization or biogenesis	1.39E-11	29
Biological Process	GO:0009056	catabolic process	3.62E-07	44
Biological Process	GO:0042866	pyruvate biosynthetic process	6.15E-07	14
Biological Process	GO:0045229	external encapsulating structure organization	0.0050282	10
Biological Process	GO:0017144	drug metabolic process	3.445E-12	42
Biological Process	GO:0072524	pyridine-containing compound metabolic process	0.0001489	14

Biological Process	GO:0072593	reactive oxygen species metabolic process	0.010697	9
Biological Process	GO:0005975	carbohydrate metabolic process	1.9168E-16	80
Biological Process	GO:1901135	carbohydrate derivative metabolic process	1.2817E-06	36
Biological Process	GO:0051186	cofactor metabolic process	7.24E-04	25
Biological Process	GO:0006091	generation of precursor metabolites and energy	1.85E-02	19
Biological Process	GO:0055114	oxidation-reduction process	6.6935E-20	137
Biological Process	GO:0043086	negative regulation of catalytic activity	2.09E-02	8
Biological Process	GO:0006979	response to oxidative stress	0.00044636	17
Biological Process	GO:0032787	monocarboxylic acid metabolic process	1.30E-02	14
Biological Process	GO:0072521	purine-containing compound metabolic process	6.92E-04	16
Biological Process	GO:0098869	cellular oxidant detoxification	6.80E-04	18
Biological Process	GO:0042221	response to chemical	6.20E-03	27
Biological Process	GO:0006032	chitin catabolic process	1.14E-14	16
Biological Process	GO:0046939	nucleotide phosphorylation	1.77E-06	14
Molecular Fuction	GO:0003700	DNA-binding transcription factor activity	1.34E-06	32
Molecular Fuction	GO:0003824	catalytic activity	7.57E-20	328
Molecular Fuction	GO:0004857	enzyme inhibitor activity	7.03E-03	8
Molecular Fuction	GO:0016701	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	2.37E-17	24
Molecular Fuction	GO:0050662	coenzyme binding	2.22E-12	44
Molecular Fuction	GO:0016157	sucrose synthase activity	2.73E-05	6
Molecular Fuction	GO:0004568	chitinase activity	2.06E-16	16
Molecular Fuction	GO:0016841	ammonia-lyase activity	1.31E-02	3
Molecular Fuction	GO:0010333	terpene synthase activity	4.29E-03	8
Molecular Fuction	GO:0004634	phosphopyruvate hydratase activity	4.82E-02	2
Molecular Fuction	GO:0008061	chitin binding	3.16E-10	9
Molecular Fuction	GO:0016491	oxidoreductase activity	2.25E-27	128
Molecular Fuction	GO:0019842	vitamin binding	2.35E-03	11
Molecular Fuction	GO:0020037	heme binding	1.28E-05	33
Molecular Fuction	GO:0000287	magnesium ion binding	9.73E-05	15
Molecular Fuction	GO:0004618	phosphoglycerate kinase activity	3.25E-04	4
Molecular Fuction	GO:0016798	hydrolase activity, acting on glycosyl bonds	2.25E-13	49
Molecular Fuction	GO:0030599	pectinesterase activity	5.46E-04	8

Molecular Fuction	GO:0016757	transferase activity, transferring glycosyl groups	1.32E-03	31
Molecular Fuction	GO:0043169	cation binding	1.91E-03	89
Molecular Fuction	GO:0003955	NAD(P)H dehydrogenase (quinone) activity	3.22E-02	2
Molecular Fuction	GO:0030955	potassium ion binding	9.70E-03	4
Molecular Fuction	GO:0004743	pyruvate kinase activity	9.70E-03	4
Molecular Fuction	GO:0004601	peroxidase activity	1.37E-05	17
Molecular Fuction	GO:0005506	iron ion binding	7.82E-03	21
Molecular Fuction	GO:0016614	oxidoreductase activity, acting on CH-OH group of donors	4.29E-03	12
Celular Component	GO:0005576	extracellular region	8.99E-10	15
Celular Component	GO:0048046	apoplast	1.18E-03	6
Celular Component	GO:0000145	exocyst	1.18E-03	5
Celular Component	GO:0000015	phosphopyruvate hydratase complex	8.95E-03	2
Celular Component	GO:0005618	cell wall	2.49E-03	5

Table S2.8. Complete list of genes annotated with DRAGO 2 tool. (available online)

Table S2.9. Genes used for calculating average Log2(fold change) expression levels for Figures 2.5 and 2.6.

A) Hormone responsive genes

A.1) Abscisic acid response genes

Gene	Transcript ID	blastx annotation
PYL4	unigene11797	abscisic acid receptor PYL4-like [Olea europaea var. sylvestris]
PP2C	unigene2613	probable protein phosphatase 2C 51 [Selaginella moellendorffii]

A.2) Jasmonate response genes

Gene	Transcript ID	blastx annotation
MYC2	isotig31234	PREDICTED: transcription factor bHLH13-like isoform X1 [Nicotiana tabacum]
PLA2G	isotig59095	phospholipase A2-alpha-like [Olea europaea var. sylvestris]
LOX2S	isotig54243	linoleate 13S-lipoxygenase 3-1, chloroplastic [Prunus persica]
LOX2S	unigene4631	linoleate 13S-lipoxygenase 3-1, chloroplastic-like [Cajanus cajan]
LOX2S	unigene4631	linoleate 13S-lipoxygenase 3-1, chloroplastic-like [Cajanus cajan]

LOX2S	unigene7561	linoleate 13S-lipoxygenase 3-1, chloroplastic-like [Coffea arabica]
AOS1.1	unigene130183	allene oxide synthase-like [Selaginella moellendorffii]
AOS1.1	unigene35275	allene oxide synthase 1, chloroplastic-like [Cucurbita moschata]
AOS1.2	isotig49985	PREDICTED: allene oxide synthase 1, chloroplastic-like [Gossypium arboreum]
AOS3	isotig46612	allene oxide synthase 3-like [Vigna radiata var. radiata]
AOS3	isotig49547	allene oxide synthase 3-like [Cucurbita moschata]
OPR	unigene13678	hypothetical protein PHAVU_003G131500g [Phaseolus vulgaris]
OPR	unigene71935	hypothetical protein PHAVU_003G131500g [Phaseolus vulgaris]
OPR1	isotig30320	12-oxophytodienoate reductase 1-like [Cucurbita pepo subsp. pepo]
OPR2	isotig101377	12-oxophytodienoate reductase 2 [Ricinus communis]
OPR3	unigene107479	12-oxophytodienoate reductase 3 [Medicago truncatula]
OPR3	unigene132639	PREDICTED: 12-oxophytodienoate reductase 3 [Tarenaya hassleriana]
OPR3	unigene167462	12-oxophytodienoate reductase 3-like [Cajanus cajan]
OPR3	unigene18959	PREDICTED: 12-oxophytodienoate reductase 3 [Tarenaya hassleriana]
OPR7	unigene129685	12-oxophytodienoate reductase 7 [Amborella trichopoda]
OPR7	unigene68423	12-oxophytodienoate reductase 7 [Amborella trichopoda]
OPR7	unigene98191	12-oxophytodienoate reductase 7 [Amborella trichopoda]
OPR11	isotig35882	putative 12-oxophytodienoate reductase 11 [Coffea eugenioides]

A.3) Salicylic acid response genes

Gene	Transcript ID	blastx annotation
EDS1	isotig48001	protein EDS1L-like [Cynara cardunculus var. scolymus]
EDS1	isotig52813	protein EDS1B-like [Manihot esculenta]
EDS1	isotig54396	protein EDS1B-like [Phoenix dactylifera]
EDS1	unigene146318	protein EDS1L-like [Ananas comosus]
EDS1	unigene147115	protein EDS1L [Medicago truncatula]
EDS1	unigene21363	protein EDS1L-like [Cynara cardunculus var. scolymus]
PAD4	isotig32554	PREDICTED: lipase-like PAD4 [Nicotiana tabacum]
PAD4	isotig80552	PREDICTED: lipase-like PAD4 [Populus euphratica]
PAD4	unigene146306	PREDICTED: lipase-like PAD4 [Elaeis guineensis]
PAD4	unigene31706	PREDICTED: lipase-like PAD4 [Elaeis guineensis]
SAG101	unigene146258	senescence-associated carboxylesterase 101-like [Lactuca sativa]

MKS1	TRINITY_DN37646_c0_g1_i1	PREDICTED: protein MKS1 [Eucalyptus grandis]

B) *Pathogenesis-related protein* and *chitinase* genes

Gene family	Transcript ID	blastx annotation
PR-1	isotig35840	PREDICTED: pathogenesis-related protein 1-like [Musa acuminata subsp. malaccensis]
PR-1	unigene1468	pathogenesis-related protein PRB1-3-like [Ananas comosus]
PR-1	unigene17284	pathogenesis-related protein PRB1-3-like [Ananas comosus]
PR-1	unigene21878	pathogenesis-related protein PRB1-3-like [Ananas comosus]
PR-4	isotig64114	pathogenesis-related protein PR-4 [Selaginella moellendorffii]
PR-4	unigene30409	pathogenesis-related protein PR-4 [Selaginella moellendorffii]
PR-4	unigene33288	pathogenesis-related protein PR-4 [Selaginella moellendorffii]
PR-5	isotig10512	thaumatin-like pathogenesis-related protein 4 [Medicago truncatula]
PR-5	isotig57101	thaumatin-like protein L3 [Pinus monticola]
PR-5	isotig57379	thaumatin-like protein L6 [Pinus monticola]
PR-5	isotig57546	thaumatin-like protein L4 [Pinus monticola]
PR-5	isotig58934	thaumatin-like protein [Oryza sativa Japonica Group]
PR-5	isotig75533	pathogenesis-related protein PR-4-like [Ziziphus jujuba]
PR-5	isotig95331	thaumatin-like protein, partial [Picea sitchensis]
PR-5	unigene126502	thaumatin-like protein 1 [Cinnamomum micranthum f. kanehirae]
PR-5	unigene17357	thaumatin-like protein L6 [Pinus monticola]
PR-5	unigene22112	thaumatin-like pathogenesis-related protein 4 [Medicago truncatula]
PR-5	unigene29681	thaumatin-like protein [Panicum hallii]
PR-5	unigene31714	pathogenesis-related protein 5-like [Arachis duranensis]
PR-5	unigene33770	thaumatin-like pathogenesis-related protein 4 [Medicago truncatula]
PR-5	unigene3797	thaumatin-like protein L2 [Pinus monticola]
PR-5	unigene44676	thaumatin-like protein 1 [Amborella trichopoda]
PR-5	unigene44846	PREDICTED: pathogenesis-related protein 5 [Camelina sativa]
chitinase	isotig108630	PREDICTED: endochitinase EP3-like [Nicotiana attenuata]
chitinase	isotig23174	class V chitinase [Pinus banksiana]
chitinase	isotig23739	endochitinase EP3-like [Rosa chinensis]
chitinase	isotig36402	endochitinase EP3-like [Rosa chinensis]
chitinase	isotig45495	class V chitinase, partial [Pinus banksiana]
chitinase	isotig49023	class V chitinase, partial [Pinus banksiana]

chitinase	isotig49993	chitinase 2-like [Phoenix dactylifera]
chitinase	isotig51450	chitinase 1 [Setaria italica]
chitinase	isotig52008	endochitinase EP3-like [Rosa chinensis]
chitinase	isotig52920	chitinase 1 [Setaria italica]
chitinase	isotig53068	endochitinase A [Setaria italica]
chitinase	isotig54670	chitinase 11-like [Ananas comosus]
chitinase	isotig54940	hevein-like preproprotein [Citrus sinensis]
chitinase	isotig55420	PREDICTED: endochitinase EP3-like [Pyrus x bretschneideri]
chitinase	isotig56602	chitinase 2-like [Phoenix dactylifera]
chitinase	isotig58212	PREDICTED: endochitinase EP3-like [Pyrus x bretschneideri]
chitinase	isotig74797	class V chitinase, partial [Pinus banksiana]
chitinase	unigene11508	class V chitinase [Glycine max]
chitinase	unigene128225	class I chitinase [Pinus banksiana]
chitinase	unigene13385	class V chitinase [Pinus banksiana]
chitinase	unigene17114	PREDICTED: chitinase 1 isoform X2 [Musa acuminata subsp. malaccensis]
chitinase	unigene210324	endochitinase EP3-like [Rosa chinensis]
chitinase	unigene33122	endochitinase A [Setaria italica]
chitinase	unigene3850	PREDICTED: basic endochitinase-like [Ipomoea nil]
chitinase	unigene56017	endochitinase A [Sorghum bicolor]

Chapter 3

MicroRNA-mediated post-transcriptional regulation of *Pinus pinaster* response and resistance to pinewood nematode

This chapter has been published in *Scientific Reports*:

Modesto, I., Inácio, V., Van de Peer, Y. & Miguel, C. M. (2022) MicroRNA-mediated post-transcriptional regulation of *Pinus pinaster* response and resistance to pinewood nematode. *Scientific Reports*, 12. https://doi.org/10.1038/S41598-022-09163-3.

Inês Modesto participated in the conceptualization of the experiments, performed the RNA extractions, analysed and interpreted the data, prepared the figures and tables, and wrote the original draft of the manuscript.

3. MicroRNA-mediated post-transcriptional regulation of *Pinus pinaster* response and resistance to pinewood nematode

3.1. Abstract

Pine wilt disease (PWD), caused by the parasitic nematode *Bursaphelenchus xylophilus*, or pinewood nematode (PWN), is a serious threat to pine forests in Europe. *Pinus pinaster* is highly susceptible to the disease and it is currently the most affected European pine species. In this work, we investigated the role of small RNAs (sRNAs) in regulating *P. pinaster*-PWN interaction in an early stage of infection. After performing an artificial PWN inoculation assay, we have identified 105 plant microRNAs (miRNAs) responsive to PWN. Based on their predicted targets, part of these miRNAs was associated with roles in jasmonate-response pathway, ROS detoxification, and terpenoid biosynthesis. Furthermore, by comparing resistant and susceptible plants, eight miRNAs with putative functions in plant defence and resistance to PWN have been identified. Finally, we explored the possibility of bidirectional trans-kingdom RNA silencing, identifying several *P. pinaster* genes putatively targeted by PWN miRNAs, which was supported by degradome analysis. Targets for *P. pinaster* miRNAs were also predicted in PWN, suggesting a role for trans-kingdom miRNA transfer and gene silencing both in PWN parasitism as in *P. pinaster* resistance to PWD. Our results provide new insights into previously unexplored roles of sRNA post-transcriptional regulation in *P. pinaster* response and resistance to PWN.

3.2. Introduction

Pinewood nematode (PWN), or *Bushaphelenchus xylophilus*, is a migratory plant-parasitic nematode that causes pine wilt disease (PWD) in several conifer species. PWN is transmitted to healthy trees through the insect vector *Monochamus* spp. while it feeds on the tree's bark (Kim et al., 2020;

Vicente et al., 2012). This nematode infects the tree stem, migrating through resin canals and feeding on plant tissues. The progressive destruction of stem tissues leads to the disruption of water flow, causing the wilting and death of the tree.

PWD has become an increasing threat to worldwide conifer forests, especially in Asia and South-eastern Europe, causing economic losses in the forestry industry and having a severe environmental impact (Webster and Mota, 2008). In Europe, PWD was first detected in Portugal in 1999 (Mota et al., 1999) and has since spread to Spain, despite the sanitary measures implemented (Abelleira et al., 2011). *Pinus pinaster* is the mainly affected species in this region (Vicente et al., 2012; Webster and Mota, 2008).

As a strategy to help mitigate the spreading and damage of PWD, resistant varieties of susceptible *Pinus* species have been developed (Toda and Kurinobu, 2002; Xu et al., 2012). Breeding programs were successfully implemented for *Pinus thunbergii*, *Pinus densiflora*, and *Pinus massoniana* (Toda and Kurinobu, 2002; Xu et al., 2012). For *P. pinaster*, the first steps were given in order to select individuals with increased PWN resistance (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017).

Plant defence response initiates after the recognition of the pathogen by cell membrane receptor-like kinases (RLKs) or receptor-like proteins (RLPs), activating the pattern-triggered immunity (PTI) (Miller et al., 2017). Pathogens and pests can, however, produce effectors that suppress PTI. In turn, plants may recognize these effectors through nucleotide-binding/leucine-rich-repeat (NLR) receptors, initiating the more robust effector-triggered immunity (ETI) (Miller et al., 2017). The activation of PTI and ETI trigger immune responses controlled by plant hormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) or abscisic acid (ABA) (Buscaill and Rivas, 2014; Miller et al., 2017). In response to PWN inoculation, a transcriptional reprogramming was observed in *P. pinaster* stem tissues (Gaspar et al., 2017; Modesto et al., 2021). This included the

differential expression of RLK/RLP and NLR encoding genes, as well as genes involved in secondary metabolism, oxidative stress response, lignin synthesis, and phytohormones signalling pathways. An increase in JA levels was observed after inoculation, while high SA levels were associated with susceptibility. Furthermore, resistant plants showed higher lignification around the inoculation zone when compared to susceptible plants (Modesto et al., 2021).

Several studies have shown important roles for small non-coding RNAs (sRNAs) in the interaction of host plants with viruses, bacteria, fungi, nematodes, and herbivore insects (Brant and Budak, 2018; Huang et al., 2016; Rose et al., 2019). MiRNAs have been implicated in the regulation of plant hormone synthesis and signalling, callose deposition, expression of *NLR receptors*, and production of secondary metabolites. On the other hand, pathogens' and pests' effectors may suppress plant immune response by reducing the accumulation of sRNAs or interfering with the RNA silencing machinery (Brant and Budak, 2018a; Huang et al., 2016; Rose et al., 2019). Furthermore, trans-kingdom RNA silencing has been reported, in which sRNAs encoded by pathogens directly suppress host genes with roles in plant immunity (Brilli et al., 2018; Wang et al., 2017; Weiberg et al., 2013). Likewise, transgenic plants expressing exogenous sRNAs/dsRNAs can induce the silencing of genes in pathogens or pests, in a process called host-induced gene silencing (HIGS) (Brant and Budak, 2018; Huang et al., 2016). Recent studies suggest that naturally occurring plant miRNAs may also be transferred to pathogens and target their genes in order to fight infection (Brilli et al., 2018; Zhang et al., 2016).

The role of miRNAs in the regulation of growth in PWN infected plants has been previously investigated in needle tissues of *P. Massoniana* (Xie et al., 2017). Plant hormone signalling genes were targeted by differentially expressed miRNAs, leading to the suppression of indole acetic acid and zeatin synthesis thus causing the inhibition of plant growth, but the role of the expressed miRNAs in regulating plant immune response was not

addressed. In *P. pinaster*, sRNAs were reported to be involved in the regulation of embryo development (Rodrigues et al., 2019) and abiotic stress response (Perdiguero et al., 2020), but their function in biotic stress has not been described.

In this study, the regulatory roles of sRNAs in *P. pinaster*-PWN interaction during an early stage of infection (72 hours post-inoculation, hpi) were investigated in PWN infected tissues (stem). While 105 pine differentially expressed (DE) miRNAs were found to be responsive to PWN and possibly regulating JA-response, ROS detoxification and terpenoid biosynthesis, only eight miRNAs were identified with predicted roles in PWN resistance. Our results suggest that post-transcriptional regulation of *RLK/RLP receptors* and *L-fucose synthase* by miRNAs might be a relevant mechanism involved in resistance to PWD. Furthermore, investigation of possible bidirectional trans-kingdom RNA silencing revealed that silencing of the host plant genes by PWN miRNAs may promote virulence, while targeting of PWN genes by the plant miRNAs may have a role in *P. pinaster* resistance to PWD.

3.3. Results

To identify sRNAs involved in *P. pinaster* response and resistance to PWN, an inoculation assay was performed with plants from a half-sib family characterized by Carrasquinho *et al.* (2018). Within this family, individuals may present resistant or susceptible phenotypes after PWN inoculation, as previously described (Carrasquinho et al., 2018). Sample collection from the stem of inoculated plants was performed at 72 hpi. After sampling, symptoms were observed weekly and plants were classified on a scale of 0 (no visible symptoms) to 4 (more than 75% of brown/wilted needles) (Modesto et al., 2021) (Table 3.1). After 210 days post-inoculation (dpi), 28% of the plants remained healthy (level 0) and were considered resistant, while 72% of the plants showed symptoms and were considered susceptible. The susceptible plants selected for RNA-seq were the first four

plants showing a level 4 of symptoms in the symptoms scale. Symptoms evaluation and progression along the experiment have been previously detailed in Modesto *et al.* (2021).

Table 3.1. Symptoms' progression in selected timepoints. Symptoms were evaluated weekly for 210 days post- inoculation (dpi) and registered according to a five-level scale based on percentage of brown/wilted needles: 0—0%; 1—1 to 25%; 2—26 to 50%; 3—51 to 75%; 4—7 to 100%.

Symptoms	Days post inoculation (dpi)					
	14 dpi	21 dpi	35 dpi	42 dpi	105 dpi	210 dpi
0	83%	72%	44%	44%	28%	28%
1	11%	17%	28%	22%	22%	17%
2	6%	11%	11%	0%	0%	5%
3	0%	0%	6%	11%	0%	0%
4	0%	0%	11%	23%	50%	50%

3.3.1. Small RNAs sequencing and identification

Small RNA libraries were sequenced for four susceptible, five resistant, and four control individuals. Small RNA sequencing data yielded approximately 23-40 million reads per sample, with sizes ranging between 18-50 bp. Since the nematode infects and migrates through stem tissues, and these tissues have been harvested during sampling, reads were mapped to both *Pinus taeda* (Zimin et al., 2017) and PWN genomes (Kikuchi et al., 2011). An average of 97% mapped reads was obtained, from which 99.5% mapped to the *P. taeda* genome, and 0.5% mapped to the PWN genome (Supplementary Table S3.1). Reads mapping to different genomes were analysed separately.

An average of 18 million *P. pinaster* reads was retained per sample after initial filtering, with sizes between 18-26 nucleotides (Supplementary Table S3.1). This corresponds to 49-69% of the reads that mapped to the *P. taeda* genome, and most were 21nt (≈50%) (Supplementary Fig. S3.1). Reads were analysed to identify conserved miRNAs, novel miRNAs, and trans-acting sRNAs (tasiRNAs). A total of 4984 miRNAs were identified in all

samples (Fig. 3.1a, Supplementary Table S3.2), from which 850 were novel (Table 3.2). The conserved miRNAs belonged to 184 different families. A total of 3636 tasiRNAs were identified in all samples (Fig. 3.1b). A large part of the miRNAs (63%) and the tasiRNAs (50%) were expressed in all samples (Fig. 3.1).

Table 3.2. Numbers of small RNAs detected in *P. pinaster* and PWN, *B. xylophilus*. Values for susceptible, resistant and control samples represent the mean of the biological replicates.

	Pinus pinaster				***Bursaphelencus xylophilus***		
	Total	**Sus**	**Res**	**Cont**	**Total**	**Sus**	**Res**
Conserved miRNA families	184	143 (±2)	144 (±2)	137 (±3)	195	93 (±22)	123 (±18)
Conserved miRNA members	3506	3079 (±75)	3213 (±223)	2725 (±234)	908	329 (±91)	466 (±88)
Novel miRNAs	850	506 (±24)	529 (±30)	447 (±35)	78	41 (±6)	48 (±7)
Total miRNAs	4356	3586 (±93)	3743 (±251)	3173 (±269)	986	369 (±96)	514 (±95)
tasiRNAs	3636	2070 (±65)	2314 (±194)	1967 (±134)	-	-	-

Sus – susceptible; Res – resistant; Cont - control

For PWN originating sequences, an average of 100,000 reads with sizes between 18-26 nucleotides were retained per sample (Supplementary Table S3.1). This corresponds to 51-69% of the reads that mapped to the *B. xylophilus* genome and most of them were 21nt (≈52%; Supplementary Fig. 3.1b). Filtered reads were subsequently analysed to identify conserved miRNAs and novel miRNAs. A total of 919 miRNAs were identified in all samples (Table 3.2, Supplementary Table S3.3), from which 13 were novel. The conserved miRNAs belonged to 195 different families.

3.3.2. *P. pinaster* miRNAs responsive to PWN and their target genes

Differential expression analysis between inoculated and control plants revealed 105 DE miRNAs (adjusted p-value ≤ 0.05; Supplementary Table S3.2), from which 79 were upregulated and 26 were downregulated. The DE miRNAs included 86 conserved ones, from 29 families (Fig. 3.2a).

Some of these families had one single isoform differentially expressed (e.g. miR11428, miR11430), while 18 had two to 16 (miR529) isoforms (Supplementary Table S3.2). The mean expression for each family is shown in Figure 2a.

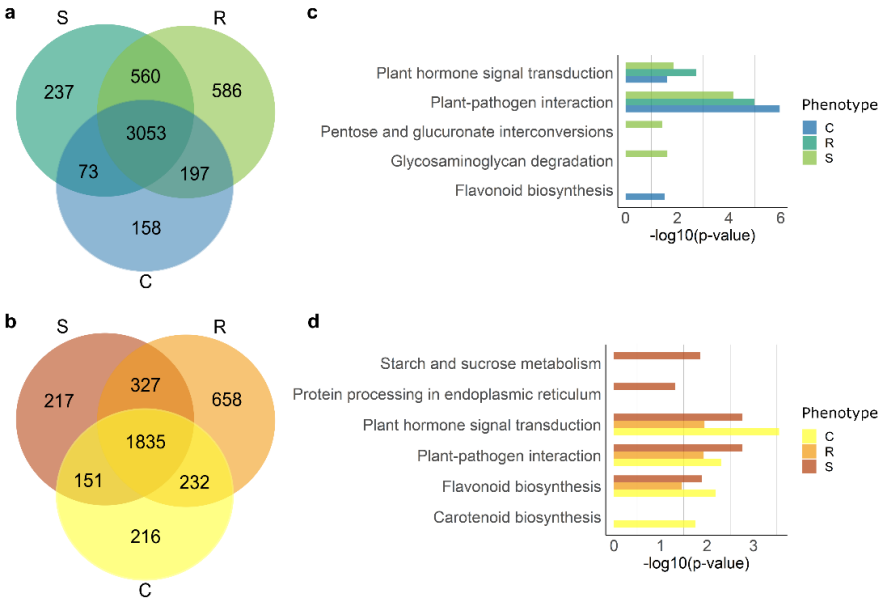


Figure 3.1. Number of expressed *Pinus pinaster* miRNAs (a) and tasiRNAs (b) in susceptible (S), resistant (R), and control (C) samples. Pathway enrichment analysis of predicted target genes of the expressed miRNAs **(c)** and tasiRNAs **(d)**. The x-axis represents the significance of pathway enrichment (− log10 of corrected p-values) **(c, d)**. Venn diagrams were generated online (https://bioinformatics.psb.ugent.be/webtools/Venn/) and edited with Inkscape 1.1 (https://inkscape.org/). Bar plots were generated with R 4.1.0 (https://cran.r-project.org/) ggplot2 package (https://ggplot2.tidyverse.org/).

To explore the putative function of the DE miRNAs, their target genes were predicted using psRNATarget and the transcriptome. Taking advantage of the transcriptomics data available for the same samples (Modesto et al., 2021), the analysis of negative correlations of gene expression levels between miRNAs and mRNAs was performed. In this way, it was possible to identify 1682 target genes (Pearson's correlation R ≤ -0.65; Supplementary Table S3.4).

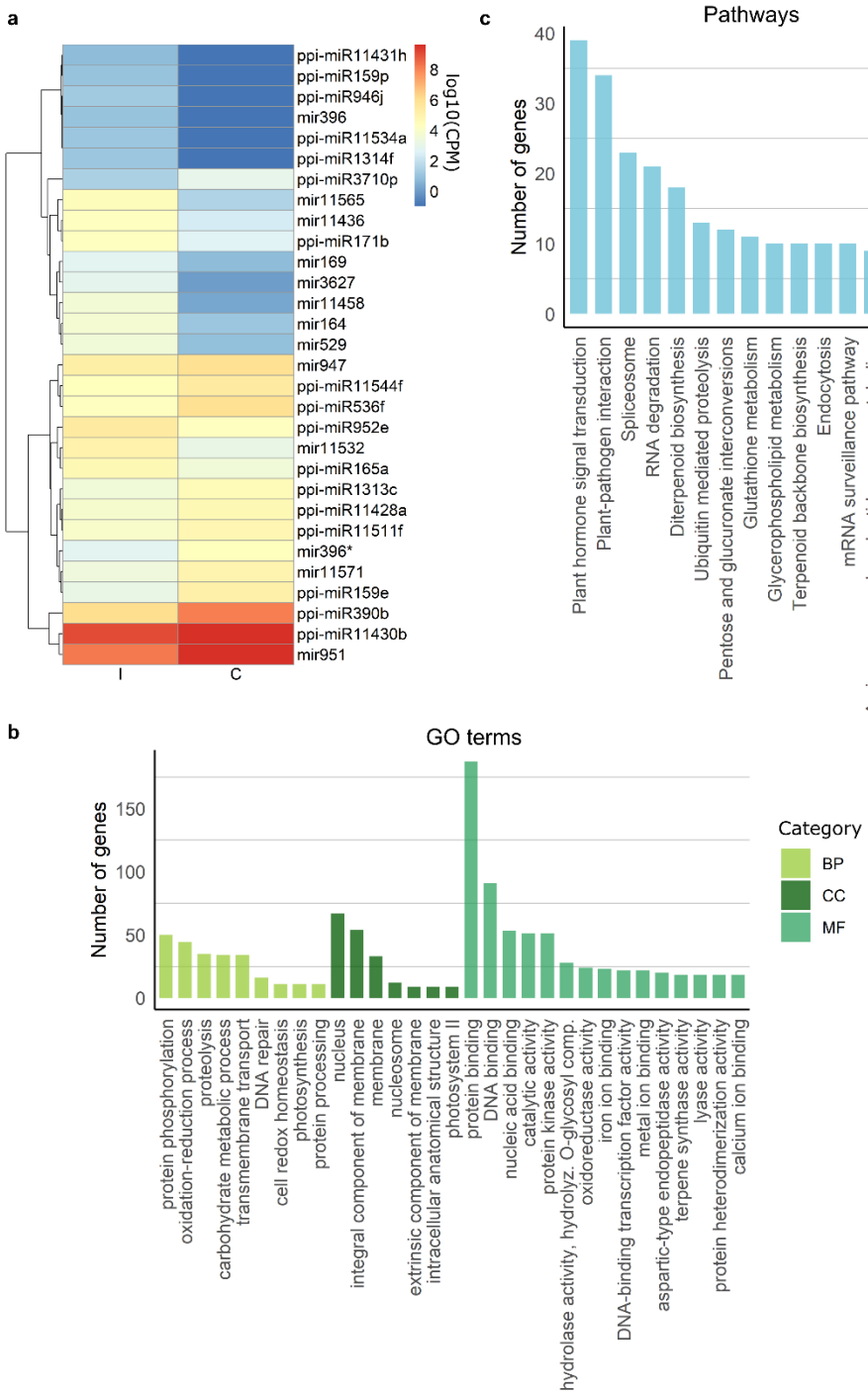

a
ppi-miR11431h
ppi-miR159p
ppi-miR946j
mir396
ppi-miR11534a
ppi-miR1314f
ppi-miR3710p
mir11565
mir11436
ppi-miR171b
mir169
mir3627
mir11458
mir164
mir529
mir947
ppi-miR11544f
ppi-miR536f
ppi-miR952e
mir11532
ppi-miR165a
ppi-miR1313c
ppi-miR11428a
ppi-miR11511f
mir396*
mir11571
ppi-miR159e
ppi-miR390b
ppi-miR11430b
mir951
I
C
log10(CPM)
8
6
4
2
0
c
Pathways
Number of genes
40
30
20
10
0
Plant hormone signal transduction
Plant-pathogen interaction
Spliceosome
RNA degradation
Diterpenoid biosynthesis
Ubiquitin mediated proteolysis
Pentose and glucuronate interconversions
Glutathione metabolism
Glycerophospholipid metabolism
Terpenoid backbone biosynthesis
Endocytosis
mRNA surveillance pathway
Amino sugar and nucleotide sugar metabolism
Lysosome
Purine metabolism
b
GO terms
Number of genes
150
100
50
0
protein phosphorylation
oxidation-reduction process
proteolysis
carbohydrate metabolic process
transmembrane transport
DNA repair
cell redox homeostasis
photosynthesis
protein processing
nucleus
integral component of membrane
membrane
nucleosome
extrinsic component of membrane
intracellular anatomical structure
photosystem II
protein binding
DNA binding
nucleic acid binding
catalytic activity
protein kinase activity
hydrolase activity, hydrolyz. O-glycosyl comp.
oxidoreductase activity
iron ion binding
DNA-binding transcription factor activity
metal ion binding
aspartic-type endopeptidase activity
terpene synthase activity
lyase activity
protein heterodimerization activity
calcium ion binding
Category
BP
CC
MF

Figure 3.2. miRNAs differentially expressed between inoculated (I) and control plants (C) and functional analysis of their target genes. (a) Average expression [log10(CPM)] for each conserved DE miRNA family, except for families where the miRNAs presented opposite expression patterns to each other, in which case isoform expression is represented. **(b)** GO terms and **(c)** pathways most represented in *P. pinaster* predicted target genes for the DE miRNAs. The y-axis represents the number of genes within each KEGG pathway 26,27 or GO term. BP biological process, CC cellular component, MF molecular function. Plots were generated with R 4.1.0 (https://cran.r-project.org/) pheatmap package (https://cran.r-project.org/web/packages/pheatmap/) and ggplot2 package (https://ggplot2.tidyverse.org/). Inkscape 1.1 (https://inkscape.org/) was used to assemble the final figure.

After redundancy reduction, 184 GO terms were attributed to target genes (Supplementary Table S3.5). Doing a gene set enrichment analysis, only the biological process (BP) terms macromolecule modification and response to stimulus were significantly enriched ($p \leq 0.05$). Within the most represented GO terms (Fig. 3.2b) were the BPs oxidation-reduction process and cell redox homeostasis, the cellular components (CCs) nucleus and integral component of membrane, and the molecular functions (MFs) DNA binding, protein kinase activity, and terpene synthase activity. Protein phosphorylation and protein binding were also highly represented in the analysis.

Regarding KEGG annotation (Kanehisa, 2019; Kanehisa and Goto, 2000), 71 pathways were assigned to target genes (Supplementary Table S3.5). Plant hormone signal transduction was significantly enriched ($p \leq 0.05$) in the targets of the DE miRNAs. The most represented pathways included plant-pathogen interaction, diterpenoid biosynthesis, and terpenoid backbone biosynthesis (Fig. 3.2c). Within the pathway plant hormone signal transduction were several jasmonate responsive genes, such as *JAZ/Tify* and *MYC4* transcription factors. The miRNAs targeting these genes were downregulated after inoculation (Table 3.3), suggesting an activation of the JA pathway. Plant-pathogen interaction genes, such as *WRKY* transcription factors, disease resistance proteins (*RLPs/RLKs*), and calcium-dependent protein kinase *CPK28*, were targeted by upregulated miRNAs (Table 3.3). Among the target genes, it was also possible to identify *terpene synthase*

genes, such as *bifunctional abietadiene synthase* (*AS*) and *bifunctional levopimaradiene synthase* (*LPS*) (Table 3.3). Genes involved in detoxification of ROS were targeted by several upregulated miRNAs, including *peroxiredoxins*, *superoxide dismutase* (*MSD1*), or *thioredoxin* (Table 3.3).

Five Pfam protein domains were enriched in *P. pinaster* DE miRNAs predicted target genes ($p \leq 0.05$; Supplementary Table S3.5), including F-box domain, which mediates protein-protein interactions, and SBP domain, found in transcription factors.

RT-qPCR analysis of five DE miRNAs showed similar expression trends as the small RNA-seq results (Pearson's correlation R = 0.77, $p = 0.009$; Supplementary Fig. S3.2). For each of these miRNAs, RT-qPCR analysis was performed for one predicted target gene and a strong positive correlation was found between RT-qPCR and RNA-seq results (Pearson's correlation R = 0.97, $p = 1.8e-06$; Supplementary Fig. S3.2). A correlation analysis was also made between the RT-qPCR values of the miRNAs and respective predicted target gene. For two pairs of miRNA-target gene a high negative correlation value, although not significant, was obtained (miRNovel-*RPP13* Pearson's R = -0.78; miR11436b-*RLK3* Pearson's R = -0.61), while for the remaining pairs low or positive correlation coefficients were obtained (Supplementary Table S3.6).

3.3.3. miRNAs associated with PWN resistance and their target genes

To identify miRNAs that may have a role in resistance to PWN, resistant and susceptible samples were compared, revealing eight miRNAs DE between these two groups (adjusted *p*-value ≤ 0.05; Fig. 3.3a). From these, seven were conserved miRNAs, corresponding to five families (Fig. 3.3a): miR166, miR947, miR951, miR3627, and miR11511. These families were also, as previously mentioned, differentially expressed after inoculation (Fig. 3.2a), although the isoforms detected as significantly differentially expressed were distinct (Supplementary Table S3.2).

Table 3.3. Selected differential expressed *P. pinaster* miRNAs and predicted target genes.

miRNA	Exp. pattern	Log2FC	Target ID	Target annotation	GO terms/Pathways
DE miRNAs Inoculated vs Control					
ppi-miR166f	down	-0.623	unigene 8322	*protein TIFY 6B-like*	Plant hormone signal transduction
			unigene 942	*protein TIFY 6B*	Plant hormone signal transduction
ppi-miR947e	down	-1.214	unigene 105220	*protein TIFY 10A*	Plant hormone signal transduction
ppi-miRnovel43f	down	-1.541	unigene 26097	*transcription factor MYC4-like*	Plant hormone signal transduction
ppi-miR390b	down	-2.065	unigene 3146	*nematode resistance protein-like HSPRO1*	defence response
ppi-miR11565a-i	up	3.734 (±1.83)	isotig42 180	*WRKY transcription factor 20*	Plant-pathogen interaction
			unigene 650	*WRKY transcription factor 44*	Plant-pathogen interaction
ppi-miRnovel81 6	up	1.754	isotig42 166	*calcium-dependent protein kinase 28 (CPK28)*	Plant-pathogen interaction
ppi-miR11565h	up	5.948	unigene 12702	*disease resistance RPP13-like protein 4*	Plant-pathogen interaction
ppi-miR11458e	up	6.313	isotig49 219	*disease resistance protein At1g61300*	Plant-pathogen interaction
ppi-miR11458f	up	5.155	unigene 57660	*disease resistance protein RPS2*	Plant-pathogen interaction
ppi-miR3627u	up	5.053	isotig51 344	*disease resistance protein RPS2-like*	Plant-pathogen interaction
ppi-miR529l	up	5.121	unigene 116482	*probable RLK*	Plant-pathogen interaction
ppi-miR946j	up	5.178	isotig75 044	*disease resistance RPP13-like protein 4*	Plant-pathogen interaction
ppi-miR396j	up	4.923	unigene 75931	*disease resistance protein At4g27190-like*	Plant-pathogen interaction
			isotig42 452	*bifunctional levopimaradiene synthase (LPS)*	Diterpenoid biosynthesis
Novel_1871	up	1.887	unigene 31062	*bifunctional abietadiene synthase (AS)*	Diterpenoid biosynthesis
ppi-miR11436b, f-k, m	up	2.428 (±1.86)	unigene 2998	*bifunctional abietadiene synthase (AS)*	Diterpenoid biosynthesis
			unigene 9633	*bifunctional abietadiene synthase (AS)*	Diterpenoid biosynthesis
ppi-miR11436b, f-m	up	2.754 (±2.00)	isotig44 195	*4-hydroxy-3-methylbut-2-enyl diphosphate reductase*	Terpenoid backbone biosynthesis
ppi-miRnovel12 51	up	4.890	unigene 97227	*bifunctional levopimaradiene synthase (LPS)*	Monoterpenoid biosynthesis

ppi-miR3627s	up	5.761	isotig56835	*short-chain dehydrogenase/reductase 2b-like*	Monoterpenoid biosynthesis
ppi-miR11436b, f, g, j, k, m	up	2.725 (±2.11)	isotig34808	*peroxiredoxin Q*	Cell redox homeostasis
ppi-miR1314f	up	5.123	isotig25066	*peroxiredoxin-2E*	Cell redox homeostasis
ppi-miR3627l	up	3.980	isotig25066	*peroxiredoxin-2E*	Cell redox homeostasis
ppi-miR529c, y	up	4.383 (±2.91)	isotig12834	*thioredoxin F-type*	Cell redox homeostasis
DE miRNAs Resistance vs Susceptible					
ppi-miR166h	R > S	6.418	unigene67614	*putative RLK*	protein serine/threonine kinase activity
ppi-miR951f	R < S	-1.329	unigene93826	*putative RLK*	protein serine/threonine kinase activity
			unigene5558	*putative RLK*	protein kinase activity
ppi-miR947f	R > S	5.163	isotig45349	*GDP-L-fucose synthase 2*	Amino sugar and nucleotide sugar metabolism
Novel_110	R < S	-1.550	isotig51371	*protein COBRA-like*	cellulose microfibril organization
			unigene925	*Ninja family protein*	signal transduction
ppi-miR3627m	R < S	-3.661	unigene82871	*short-chain dehydrogenase reductase 2a-like*	oxidoreductase activity

Exp. Pattern – expression pattern; Log2FC – Log2(fold change); up – upregulated; down – downregulated; R – resistant; S - susceptible

Negative correlations of expression levels between miRNAs and predicted targets (Modesto et al., 2021), led to the detection of 37 target genes (Pearson's correlation R ≤ -0.65; Supplementary Table S3.7). After redundancy reduction, 24 GO terms were attributed to these target genes (Fig. 3.3b), including the BPs oxidation-reduction process, signal transduction, and the MF protein kinase activity. KEGG pathway terms (Kanehisa, 2019; Kanehisa and Goto, 2000) were attributed only to six of the target genes and included endocytosis, phagosome, amino sugar and nucleotide sugar metabolism, proteasome, lysine degradation, and pyrimidine metabolism (Supplementary Table S3.7).

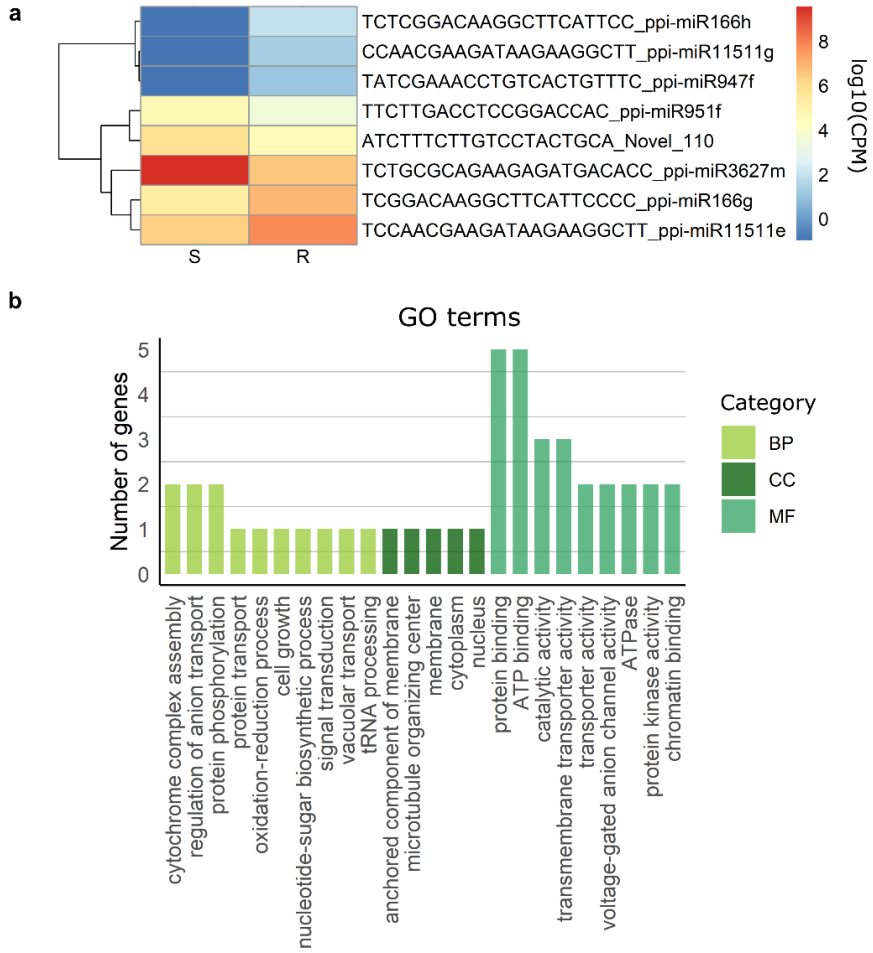


Figure 3.3. miRNAs differentially expressed between resistant (R) and susceptible (S) plants (a) and functional analysis of their target genes (b). (a) The heatmap represents average log10(CPM) values for each miRNA. **(b)** GO terms represented in the predicted target genes for the DE miRNAs. The y-axis represents the number of genes within each GO terms. BP biological process, CC cellular component, MF molecular function. Plots were generated with R 4.1.0 (https://cran.r-project.org/) pheatmap package (https://cran.r-project.org/web/packages/pheatmap/) and ggplot2 package (https://ggplot2.tidyverse.org/). Inkscape 1.1 (https://inkscape.org/) was used to assemble the final figure.

Within the target genes, it was possible to identify three *RLK*s (Table 3.3). One of these genes was targeted by miR166h, which was expressed at higher levels in resistant plants, while the other two were targeted by miR951f, which were both expressed at higher levels in susceptible plants. *GDP-L-fucose synthase 2* was targeted by miR947f, which was more expressed in resistant plants (Table 3.3). The miRNAs miR3627m and Novel_110, which showed increased expression in susceptible plants, targeted a gene with oxidoreductase activity and a COBRA protein-encoding gene, involved in cellulose deposition, respectively. Novel_110 also targeted a gene encoding for a Ninja family protein, which negatively regulates the JA defence response (Table 3.3).

3.3.4. Differentially expressed miRNAs and tasiRNA production

Several of the DE miRNAs here detected have been previously identified as leading to the production of tasiRNAs in *Picea abies* (Xia et al., 2015). These miRNAs targeted *NB-LRR* resistance genes, non-coding RNAs, and genes of unknown function. TasiRNAs commonly originate also from genes of the *pentatricopeptide repeat-containing protein* (*PPR*) family (Fei et al., 2013). Here, it was possible to identify targets with similar annotations for five miRNAs of the families miR947, miR3627, and miR11532 (Supplementary Table S3.8). Four of these transcripts were indeed predicted to originate sequences of tasiRNAs in the analysed *P. pinaster* samples. Three of these transcripts encode NB-LRR resistance proteins, targets of the miR11532 family, and one encodes a gene of unknown function, targeted by miR947f (Supplementary Table S3.8). Predicted targets of tasiRNAs included genes involved in plant hormone signal transduction, plant-pathogen interaction, and flavonoid biosynthesis pathways in all three groups of samples (Fig. 3.1d).

3.3.5. Investigation of miRNA mediated trans-kingdom interaction

As interactions between the miRNAs of parasites and the transcripts of their host plants have been previously reported (Brant and Budak, 2018a; Huang et al., 2016b; Rose et al., 2019), we searched for possible targets of PWN miRNAs in the *P. pinaster* transcriptome. Only predicted targets with an expression that correlated negatively with the expression of the PWN miRNAs were maintained. Remarkably, this led to the detection of 2515 target genes (Pearson's correlation $R \leq -0.65$; Supplementary Table S3.9).

Gene set enrichment analysis revealed 39 enriched GO terms after redundancy reduction (Supplementary Table S3.10) and included general BPs like protein refolding, protein phosphorylation, and RNA processing, as well as MFs such as ATP binding, transferase activity, and protein binding (Fig. 3.4a). On the other hand, some of the target genes seem to be involved in BPs more directly connected to plant defence response, such as isoprenoid biosynthetic process and regulation of abscisic acid-activated signalling pathway (Fig. 3.4a). The most represented pathways included spliceosome, ribosome, and mRNA surveillance pathway, but also plant hormone signal transduction, terpenoid backbone biosynthesis, and MAPK signalling pathway (Supplemental Table S3.9). The Pfam protein kinase domain was also significantly enriched (Supplemental Table S3.10).

The use of degradome data to further support the targeting of *P. pinaster* transcripts by PWN miRNAs allowed for the identification of 116 target regions (Supplementary Table S3.11). When applying stricter filters, such as selecting only regions with a score higher than three (more than one degradome read in the position, but lower coverage than the average of the corresponding transcript) or than two (coverage on the site is higher than the average of the corresponding transcript), 60 and 41 target regions, respectively, were still retained. From the 116 target regions, only 12 were predicted to be also targeted by *P. pinaster* sRNAs (Supplementary Table S3.12).

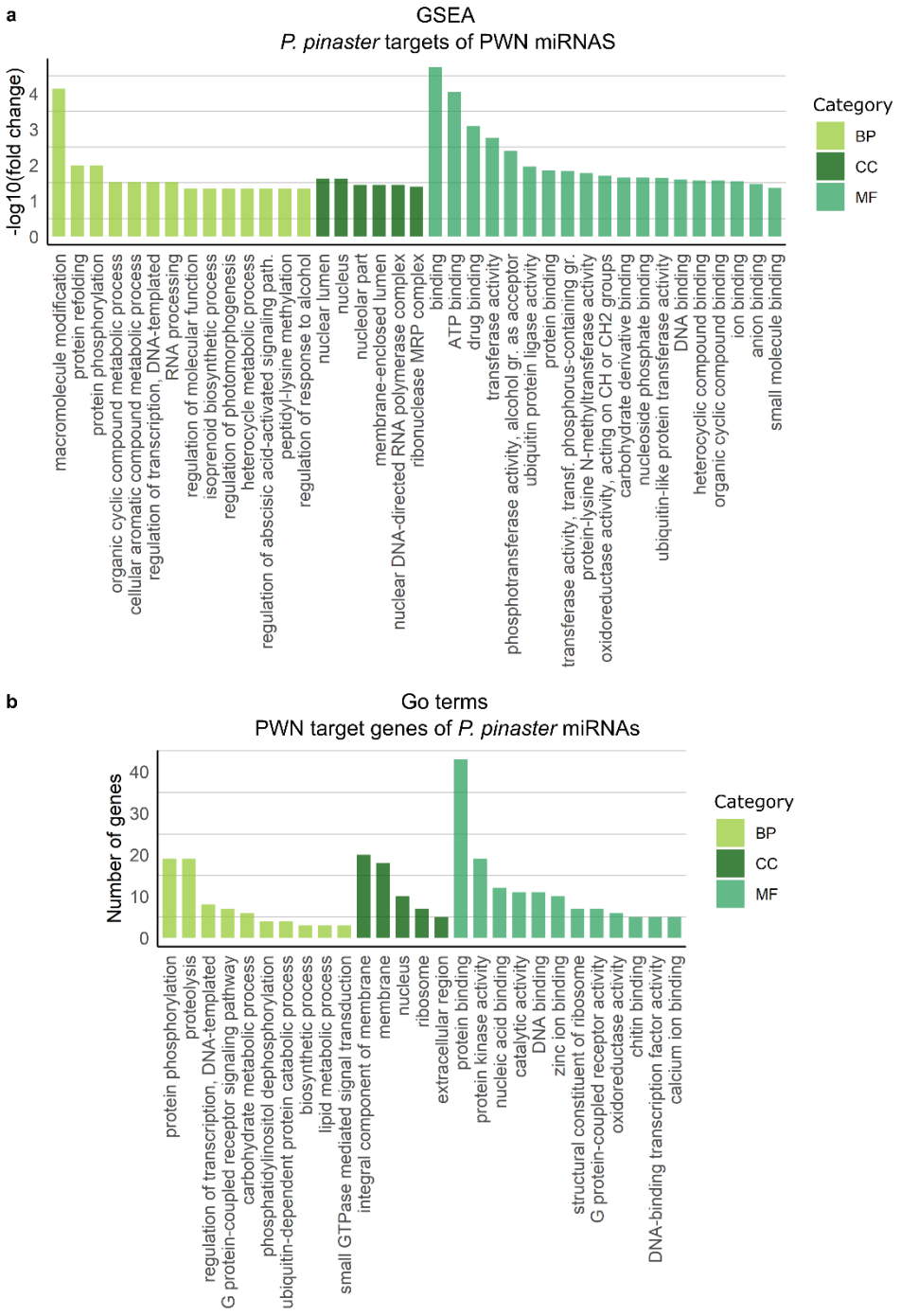

a
GSEA
P. pinaster targets of PWN miRNAS
-log10(fold change)
0
1
2
3
4
Category
BP
CC
MF
macromolecule modification
protein refolding
protein phosphorylation
organic cyclic compound metabolic process
cellular aromatic compound metabolic process
regulation of transcription, DNA-templated
RNA processing
regulation of molecular function
isoprenoid biosynthetic process
regulation of photomorphogenesis
heterocycle metabolic process
regulation of abscisic acid-activated signaling path.
peptidyl-lysine methylation
regulation of response to alcohol
nuclear lumen
nucleus
nucleolar part
membrane-enclosed lumen
nuclear DNA-directed RNA polymerase complex
ribonuclease MRP complex
binding
ATP binding
drug binding
transferase activity
phosphotransferase activity, alcohol gr. as acceptor
ubiquitin protein ligase activity
protein binding
transferase activity, transf. phosphorus-containing gr.
protein-lysine N-methyltransferase activity
oxidoreductase activity, acting on CH or CH2 groups
carbohydrate derivative binding
nucleoside phosphate binding
ubiquitin-like protein transferase activity
DNA binding
heterocyclic compound binding
organic cyclic compound binding
ion binding
anion binding
small molecule binding
b
Go terms
PWN target genes of P. pinaster miRNAs
Number of genes
0
10
20
30
40
Category
BP
CC
MF
protein phosphorylation
proteolysis
regulation of transcription, DNA-templated
G protein-coupled receptor signaling pathway
carbohydrate metabolic process
phosphatidylinositol dephosphorylation
ubiquitin-dependent protein catabolic process
biosynthetic process
lipid metabolic process
small GTPase mediated signal transduction
integral component of membrane
membrane
nucleus
ribosome
extracellular region
protein binding
protein kinase activity
nucleic acid binding
catalytic activity
DNA binding
zinc ion binding
structural constituent of ribosome
G protein-coupled receptor activity
oxidoreductase activity
chitin binding
DNA-binding transcription factor activity
calcium ion binding

Figure 3.4. Functional analysis of the predicted target genes of miRNAs possibly involved in trans-kingdom interaction. (a) Gene set enrichment analysis (GSEA) of GO terms represented in *P. pinaster* predicted targets for PWN miRNAs. The y-axis represents the significance of pathway enrichment (-log10 of corrected p-values). **(b)** GO terms represented in PWN predicted target genes for *P. pinaster* miRNAs. The y-axis represents the number of genes within each GO terms. Plots were generated with R 4.1.0 (https://cran.r-project.org/) ggplot2 package (https://ggplot2.tidyverse.org/). Inkscape 1.1 (https://inkscape.org/) was used to assemble the final figure.

Target genes identified in the degradome have GO annotations ranging from photosynthesis, structural constituent of ribosome, and ATP binding (Supplementary Table S3.13) to defence response to fungus and oxidation-reduction process. Target genes with known roles in plant defence response included *thaumatin-like proteins*, *PR-4*, *RLK*, genes involved in flavonoid biosynthesis (*chalcone synthase 1* and *caffeoyl-CoA O-methyltransferase*), and *thioredoxin H4-1*, involved in cell redox homeostasis (Supplementary Table S3.11).

Trans-kingdom interactions through sRNAs have been described in both directions, this is, sRNAs from plants may also target pathogens or parasites genes (Brilli et al., 2018; Zhang et al., 2016). Therefore, targets for *P. pinaster* miRNAs DE between susceptible and resistant plants were predicted in the PWN transcriptome and led to the identification of 552 target regions in 487 PWN genes (Supplementary Table S3.14). Analysis of the targets' GO annotations and pathways (Fig. 3.4b, Supplementary Table S3.15) reveal that *P. pinaster* miRNAs may target genes important for PWN response to stimuli (e.g. MF protein kinase activity; BP G protein-coupled receptor signalling pathway), transcriptional response (e.g. BP regulation of transcription, DNA-templated; pathways spliceosome and ribosome), detoxification of plant xenobiotic compounds (e.g. MF oxidoreductase activity; pathway metabolism of xenobiotics by cytochrome P450), and digestion of plant tissues (e.g. BPs proteolysis and carbohydrate metabolic process; pathways lysosome or protein digestion and absorption).

3.4. Discussion

The importance of miRNAs in plant response to biotic and abiotic stresses has been repeatedly demonstrated in the last years (Rose et al., 2019; Song et al., 2019). Several studies have shown an important regulatory role of miRNAs in plant response to parasitic nematodes (Jaubert-Possamai et al., 2019). However, the role of miRNAs in the defence response to PWN has not been previously reported and few studies focussed on defence response in conifer species (Krivmane et al., 2020; Lu et al., 2007). The expression of miRNAs after PWN inoculation was previously analysed in *P. massoniana* (Xie et al., 2017), but this analysis was made in needles to study regulation of plant growth and no insights are currently available regarding the post-transcriptional regulation of genes or pathways possibly involved in defence response against PWN. In this study, we investigated the role of miRNAs in the regulation of *P. pinaster* defence response to PWN inoculation, explored their involvement in resistance to PWD and, finally, identified miRNAs that may have an important role in sRNA mediated trans-kingdom interaction.

MiRNAs can regulate gene expression by mRNA cleavage or translation inhibition (Yu et al., 2017). In plants, the most common mechanism is target cleavage (Yu et al., 2017), in which case the expression of a miRNA and its respective targets is expected to correlate negatively. Taking this into account, we combined the miRNA data here obtained with mRNA expression data of the same samples previously described in Modesto *et al.* (2021). This approach allowed us to narrow down significantly an extensive list of possible gene targets and increase the reliability of the final targets list. RT-qPCR results supported a strong negative correlation between the expression of two of the five miRNA-target gene pairs tested. For the remaining pairs, it is possible that the expression of other predicted targets not tested here may show high negative correlation, but also alternative miRNA regulatory mechanisms beyond mRNA target cleavage

should not be excluded. A more extensive testing would be necessary to have additional insights into the miRNA-target regulatory relation.

Analysing the DE miRNAs between inoculated and control samples, it was possible to identify a set of *P. pinaster* miRNAs involved in response to PWN inoculation. Several of the conserved miRNAs families have been described as involved in response to root-knot nematode or cyst nematode in Arabidopsis (Hewezi et al., 2008), cotton (Cai et al., 2021), tomato (Koter et al., 2018) and soybean (Jaubert-Possamai et al., 2019; Tian et al., 2017), including miR159, miR390, miR396, miR164, miR166, and miR3627. In such interactions, the expression of these miRNAs has been associated with cyst or gall formation. As PWN life strategy is different from sedentary nematodes, and their survival does not depend on the formation of those specialised feeding structures, the role of these miRNAs in response to PWN is likely different. The predicted targets having a negatively correlated expression with these miRNAs were, in fact, distinct from what is described in the literature (Cai et al., 2021; Hewezi et al., 2008; Jaubert-Possamai et al., 2019; Koter et al., 2018; Tian et al., 2017). For instance, while several MYB transcription factors were predicted for miR159, as described for other nematode-plant interactions (Jaubert-Possamai et al., 2019), the expression of the miRNAs and respective target transcripts were not negatively correlated. This suggests that miR159, as well as the other mentioned conserved miRNAs, regulate different genes and pathways in *P. pinaster* response to PWN, when compared to the response to sedentary nematodes in angiosperms.

Several of the identified PWN responsive miRNAs were previously described as involved in *P. taeda* response to fusiform rust (Lu et al., 2007). This includes the conserved families miR159, miR166, miR171, miR390 or miR396, and Pinaceae specific conserved families miR946, miR947, miR951, and miR952. However, the *P. pinaster* targets here predicted for these miRNAs were different from *P. taeda* targets or were not negatively correlated with the corresponding miRNA expression. Therefore, although

the miRNAs involved in response to pathogens and parasite nematodes seem to be partly conserved, both between angiosperms and gymnosperms, as well as between these two closely related *Pinus* species, they may regulate different defence mechanisms. The defence mechanisms induced by biotrophic pathogens, such as fusiform rust fungus or sedentary nematodes, and migratory nematodes or herbivore insects are often described as antagonistic (Caarls et al., 2015).

Part of the miRNA families here detected as differentially expressed after PWN inoculation were also responsive to drought stress in *P. pinaster* (Perdiguero et al., 2020) (miR159, miR164, miR166, miR169, miR396, miR529, miR1313, miR3627, miRnovel578), suggesting these families may have a role in the regulation of stress responses in general.

In this work, we showed that some of the pathways previously pointed out as relevant for *P. pinaster* response to PWN inoculation (Gaspar et al., 2017; Modesto et al., 2021; Rodrigues et al., 2021) seem to be post-transcriptionally regulated by miRNAs. These include plant hormone signalling pathways, of which the JA response pathway is highlighted. The induction of JA immunity has been earlier reported in *P. pinaster* in response to PWN (Gaspar et al., 2017; Modesto et al., 2021; Rodrigues et al., 2021) and associated with resistance (Modesto et al., 2021). Several miRNAs here observed to be responsive to PWN infection or associated with resistance (miR947 and miR951), belong to families previously described as responsive to methyl-jasmonate (MeJA) treatment in *Taxus chinensis* (miR164, miR169, miR390, miR396) (Qiu et al., 2009) or *Pinus sylvestris* (miR946, miR947, miR951, miR952) (Krivmane et al., 2020). Additionally, miRNAs of the families miR166 and miR947 seem to target *JAZ/Tify* transcription factors, which repress JA response, while the novel miRNA miRnovel43f seem to target *MYC4* transcription factor, which induces JA response (Wasternack and Song, 2017). The downregulation of miR166, miR947, and miRnovel43f suggests their expression is inhibited by higher levels of JA (Modesto et al., 2021), inducing the expression of

their targets. The DE of the miRNA Novel_110 and respective target, the JA defence response regulator *NINJA*, between resistant and susceptible plants indicates that this hormone has also an important role in *P. pinaster* resistance to PWN, as previously suggested (Modesto et al., 2021). JA immune response seems to be, therefore, tightly regulated during *P. pinaster* response to PWN, both at the transcriptional and post-transcriptional levels, and the results here obtain further support its important role in resistance to this nematode.

Several of the identified targets of DE miRNAs were *RLK*s or *RLP*s, involved in the activation of PTI. Noticeably, when comparing susceptible and resistant plants, the two miRNAs targeting different *RLK* genes had contrasting expression patterns, with miR166h more expressed in susceptible plants and miR951f more expressed in resistant plants. The different post-transcriptional regulation of the targeted *RLKs* in susceptible and resistant plants may lead to the activation of distinct defence pathways. A contrasting differential expression of RLK/RLP encoding genes in resistant and susceptible plants has been previously associated with *P. pinaster* resistance to PWN (Modesto et al., 2021).

ROS detoxification has been described as an important part of plant defence response (Holbein et al., 2016; Miller et al., 2017) and in particular in *Pinus* spp response to PWN infection (Gaspar et al., 2017; Hirao et al., 2012; Liu et al., 2017; Modesto et al., 2021). In this work, several genes involved in maintaining cell redox homeostasis, as *peroxiredoxins* and *thioredoxins*, seem to be regulated by miRNAs induced after PWN inoculation, supporting the importance of this mechanism in *P. pinaster* defence response to PWN. In susceptible plants, higher expression levels were observed for miR3627m, which targets a gene encoding for a protein with oxireductase activity, suggesting that susceptible plants might have lower ROS detoxification ability when compared to resistant plants. A better and more prolonged ROS detoxification was associated with PWN resistance in *P. Massoniana* (Liu et al., 2017), while higher expression of

oxidative stress response genes was observed in resistant *P. thunbergii* (Hirao et al., 2012) and *P. pinaster* (Modesto et al., 2021).

Terpenoids are important compounds in *Pinus* spp defence against several pests (Keeling and Bohlmann, 2006). Multiple genes encoding enzymes involved in terpenoid biosynthesis pathways were targeted by DE miRNAs, highlighting the importance of these compounds in response to PWN. Increased expression of terpene synthases, including *AS* and *LPS* genes, has been previously reported in *P. pinaster* (Modesto et al., 2021) and *P. massoniana*(Liu et al., 2017) response to PWN and associated with PWN resistance. Moreover, the products of two *P. massoniana* terpene synthases, α-pinene and longifolene, directly inhibited the survival rate of PWN in vitro (Liu et al., 2020), reinforcing the importance of these compounds in plant response and resistance to PWN.

The role of L-fucose biosynthesis and protein fucosylation in plant defence response has been recently highlighted in Arabidopsis (Zhang et al., 2019). In Arabidopsis, fucosylation of RLKs/RLPs was found to be essential for the normal activation of PTI and ETI. Interestingly, miR947f, differentially expressed between susceptible and resistant plants, seem to target a *GDP-L-fucose synthase*. The post-transcriptional regulation of a *GDP-L-fucose synthase* points to a relevant role of fucosylation in achieving resistance to PWN. Additional studies may clarify if an earlier activation of this gene is detected in resistant plants prior to the 72hpi for the fast activation of PTI upon inoculation.

In recent years, evidence for trans-kingdom transference of sRNAs has been accumulating (Lefebvre and Lécuyer, 2017; Rose et al., 2019), including in host-pathogen and host-parasite interactions. In plants, examples of sRNA transfer between plant and pathogenic fungi or oomycetes have been reported (Brilli et al., 2018; Wang et al., 2016; Weiberg et al., 2013; Zhang et al., 2016). For instance, *Botrytis cinerea* miRNAs targeted and silenced Arabidopsis transcripts with important roles in plant immunity, such as *MAPK*s and *WRKY* transcription factors (Wang

et al., 2017; Weiberg et al., 2013). Transference of miRNAs from plant to pathogen has also been reported (Brilli et al., 2018; Rose et al., 2019; Zhang et al., 2016). *Gossypium hirsutum* miR166 and miR159 were transferred to the fungus *Verticillium dahlia*, targeting genes essential for the virulence of this fungus (Zhang et al., 2016). Furthermore, bidirectional sRNA transfer and trans-kingdom transcript cleavage was described in the interaction between the oomycete *Plasmopara viticola* and grapevine (Brilli et al., 2018). Therefore, sRNA transference between pathogens and plant hosts seems to be an important strategy both for plant defence and resistance, as well as for pathogen virulence. Here, we report several *P. pinaster* transcripts predicted as targets of PWN miRNAs. The silencing or downregulation of many of these target genes, such as transcriptional factors, RNA processing genes, ribosomal proteins or protein folding genes, may negatively affect the plant cell transcriptional response, as well as protein synthesis and correct assembly. On the other hand, several of the predicted targets are directly involved in plant immune response, such as genes involved in plant hormone signal transduction, terpenoid backbone biosynthesis, and MAPK signalling. The simultaneous targeting of genes important for protein synthesis, synthesis of toxic compounds, as well as early initiation and onset of the plant immune response, can affect the plant capacity to set a timely and appropriate defence response to PWN and therefore may be essential for the virulence of this nematode. The silencing of *P. pinaster* transcripts by PWN was supported by degradome data obtained from similar *P. pinaster* samples inoculated with the same PWN strain and collected at the same timepoint. Accordingly, it was possible to validate several *P. pinaster* targets using this approach. A very small number of the target sites predicted using degradome data were also predicted as target sites for *P. pinaster* miRNAs, supporting that the cleavage was guided by PWN miRNAs for most of the predicted targets.

In the opposite direction, the targeting of PWN genes by *P. pinaster* sRNAs, several interactions were also predicted. Contrasting with plants, post-transcriptional regulation in animals commonly involves the inhibition of

translation of the targeted transcripts, and not their cleavage (Gebert and MacRae, 2019). In this way, validating this interaction is not possible through degradome analysis. Nevertheless, analysing PWN genes targeted by *P. pinaster* miRNAs differentially expressed between resistant and susceptible plants may give us important information about resistance mechanisms. The miRNAs differentially expressed between resistant and susceptible plants were predicted to target several genes expressed in PWN pharyngeal gland cells and intestine (Espada et al., 2016), several of which encode proteins previously detected in PWN secretome (Shinya et al., 2013). These genes are important for PWN evasion of plant defence response, PWN migration through plant tissues, and feeding. For instance, genes like *cytochrome P450* or *epoxide hydrolase* encode enzymes that degrade xenobiotic compounds produced by the plant host, allowing for the PWN to survive in the hostile environment. On the other hand, *peptidases* may be involved in the degradation of plant defence proteins and the digestion of plant tissues, which allows for migration throughout the plant and nutrients uptake, but can also be essential for embryogenesis and larval development (Kikuchi et al., 2011). Lysosomal enzymes may also play an important role in the digestion of ingested proteins in PWN, as it was observed in *C. elegans* intestine-specific secondary lysosomes (Kikuchi et al., 2011). In this way, the differential targeting of these genes by *P. pinaster* miRNAs in resistant and susceptible plants may affect PWN survival and development, contributing to the contrasting observed phenotypes.

Although no naturally occurring transference of sRNAs has been described in nematode-plant interactions, host-induced gene silencing (HIGS) has been shown to be an efficient method to manage these parasites (Lefebvre and Lécuyer, 2017; Rose et al., 2019). This strategy involves the engineering of plant hosts to express RNA interference (RNAi)-inducing dsRNA that target and silence, in this case, nematode genes important for their growth, development or pathogenicity (Ghag, 2017). For instance, the transformation of potato plants (*Solanum tuberosum*) with an RNAi

construct complementary to a root-knot nematode (*Meloidogyne chitwoodi*) effector gene increased plant resistance to this nematode (Dinh et al., 2014). In soybean, RNAi constructs targeting two genes potentially essential to root-knot nematode (*Meloidogyne incognita*) survival restricted greatly the number of galls formed in the plant roots (Ibrahim et al., 2011). Therefore, the uptake by the nematode of these dsRNAs or RNAi produced by the host conferred resistance in the transgenic plants. Although the process of sRNA translocation between organisms is not yet clear, sRNAs or sRNA-protein complexes seem to be more likely transported by extracellular vesicles (Lefebvre and Lécuyer, 2017; Rose et al., 2019). Trans-kingdom RNA silencing can open new perspectives of fighting PWN through the development of HIGS, which was shown to be an ecological and efficient method for parasite management (Lefebvre and Lécuyer, 2017; Rose et al., 2019).

In conclusion, this work provides new insights into the relevance of post-transcriptional regulation in *P. pinaster*-PWN interaction during the early stages of infection. The set of candidate miRNA-target nodes identified here represents an important foundation for future functional characterization studies in the context of PWD and PWN resistance. Furthermore, a possible role for trans-kingdom miRNA transfer and gene silencing was revealed, both for PWN parasitism and *P. pinaster* resistance. Although degradome analysis experimentally supported the silencing of *P. pinaster* genes by PWN miRNAs, further experimental work confirming the transference of miRNAs between organisms, the physical interaction between miRNA-target genes and subsequent gene silencing, would be of great relevance to better understand the significance of this bidirectional interaction in PWD and PWN resistance.

3.5. Materials and Methods

3.5.1. Plant material and PWN inoculum

The *P. pinaster* half-sib family 440 was previously evaluated regarding the genetic effects on survival after PWN inoculation of 2-year-old plants (Carrasquinho et al., 2018), showing a predicted survival mean of 15% (in a range of 6%-23%). Seeds, provided by Dr. Isabel Carrasquinho (INIAV, Portugal), were collected from the mother tree 440, which is included in the reference population for PWD resistance (Ribeiro et al., 2012), located in "Herdade da Comporta" (38◦21′28.52″N, 8◦45′49.89″W) in southern Portugal. The necessary permissions were obtained for the collection and use of the seeds. Relevant institutional, national, and international guidelines for plant material collection and experimental work were followed. Four-year-old plants, germinated from the collected seeds, were maintained in 4L pots in a greenhouse and placed according to a completely randomized experimental design.

B. xylophilus isolate Bx013.003 (Carrasquinho et al., 2018; Modesto et al., 2021; Rodrigues et al., 2021), obtained from an infected *P. pinaster* tree in a field in central Portugal (39°43'33.8''N, 9°01'55.7''W) and included in INIAV's Nematology Laboratory collection (Oeiras, Portugal), was used for the inoculation assay. The sequence of the ITS region of this isolate is available in GenBank (NCBI) with the accession number MF611984.1. PWNs were maintained in culture at 25±1°C on a non-sporulating *Botrytis cinerea* strain grown on autoclaved barley grains. Previous to inoculation, nematodes were allowed to grow on sterilized wood and then isolated using the "tray" method (Whitehead and Hemming, 1965). Nematodes were suspended in water at a concentration of 1000 PWN/mL.

3.5.2. Inoculation with PWN, sample collection, and evaluation of symptoms

Twenty-three plants were inoculated in September 2016 using the method described in Futai and Furuno (1979). Eighteen plants were inoculated with a suspension of 500 nematodes at mixed developmental stages, while five control plants were inoculated with sterile water. The inoculum was pipetted into a small longitudinal wound made in the main stem with a sterile scalpel below the apical shoot region (Modesto et al., 2021). Stem samples of approximately 5 cm, including the inoculation zone, were collected 72 hpi and immediately frozen in liquid nitrogen. The remaining part of each plant, below the inoculation zone, was kept in the greenhouse and observed weekly for 210 days. The progression of symptoms was registered by classifying the plants on a scale of 0 (no visible symptoms) to 4 (more than 75% of needles brown/wilted) in each observation point (Table 3.1). The first symptoms were observed 14 days post-inoculation (dpi) and evolved progressively until the end of the experiment. Plants that presented symptoms (1-4 on the scale) were classified as susceptible, while plants that did not present any symptoms (0 on the scale) were classified as resistant. This classification was based on external symptoms only and it is unknown if PWN multiplication was impaired in plants without symptoms, showing true resistance, or if plants maintained a healthy phenotype despite PWN multiplication, showing tolerance instead (Trudgill, 1991).

3.5.3. RNA extraction and sRNA sequencing

Five resistant, four susceptible, and four control plants were selected for sequencing. The four chosen susceptible plants were the first presenting the maximum level of symptoms (level 4). Total RNA, including the small RNA fraction, was extracted from stem samples after debarking using the method described in Le Provost *et al.* (2007). RNA and miRNA concentrations were determined using Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA USA) with the RNA BR Assay Kit and

miRNA Assay Kit. RNA integrity was checked with LabChip GX (PerkinElmer, Hopkinton, MA USA). Libraries were prepared with the Illumina TruSeq Small mRNA protocol and sequenced on Illumina HiSeq 2500 (Fasteris, Switzerland), providing 50 bp single-end reads. Each sample was run in two independent lanes.

3.5.4. Identification of small RNAs and differential expression analysis

The quality of the small RNA-seq data was checked using FastQC v 0.11.4(Andrews, 2010). Adapter and quality trimming was performed using Trimmomatic (Bolger et al., 2014). As samples included *P. pinaster* and PWN RNA, to be able to distinguish between sequences originating from each organism reads were mapped to *Pinus taeda* (Zimin et al., 2017) and PWN (Kikuchi et al., 2011) genomes using BWA alignment software v0.7.17 (BWA-backtrack algorithm) (Li and Durbin, 2009). Separate fastq files were prepared with reads originating from plant or nematode.

Reads were then processed with the sRNA analysis pipeline miRPursuit (Chaves et al., 2017). In an initial step, data was filtered to remove t/rRNAs, low complexity reads, reads with an absolute abundance <= 5, and reads outside the range of 18-26 nucleotides. For *P. pinaster* originating reads, conserved miRNAs were identified by comparing the reads with mature plant miRNAs from the miRBase v22 database (www.mirbase.org), allowing for up to 3 mismatches. Novel miRNAs and tasiRNAs were predicted using default parameters. For PWN, conserved reads were annotated by comparing with previously described PWN miRNAs (Ding et al., 2015), allowing for the maximum of 2 mismatches. Novel miRNAs were predicted using a minimum hairpin length of 50.

Differential expression analysis was performed for *P. pinaster* miRNAs using DESeq2 (Love et al., 2014) with a 0.05 false discovery rate (FDR) threshold. To identify miRNAs responsive to PWN inoculation, inoculated plants were compared to control plants, while to identify miRNAs possibly involved in resistance, susceptible samples were compared to resistant

ones. CPMs (Counts Per Million) were calculated for each sample by normalizing against the total number of reads in each library and multiplying by a factor of 10^6. These CPMs were used to create expression heatmaps in R v4.1.0 (https://www.R-project.org/).

3.5.5. Target prediction and enrichment analysis

Prediction of miRNA targets in *P. pinaster* was performed using the online tool psRNTarget (Dai et al., 2018) with default parameters (except for *HSP size* = 18), and *P. pinaster* transcriptome, containing only transcripts with predicted coding sequences (Modesto et al., 2021). As mRNA transcription data was available for the same samples as the ones analysed in this paper (Modesto et al., 2021), it was possible to correlate the expression of the sRNAs and their predicted target genes. Pearson correlations were calculated using R and only pairs of sRNA-targets with expressions negatively correlated ($R < -0.65$) were retained. Targets were predicted for *P. pinaster* miRNAs and tasiRNAs, as well as PWN miRNAs.

To validate the targeting of *P. pinaster* transcripts by PWN miRNAs, degradome sequencing data available in European Nucleotide Archive (ENA) database (PRJEB48279) was used. These data consist of two libraries containing a pool of RNA extracted from stem samples of four resistant and four susceptible *P. pinaster* samples at 72 hpi. Although these samples belong to a different family than the one used in the present study, family 465 (Carrasquinho et al., 2018), the inoculum used and the collection timepoint were the same and therefore, variation in PWN miRNA expression is expected to be low. Degradome sequencing data, PWN detected miRNAs, and *P. pinaster* transcriptome (Modesto et al., 2021) were used as input for CleaveLand4 v4.5 (Addo-Quaye et al., 2009) to detect cleaved sRNA targets.

Target genes were predicted in PWN using miRanda v3.3a (Enright et al., 2004), with a minimum score of 120 and maximum energy of -20. For this analysis, only 3'UTR sequences were used (up to 800bp upstream from the

predicted coding sequences), as in animals miRNAs target primarily these regions, and not the entire gene (Merritt et al., 2008). Targets were predicted for *P. pinaster* DE miRNAs.

PWN genome was functionally annotated by aligning sequences with NCBI RefSeq Invertebrate database (accessed May 2021) using BLASTx in DIAMOND v2.0.9 (Buchfink et al., 2021). InterProScan was used to attribute gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa, 2019; Kanehisa and Goto, 2000). KEGG annotation was further improved by using KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007). GO and KEGG annotations for *P. pinaster* transcriptome were obtained from Modesto *et al.* (2021).

For the predicted target genes, for both *P. pinaster* and PWN, gene set enrichment analysis was performed with BiNGO plugin (Maere et al., 2005) for Cytoscape (Shannon et al., 2003), using the hypergeometric statistical test and Benjamini and Hochberg FDR for multi testing correction (*p*-value ≤0.05). Gene ontology redundancy was reduced using the online tool Revigo (Supek et al., 2011) with a trim threshold of 50%. Pathway enrichment analysis and Pfam enrichment analysis were made with BiNGO using the same parameters as described above.

3.5.6. RT-qPCR

Five *P. pinaster* miRNAs DE between inoculated and control samples were selected for expression profile validation, together with five predicted target genes negatively correlated with these miRNAs, according to previously published RNA-seq data (Modesto et al., 2021). cDNA of three resistant, three susceptible, and three control samples was synthesized using Mir-X miRNA First-Strand Synthesis Kit (Takara Bio, USA). Forward primers were manually designed to match the entire sequence of the miRNA to be amplified (Supplementary Table S3.16), while the reverse primer used was the universal mRQ 3' primer supplied with the kit. For the target genes, primers were designed with PerlPrimer v1.1.21 (Marshall, 2004)

(Supplementary Table S3.16). RT-qPCR was run in a LightCycler 480 Instrument II (Roche, Switzerland) using SYBR Green I Master (Roche) and the following conditions: 5min at 95°C, 40 cycles of 95°C for 10 s, 61–66°C for 15 s (Supplementary Table S3.16), and 72◦C for 12 s. Primer specificity was monitored by analysing the melting curves. Three technical replicates were performed for each biological replicate. Expression profiles were normalized using *5S rRNA* as a reference for miRNAs, while *actin*, *40S rRNA* (Pascual et al., 2015) and *histone H3* (de Vega-Bartol et al., 2013) were used for the target genes. Relative expression levels were calculated with the Pfaffl method (Pfaffl, 2001). Pearson's correlation coefficient was calculated between RNA-seq and RT-qPCR expression levels [Log2(fold change)] in R, for both miRNAs and target genes. Correlation analysis was also performed between the RT-qPCR expression levels [Log2(fold change)] of miRNAs and respective predicted target genes. Significance of these results was obtained through a correlation test (*t*-test) in R.

3.6. Data availability

The sequence data for this study has been submitted to the European Nucleotide Archive (ENA) under accession number PRJEB48441 (https://www.ebi.ac.uk/ena/browser/view/PRJEB48441).

3.7. Acknowledgements

We thank Lurdes Inácio (INIAV) for providing the nematode cultures, Isabel Carrasquinho (INIAV) for the plant material and Hugo Matias (ITQB NOVA) for all the technical support in the greenhouse. This work was supported by Fundação para a Ciência e a Tecnologia, I.P. (FCT/MCTES), through Grants GREEN-it (UID/ Multi/04551/2013), BioISI (UIDB/04046/2020 and UIDP/04046/2020) and the doctoral fellowship SFRH/ BD/111687/2015 (to IM). Support was also provided by FCT/MCTES through national funds (PIDDAC) and co-financed by Fundo Europeu de Desenvolvimento Regional (FEDER) of the EU, through Programa Opera- cional Regional de

Lisboa do Portugal 2020 or other programs that may succeed - PTDC/BAA-MOL/28379/2017, LISBOA-01-0145-FEDER-028379.

3.7. Author contributions

I.M. and C.M.M. conceived the study and designed the experiments. I.M. performed the RNA extractions and data analysis. V.I. performed the RT-qPCR experiments. I.M. and C.M.M. interpreted data and prepared the manuscript. All authors discussed the results and reviewed the manuscript.

3.8. Competing interests

The authors declare no competing interests.

3.9. References

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3.10. Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-09163-3.

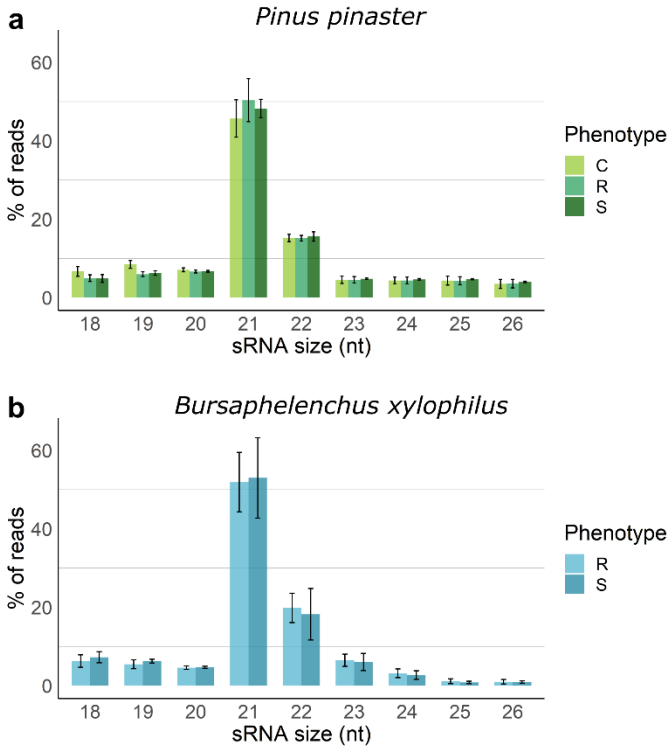

Figure S3.1. Sizes of *P. pinaster* (a) and PWN (*B. xylophilus*) (b) sRNAs after filtering the reads, in resistant (R), susceptible (S), and control (C) samples. The y-axis represents the percentage of reads in each size (in nucleotides, nt) category. Plots were generated with R 4.1.0 (https://cran.r-project.org/) ggplot2 package (https://ggplot2.tidyverse.org/). Inkscape 1.1 (https://inkscape.org/) was used to assemble the final figure.

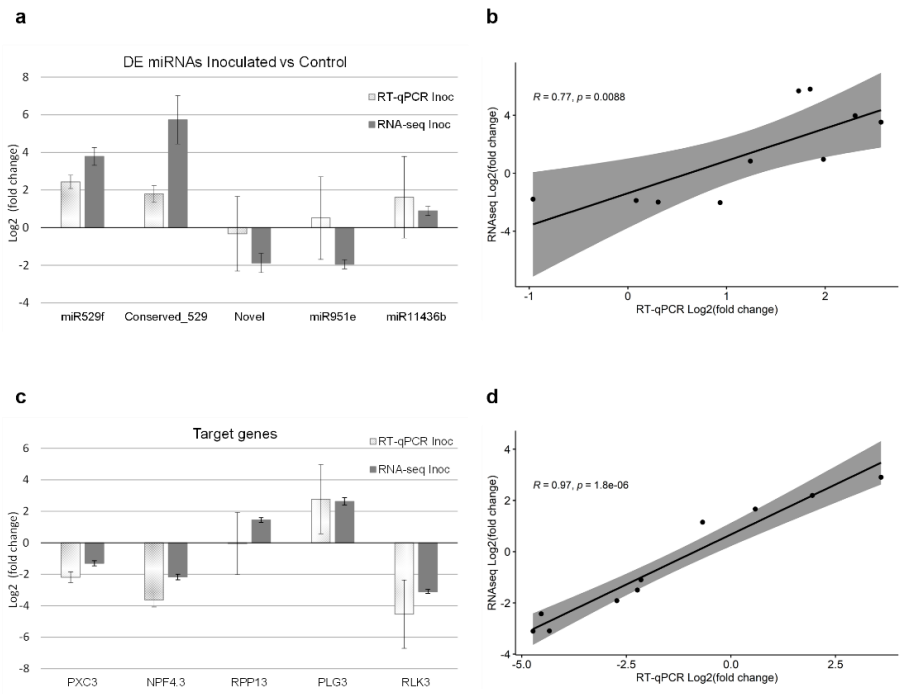

Figure S3.2. . RT-qPCR analysis of 5 DE miRNAs and 5 predicted target genes. (a)(c) Bars represent differential expression levels, in Log2(fold change), of inoculated plants in comparison with controls. Results from both the RNA-seq analysis (filled color) and the RT-qPCR analysis (dots) are displayed. Error bars represent the standard error of the biological replicates used for RNA-seq (4–5) and RT-qPCR (3). **(b)(d)** Pearson's correlation analysis of expression levels [Log2 (fold change)] between RNA-Seq and RT-qPCR. Plots were generated with Image generated with Microsoft Office Excel and with R 4.1.0 (https://cran.r-project.org/) ggplot2 package (https://ggplot2.tidyverse.org/). Inkscape 1.1 (https://inkscape.org/) was used to assemble the final figure.

Table S3.1. Summary of read mapping, filtering and processing for each library and in average. Sus - susceptible; Res - resistant; Cont - control; Inoc - Inoculated; Pta - *Pinus taeda*; Bxy - *Bursaphelenchus xylophilus* (PWN). (available online)

Table S3.2. Expression values (LogCPM, Counts Per Million) for each miRNA identified from *P. pinaster* and differential expression analysis (DEA) results [Log2(fold change)]. Sus - susceptible; Res - resistant; Cont - control. (available online)

Table S3.3. PWN miRNAs identified in inoculated samples and expression values (LogCPM, Counts Per Million). Sus - susceptible; Res - resistant. (available online)

Table S3.4. *P. pinaster* target genes predicted by psRNATarget for *P. pinaster* miRNAs differentially expressed between inoculated and control samples.

Pearson's R for the correlation of expression values between miRNAs and respective target genes is also represented. (available online)

Table S3.5. GO and KEGG terms represented in *P. pinaster* predicted target genes, with the number of genes withing each term, and Pfam enriched terms. Pfam enrichment analysis was performed using the hypergeometric statistical test and results were filtered for an adjusted p-value ≤ 0.05 (FDR). (available online)

Table S3.6. Correlation test results between the RT-qPCR expression [Log2(fold change)] of 5 miRNAs and RT-qPCR expression [Log2(fold change)] of one of the predicted target genes for each miRNA.

miRNA	Target gene	Pearson's R	*p*-value
ppi-miR529f	*PXC3*	0.56	0.440
Conserved_529	*NPF4.3*	0.26	0.740
Novel	*RPP13*	-0.78	0.066
ppi-miR951e	*PGL3*	-0.32	0.530
ppi-miR11436b	*RLK3*	-0.61	0.200

Table S3.7. *P. pinaster* target genes predicted by psRNATarget for *P. pinaster* miRNAs differentially expressed between resistant and susceptible samples. Pearson's R for the correlation of expression values between miRNAs and respective target genes is also represented. (available online)

Table S3.8. Selected miRNAs and targets that may originate tasiRNAs in *P. pinaster*.

miRNA	Expression pattern	Target ID	Target annotation
DE miRNAs Inoculated vs Control			
ppi-miR11532a,i,h	upregulated	isotig15594*	*TMV resistance protein N-like*
ppi-miR11532a,h	upregulated	unigene24594*	*TMV resistance protein N*
ppi-miR11532a,i	upregulated	isotig27040*	*putative disease resistance protein RGA4*
ppi-miR11532a	upregulated	isotig45245	*pentatricopeptide repeat-containing protein*
ppi-miR11532h	upregulated	isotig44118	*pentatricopeptide repeat-containing protein*
		unigene43066	*pentatricopeptide repeat-containing protein*
ppi-miR3627u	upregulated	unigene16345	*pentatricopeptide repeat-containing protein*
DE miRNAs Resistance vs Susceptible			
ppi-miR947f	upregulated	isotig34172*	*unknown*

*Transcripts that were predicted to originate tasiRNAs in the analysed *P. pinaster* samples.

Table S3.9. *P. pinaster* target regions predicted by psRNATarget for PWN miRNAs expressed in inoculated samples. Pearson's R for the correlation of expression values between miRNAs and respective target genes is also represented. (available online)

Table S3.10. GO and Pfam terms enriched in *P. pinaster* predicted target genes for PWN miRNAs after trimming for redundancy, and KEGG terms represented with the number of genes withing each term. Gene set and Pfam enrichment analyses were performed using the hypergeometric statistical test and results were filtered for an adjusted p-value ≤ 0.05 (FDR). (available online)

Table S3.11. *P. pinaster* target regions predicted by Cleveland4 for PWN miRNAs expressed in inoculated samples. Target regions predicted also as targets of *P. pinaster* miRNAs are highlighted in yellow (see Supplementary Table S12). (available online)

Table S3.12. *P. pinaster* target regions predicted by Cleveland4 for *P. pinaster* miRNAs expressed in all samples. Target regions predicted also as targets of PWN miRNAs are highlighted in yellow. (available online)

Table S3.13. GO and KEGG terms represented in *P. pinaster* predicted target genes for PWN miRNAs with degradome analysis and number of genes withing each term. (available online)

Table S3.14. PWN target regions predicted by Miranda for *P. pinaster* miRNAs differentially expressed between resistant and susceptible samples. (available online)

Table S3.15. GO and KEGG terms represented in PWN predicted target genes, with degradome analysis, for *P. pinaster* miRNAs DE between resistant and susceptible plat, and number of genes withing each term. (available online)

Table S3.16. Primers used for RT-qPCR analysis and annealing temperature (Ta) used for cDNA amplification. (available online)

Chapter 4

SNP detection in *Pinus pinaster* transcriptome and association with resistance to pinewood nematode

This chapter has been published in *Forests*:

Modesto, I., Inácio, V., Novikova, P., Carrasquinho, I., Van de Peer, Y. & Miguel, C. M. (2022) SNP Detection in *Pinus pinaster* Transcriptome and Association with Resistance to Pinewood Nematode. *Forests*, 13, 946. https://doi.org/10.3390/f13060946.

Inês Modesto participated in the conceptualization of the experiments, performed part of the experiments, analysed and interpreted the data, prepared the figures and tables, and wrote the original draft of the manuscript.

4. SNP detection in *Pinus pinaster* transcriptome and association with resistance to pinewood nematode

4.1. Abstract

Pinewood nematode (PWN, *Bursaphelenchus xylophilus*) is the causal agent of pine wilt disease (PWD), which severely affects *Pinus pinaster* stands in southwestern Europe. Despite the high susceptibility of *P. pinaster*, individuals of selected half-sib families have shown genetic variability in survival after PWN inoculation, indicating that breeding for resistance can be a valuable strategy to control PWD. In this work, RNA-seq data from susceptible and resistant plants inoculated with PWN were used for SNP discovery and analysis. A total of 186,506 SNPs were identified, of which 31 were highly differentiated between resistant and susceptible plants, including SNPs in genes involved in cell wall lignification, a process previously linked to PWN resistance. Fifteen of these SNPs were selected for validation through Sanger sequencing and 14 were validated. To evaluate SNP-phenotype associations, 40 half-sib plants were genotyped for six validated SNPs. Associations with phenotype after PWN inoculation were found for two SNPs in two different genes (*MEE12* and *PCMP-E91*), as well as two haplotypes of *HIPP41*, although significance was not maintained following Bonferroni correction. SNPs here detected may be useful for the development of molecular markers for PWD resistance and should be further investigated in future association studies.

4.2. Introduction

Pine wilt disease (PWD) is a worldwide threat to conifer trees that has been spreading through Eastern Asia and most recently in Europe (Webster and Mota, 2008). In these regions, several pine species are highly susceptible to PWD, and large areas of forest can be severely affected. PWD is caused by the migratory plant-parasitic nematode *Bursaphelenchus xylophilus*, or pinewood nematode (PWN), which is disseminated by the insect vector

Monochamus spp. (Jones et al., 2008; Vicente et al., 2012). This nematode spreads through the resin canals in the tree's stem, feeding on plant cells and destroying the plant tissues, finally disrupting water transport and causing the wilting of the tree (Jones et al., 2008; Vicente et al., 2012). In the Iberian Peninsula, PWN was first detected in the late 1990's (Mota et al., 1999) and in this region maritime pine (*Pinus pinaster*) is the most affected species.

Pinus pinaster is naturally distributed in the western Mediterranean Basin (Eveno et al., 2008), where natural stands are of great importance for coastal protection and wildlife habitat. *Pinus pinaster* has also been widely planted for industrial exploitation and is mainly used for paper, wood, and resin production. Due to its ecological and economic relevance, the loss of *P. pinaster* trees in Iberian forests has a major impact on the local environment and economy (Vicente et al., 2012; Webster and Mota, 2008).

Despite PWD being an introduced disease, *P. pinaster* individuals show variable degrees of susceptibility once infected (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017a; Menéndez-Gutiérrez et al., 2017b). Two independent studies with large numbers of half-sibling families revealed that survival after PWN inoculation is a heritable trait (heritability of 0.37-0.59) (Carrasquinho et al., 2018; Menéndez-Gutiérrez et al., 2017a), opening the possibilities for tree breeding for PWN resistance, as it has been implemented for other pine species (Nose and Shiraishi, 2008; Toda and Kurinobu, 2002; Xu et al., 2012).

The development of molecular markers for the phenotype of interest is an important step to expedite breeding programs, by allowing for the selection of trees at an early age or to select parent trees from natural stands (Naidoo et al., 2019; Sniezko and Koch, 2017). However, association studies aiming at identifying such molecular markers for resistance to PWD are scarce (Hirao et al., 2019) and, to the best of our knowledge, not yet available for *P. pinaster*. Being a quantitative trait, resistance to PWD is likely to have a

highly polygenic basis, with many loci having small effects on the phenotype.

With the rise of next generation sequencing, the developing of molecular markers has become easier and more affordable, even for non-model species (Sniezko and Koch, 2017). RNA-seq is one of these technologies that has been frequently used for the discovery of molecular markers, such as single nucleotide polymorphisms (SNPs) and simple-sequence repeats (SSRs) (Guo et al., 2018; Liu et al., 2014; Plomion et al., 2016). As RNA-seq produces information mainly on protein coding regions, polymorphisms associated with phenotype are more easily linked to a functional effect. Therefore, RNA-seq provides an efficient approach to identifying a large number of gene-based molecular markers and functional gene variants associated with phenotypic traits in non-model species (Liu et al., 2014; Liu et al., 2016).

In this work, we aimed at finding molecular markers for PWD resistance by identifying SNPs in genes expressed during *P. pinaster* defence response to PWN. We used RNA-seq data available from PWN inoculated susceptible and resistant plants from a half-sib family previously described (Carrasquinho et al., 2018; Modesto et al., 2021) for SNP discovery. More than 186K SNPs were identified for the half-sib family 440. The divergence between susceptible and resistant groups of samples was analysed and outliers were identified. To evaluate the SNP dataset, 15 SNPs were selected for validation through Sanger sequencing. Six of the validated SNPs were then genotyped for a larger sample of the half-sib family 440 and their association with phenotype was tested. A set of candidate genes for *P. pinaster* resistance to PWD was highlighted in this work. The SNPs here detected can be a valuable resource for future association studies focusing on resistance to PWD or to other pine diseases and pests.

4.3. Materials and Methods

4.3.1. Plant Material

The *P. pinaster* half-sibling family 440 was selected for the inoculation assays (Carrasquinho et al., 2018; Modesto et al., 2021; Rodrigues et al., 2021a). This family had been previously evaluated regarding the genetic effects on survival after PWN inoculation of 2-year-old plants and had a predicted survival mean of 15% (in a range of 6–23%) (Carrasquinho et al., 2018). Seeds were collected from the mother tree 440, belonging to a reference population for PWD resistance (Ribeiro et al., 2012) located in the south of Portugal ("Herdade da Comporta", 38°21′28.52″ N, 8°45′49.89″ W). Plants germinated from these seeds were maintained in 4L pots in a greenhouse and placed according to a completely randomized experimental design.

4.3.2. PWN Inoculum

Bursaphelenchus xylophilus isolate Bx013.003 from INIAV's Nematology Laboratory collection (Oeiras, Portugal) (Carrasquinho et al., 2018; Modesto et al., 2021; Rodrigues et al., 2021a; Rodrigues et al., 2021b) was obtained from a wild population infecting a *P. pinaster* adult tree in central Portugal (39°43′33.8″ N, 9°01′55.7″ W). The sequence of the *ITS* region of this isolate is available at NCBI GenBank (ref. MF611984.1). PWNs were reproduced in flasks containing a non-sporulating *Botrytis cinerea* strain grown on autoclaved barley grains, at 25 ± 1 °C. Prior to inoculations, the isolate was allowed to grow on sterilized wood to maintain virulence. Finally PWNs were extracted from the wood using the "tray" method (Whitehead and Hemming, 1965) and suspended in water at a calibrated concentration of 2000 PWN/mL.

4.3.3. Inoculation Assays and Sample Collection

For SNP discovery, RNA-seq data were generated from plant samples collected in a previously performed inoculation assay as described by

Modesto et al. (2021). In short, 4-year-old plants were inoculated with PWN and samples from the stem were collected 72 h post inoculation (hpi). Symptoms were evaluated weekly for 210 days and classified on a scale of 0 to 4, based on the percentage of needles presenting wilting or discoloration symptoms (0—absence of symptoms; 1—1 to 25%; 2—26 to 50%; 3—51 to 75%; 4—76 to 100%). Four susceptible plants (level 4 in the symptoms scale) and five resistant plants (level 0) were sequenced through Illumina HiSeq 2500.

For the genotyping of validated SNPs in a larger sample through Sanger sequencing, 90 three-year-old plants of the half-sibling family 440 were inoculated (September 2019) with a suspension of 1000 PWNs, following the method of Futai and Furuno (Futai and Furuno, 1979), as described in Modesto et al. (2021). The inoculum was pipetted into a small longitudinal wound made in the main stem with a sterile scalpel below the apical shoot region. After inoculation, symptoms were observed weekly for 273 days post-inoculation (dpi) and registered according to the scale (0–4) used before. Plants with symptoms (levels 1 to 4) were considered susceptible, while plants without any symptoms (level 0), by the end of the observation time, were classified as resistant. Needle samples were collected prior to inoculations and stored at −80 °C.

The height and diameter at the base of the stem were measured for all plants before the inoculation assay, and significant differences between susceptible and resistant plants were evaluated with a two-sample unpaired t-test using R v4.1.0 (https://www.r-project.org, accessed on 26 June 2021).

4.3.4. RNA-Seq

RNA-seq data used for this work are available at the public database European Nucleotide Archive (ENA) at EMBL EBI under the accession number PRJEB26836 (Modesto et al., 2021). The quality of these data was evaluated with FastQC v0.11.2 (Andrews, 2010). As a reference, the *P. pinaster* transcriptome described in Cañas et al. (2017), was used, together

with 34,737 new transcripts assembled from data originating in *P. pinaster* samples inoculated with PWN (Modesto et al., 2021). Reads were mapped to *P. pinaster* and PWN transcriptomes (Kikuchi et al., 2011) using BWA alignment software v0.7.17 (BWA-MEM) (Li, 2013). Mapping results were filtered to keep only uniquely mapped reads with SAMtools v1.6 (Li et al., 2009). *Pinus pinaster* and PWN mapping results were separated in two different files and only *P. pinaster* data was used for subsequent analysis.

4.3.5. SNP Calling and Analysis

SNP calling was performed using GATK v3.7.0 (Van der Auwera and O'Connor, 2020; McKenna et al., 2010) according to the software best practices for RNA-seq short variant discovery. SNPs with missing information for more than two samples were ex-cluded and called variants were filtered using GATK hard filters (FS > 30.0, QD < 2.0, SB < −10.0, MQ < 58.0). These filters were adjusted by comparing our SNP data with an Illumina Infinium SNP array previously designed for *P. pinaster* (Plomion et al., 2016), aiming at obtaining good quality variants without excluding many SNPs present in both datasets. SNPs detected both in our data and in the SNP array were considered true SNPs. Filtered SNPs were functionally annotated using SnpEff v4.3t (Cingolani et al., 2012).

Minor allele frequencies (MAF), nucleotide diversity (π) and Tajima's *D* were calculated using VCFtools v0.1.16 (Danecek et al., 2011). For π and Tajima's *D*, a sliding window of 200 bp was used for the calculations. Genetic differentiation (F_{ST}) was estimated between susceptible and resistant groups of samples using the same soft-ware and a sliding window of 200 bp.

4.3.6. SNP Validation

Thirty-one SNPs presenting high differentiation between susceptible and resistant groups ($F_{ST} \geq 0.8$) were selected for validation through Sanger sequencing. Primers were designed for the 26 genes containing these

SNPs (Supplementary Table S4.1) using PerlPrimer v1.1.21 (Marshall, 2004) and NCBI Primer-BLAST (accessed in January 2020). For one of the genes, it was not possible to design primers to amplify the region containing the SNP.

DNA was extracted from the needles of the same samples used for RNA-seq using the CTAB method (Doyle, 1991) with minor modifications: 1% PVP-40 in the extraction buffer, no ammonium acetate in the washing buffer, and 0.1 vol. 3M sodium acetate in the final DNA precipitation. The DNA was amplified with GoTaq DNA Polymerase (Promega) according to the manufacturer's recommendations and using optimized annealing temperatures (Supplementary Table S4.1). Amplified gene fragments were purified using SureClean (Bioline) (directly) or High Pure PCR purification kit (Roche) (from 1% agarose gel) and sequenced on an ABI 3730xl (Macrogen, Spain). The obtained sequences were checked and aligned on ChromasPro v2.1.9 (Technelysium) and the presence or absence of the SNPs was confirmed.

4.3.7. SNP Genotyping and Sequence Analysis

Genotyping of a larger sample was performed for six genes containing validated SNPs (Supplementary Table S4.1). For this, 40 samples from the inoculation assay described above were used. The first 20 samples reaching level 4 in the symptoms scale were selected as susceptible plants for genotyping, while 20 random healthy plants (level 0) were selected as resistant plants. DNA was extracted and amplified as described above (Supplementary Table S4.1). PCR products were purified using SureClean and sequenced on an ABI 3730xl (Macrogen, Spain). The obtained sequences were checked and aligned on ChromasPro v2.1.9 and all SNPs in each gene fragment were identified. Sequences were deposited in the European Nucleotide Archive (ENA) at EMBL EBI under accession number PRJEB51636.

Sequences were aligned with ClustalW (Thompson et al., 1994) for each gene. For sequences with heterozygous SNPs, the haplotypes were reconstructed using PHASE v2.1.1 (Stephens et al., 2001; Stephens and Scheet, 2005). Nucleotide diversity (π), diversity at nonsynonymous sites (π_N), diversity at synonymous sites (π_S), haplotype diversity (*H*), and Tajima's *D* neutrality test were estimated with DnaSP v6.12.03 (Rozas et al., 2017) for each gene.

4.3.8. Association Analysis

Association analysis was performed using the R package SNPassoc v2.0-11 (González et al., 2007) in R. Genotyping data were filtered to exclude SNPs with a minor allele frequency below 0.05 and SNPs outside of Hardy–Weinberg equilibrium ($p \leq 0.001$). Logistic regression was performed to assess the association between SNPs or haplotypes and phenotypes, considering resistance as case (1) and susceptibility as control (0). Diameter at the base of the stem and plant height were included as covariates, as they were shown before to influence the plant outcome after PWN inoculation (Carrasquinho et al., 2018). The null hypothesis (absence of association) was rejected at a 5% significance level. The Bonferroni method was used to correct the statistical threshold.

4.4. Results

4.4.1. RNA-Seq, SNP Discovery and SNP Annotation

To identify SNPs primarily in *P. pinaster* coding genes, RNA-seq data available for a set of nine *P. pinaster* samples were used (Modesto et al., 2021). These data were generated during a previous gene expression study, where plants were inoculated with PWN and stem samples were collected at 72 hpi. Five resistant plants and four susceptible plants were sequenced by RNA-seq. A detailed description of the symptom's progression can be found in Modesto et al. (2021).

After quality control and read filtering, 17–20 million reads were obtained per sample, with sizes ranging between 70–125 bp. An average mapping ratio of 97.8% (±0.1) was obtained, from which 57.8% (±0.8) were uniquely mapped (Supplementary Table S4.2). From these, 99.3% (±0.4) of the reads were mapped to *P. pinaster* transcriptome, while 0.7% (±0.4) were mapped to PWN transcriptome. Only the reads uniquely mapped to *P. pinaster* were kept for SNP discovery.

For the nine samples analysed, it was possible to identify a total of 414,443 SNPs before applying any filter, from which 2,569 SNPs were also present in an Illumina SNP array developed for *P. pinaster* (Plomion et al., 2016). After filtering this dataset in order to exclude low quality SNPs (see Materials and Methods), 186,506 SNPs were retained (Supplementary Table S4.3), including 2,297 SNPs that were previously reported (Plomion et al., 2016). Most of these SNPs corresponded to transitions (58.4%) (Supplementary Figure S4.1), with a transition/transversion ratio (Ts/Tv) of 1.41, similar to what was previously observed for *P. pinaster* (59.3% transitions and 1.46 Ts/Tv ratio) (Plomion et al., 2016). Ts/Tv ratio was similar in susceptible and resistant groups of samples (Table 4.1).

Table 4.1. Number of SNPs and genetic diversity estimates for all samples, for pinewood nematode susceptible samples, and for resistant samples.

	N	**SNPs**	**Transc.**	**Ts/ Tv**	**Syn**	**Non Syn**	**π**	**MAF**
All samples	9	186,506	25,857	1.41	48,992	52,882	0.003282 (±0.036491)	0.274 (±0.147)
Susceptible	4	164,416	24,206	1.41	43,312	46,784	0.003396 (±0.039505)	0.304 (±0.140)
Resistant	5	166,979	24,514	1.40	43,809	47,685	0.003250 (±0.037789)	0.294 (±0.142)

N − number of samples; Transc. − number of transcripts; Ts/Tv − transitions/tranversions ratio; Syn − synonymous SNPs; NonSyn − nonsynonymous SNPs; π − nucleotide diversity; MAF − minor allele frequency.

Most of the SNPs (86.4%) were detected in transcripts with a predicted protein-coding sequence (CDS), while the remaining (13.6%) were located in transcripts without a predicted CDS that were considered noncoding (Figure 4.1a). From the SNPs comprised in coding regions, 52,121 (52%)

were classified as nonsynonymous, resulting in amino acid changes (missense SNP) or premature stop codons (nonsense SNP).

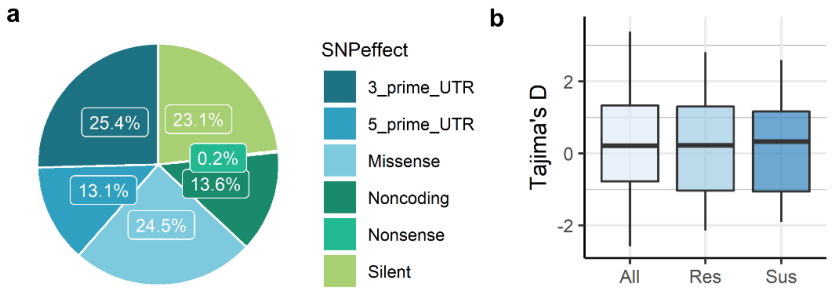

Figure 4.1. Summary of SNP effects (a) and Tajima's *D* estimation for all transcripts (b). Tajima's *D* was calculated using a sliding window of 200 bp. All − all samples; Res − resistant; Sus − susceptible.

4.4.2. Genetic Diversity and Differentiation

The 186,506 SNPs identified were located in 25,857 transcripts, in an average of one SNP every 192 bp. From these SNPs, 18,997 were singletons, existing in only one sample. Minor allele frequencies were similar between susceptible (MAF = 0.30 ± 0.14) and resistant (MAF = 0.29 ± 0.14) groups of individuals (Table 4.1), as well as the mean nucleotide diversity values (π = 0.0034 ± 0.0395 for susceptible samples; π = 0.0033 ± 0.0378 for resistant samples). Tajima's *D* median values were close to zero, showing no indication of population decline or population expansion (Figure 4.1b).

Genetic differentiation between susceptible and resistant groups was very low (F_{ST} = 0.00 ± 0.12), as expected for samples of the same half-sib family. However, several SNPs presented high differentiation between groups, including 31 SNPs with an F_{ST} above 0.80 (Figure 4.2a, Supplementary Table S4.4), and may be associated with the observed phenotypes. These SNPs were located in 26 transcripts and included 14 SNPs found in transcripts with no predicted CDS, four synonymous, and four

nonsynonymous SNPs (Figure 4.2b). The remaining were located in the 3′-untranslated regions (UTRs; six) or 5′-UTRs (three). Median nucleotide diversity (π) of the regions containing these SNPs was higher in resistant samples (π = 0.0041) than in susceptible plants (π = 0.0023) (Figure 4.2c).

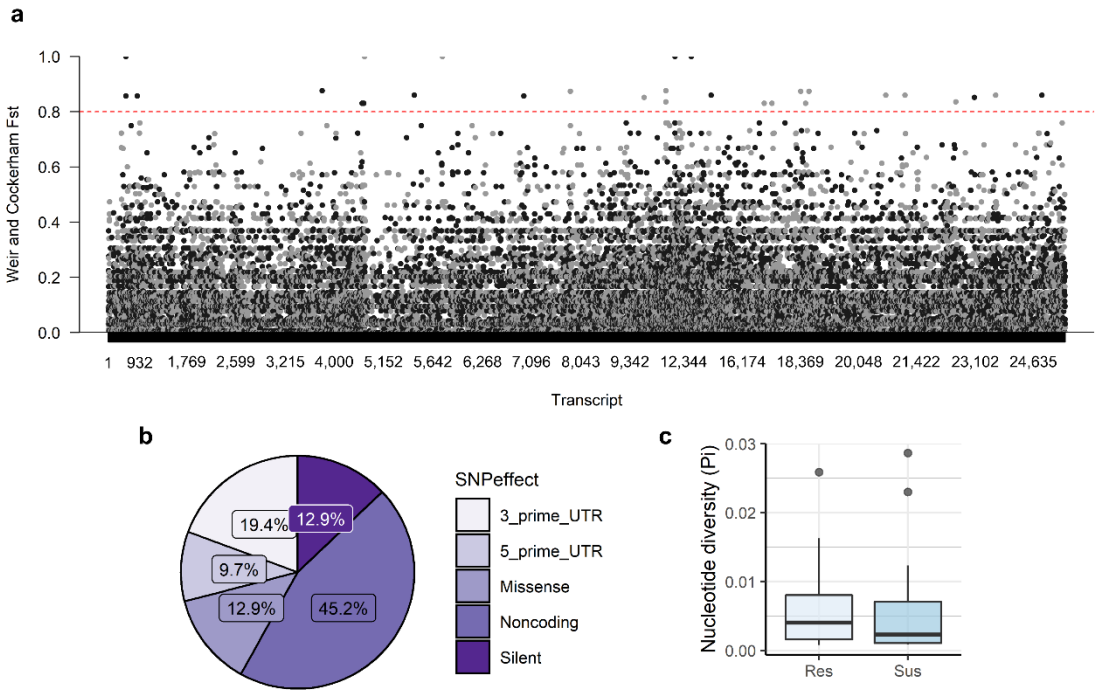

Figure 4.2. Genetic differentiation (F_{ST}) between susceptible and resistant groups (a) and characterization of the SNPs with $F_{ST} \geq 0.80$ (b,c). (a) Manhattan plot of F_{ST} values obtained between susceptible and resistant samples. The red intermittent line indicates $F_{ST} = 0.80$. **(b)** SNP effects of the SNPs with $F_{ST} \geq 0.80$. **(c)** Nucleotide diversity (π) of the regions containing the SNPs with $F_{ST} \geq 0.80$, calculated using a sliding window of 200 bp. Res − resistant; Sus − susceptible.

Within the transcripts containing SNPs with $F_{ST} \geq 0.80$, it was possible to identify two genes that may be involved in lignin biosynthesis (*peroxidase 31* and *laccase-3*), a gene involved in the synthesis of phenolic compounds (*UGT5*), a probable resistance gene (*isotig35427*), and a Myb transcription factor (*isotig42428*) (Supplementary Table S4.4). However, 12 transcripts have unknown function (five) or were not annotated (seven).

Table 4.2. Summary of the SNP validation.

Transcript	SNP pos.	SNP Annotation	Gene Annotation	Geno. RNA-seq		Geno. Sanger		Val.	Gen.	Additional SNPs
				Sus	Res	Sus	Res			
isotig67703	386	3′-UTR	pentatricopeptide repeat-containing protein At2g27610 [Quercus suber] (PCMP)	AA	GG	AA	GG	✓	✓	304CT; 320CT
isotig30230	197	5′-UTR	maternal effect embryo arrest 12 [Arabidopsis thaliana] (MEE12)	AA	CC	AA	CC	✓	✓	-
isotig42428	236	3′-UTR	protein PHOSPHATE STARVATION RESPONSE 1 [Quercus suber] (PHR1)	AA	GG	AA	GG	✓	✓	-
isotig53013	453	Syn	pentatricopeptide repeat-containing protein At4g21065 [Elaeis guineensis] (PCMP-H28)	CC	GG	CC	GG	✓	✓	594TA; 651AC
unigene161	348	Syn	kinesin-like protein KIN-12F [Nelumbo nucifera] (KIN12)	AA	GG	AA	GG	✓	✓	-
unigene8832	646	Syn	heavy metal-associated isoprenylated plant protein 41-like [Elaeis guineensis] (HIPP41)	TT	CC	TT	CC	✓	✓	-
unigene52225	105	Noncoding	unknown [Picea sitchensis] (ung52225)	CC	TT	CC	TT	✓	✓	145CT; 171GA
isotig37698	586	NonSyn	UDP-glycosyltransferase UGT5 [Picea glauca] (UGT5)	GG	CC	GC	CC	✓	×	505CT; 577AT; 739TG; 745TC
unigene58419	178	NonSyn	pentatricopeptide repeat-containing protein At3g16610 [Prunus mume] (PCMP-E91)	GG	AA	GG	AG	✓	×	-
unigene188104	297	Noncoding	no annotation (ung188104)	CC	GG	CC	GG	✓	✓	-
	298	Noncoding		GG	CC	GG	CC	✓	✓	-
	305	Noncoding		TT	GG	TT	GG	✓	✓	-
isotig09645	590	5′-UTR	Guanine nucleotide-binding protein, beta subunit [Parasponia andersonii] (GB1)	AA	GG	AA	GG	✓	✓	780AG; 804CT
	620	5′-UTR		TT	AA	TT	AA	✓	✓	
isotig46969	1304	NonSyn	hypothetical protein PHAVU_003G104100g [Phaseolus vulgaris] (HP)	GG	CC	CC	CC	×	×	-

SNP pos. − SNP position; Geno. − genotype; Val. − Validated; Gen. − Correctly genotyped ; Sus − susceptible; Res − resistant; Noncoding −SNPs in noncoding regions; Syn − synonymous SNPs; NonSyn − nonsynonymous SNPs.

4.4.3. SNP Validation through Sanger Sequencing

SNPs with high differentiation ($F_{ST} \geq 0.8$) between resistant and susceptible groups of samples were selected for validation. For 14 out of 26 transcripts comprising these SNPs, it was not possible to design primers to amplify a fragment including the SNPs (one) or the amplifications failed (13). Therefore, 12 transcripts comprising 15 SNPs were sequenced and the presence or absence of these SNPs was observed (Table 4.2). Fourteen of these SNPs were validated (93%), while one was not (7%) (Table 4.2). However, the genotype was miscalled in the RNA-seq analysis for two of the validated SNPs, for at least one of the sequenced samples (Table 4.2), giving a rate of 80% of validated and correctly genotyped SNPs.

On the other hand, it was possible to detect 12 more SNPs by Sanger sequencing than previously detected by the RNA-seq analysis. Ten of these SNPs were excluded by the hard filters applied in the RNA-seq data analysis, with four being excluded by the mapping quality (MQ) filter and six SNPs located in regions without read coverage in more than two samples. The two remaining SNPs were not detected in the RNA-seq analysis, probably due to low depth coverage (one to eight reads) of the regions where the SNPs were located in all samples.

4.4.4. Inoculation Assay, Genotyping, and Sequence Analysis

To assess if there is an association between the validated SNPs and the plants' phenotypes in a larger dataset, the genotyping of six gene fragments (Table 4.3) was performed for 40 individuals (20 resistant and 20 susceptible). To do this, a new inoculation assay was performed with 3-year-old plants from the half-sib family 440, the same used for the RNA-seq. The first symptoms appeared at 14 dpi and progressed gradually until the end of the experiment (Figure 4.3). At 273 dpi, 48% of the plants presented symptoms, while 52% remained healthy. No significant differences were found in height and diameter at the base of the stem

between resistant and susceptible groups of plants (Supplementary Figure S4.2).

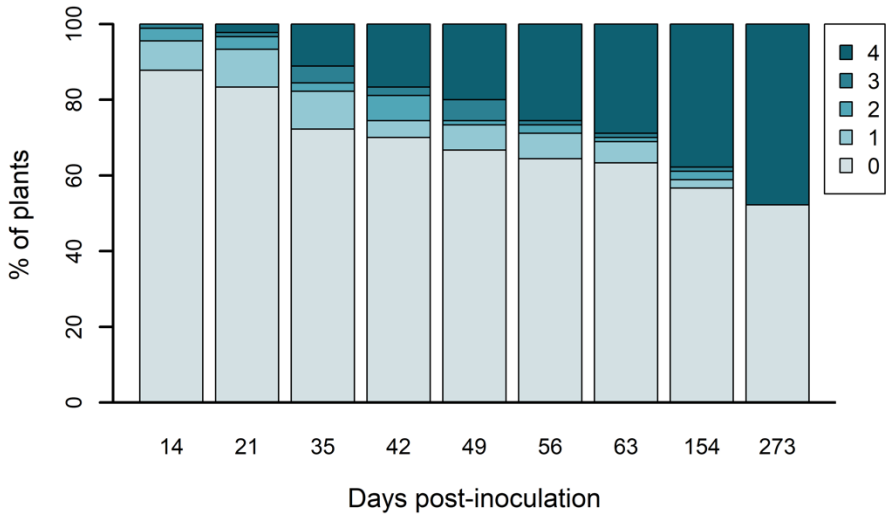

Figure 4.3. Pine wilt disease symptoms progression in *Pinus pinaster* plants according to a symptoms scale of 0 to 4. This scale is based on the percentage of wilting or brown needles in each observed plant: 0−0% of the needles presented symptoms; 1−1 to 25%; 2− 26 to 50%; 3−51 to 75%; 4−76 to 100%. Dpi − days post-inoculation.

The sequenced fragments included coding (exons) and noncoding (introns, 3′-UTR, and 5′-UTR) regions (Table 4.3) in a total of 2359 bp. These fragments contained 20 SNPs, including the six previously validated. Seven of these SNPs were synonymous, seven were nonsynonymous, and six were in noncoding regions. Nucleotide diversity (π) ranged between 0.00091 (*KIN12*) and 0.00984 (*PCMP-E91*), being similar between susceptible and resistant groups, with the exception of *PCMP-E91* and *KIN12*, for which susceptible plants presented higher values. For *PCMP-E91*, nucleotide diversity at nonsynonymous sites (π_N) was higher than nucleotide diversity at synonymous sites (π_S) (Table 4.3).

Table 4.3. Summary of genetic diversity estimates for the six sequenced gene fragments.

Gene	Frag Size (bp)	Group	Seq	Regions	SNPs	SNP Effect			π (±SD)	π_S	π_N	Hap	*H* (±SD)	Tajima's *D*
						Non cod	Syn	Non Syn						
HIPP41	673	All	80	3 exons; 2 introns	6	2	2	2	0.00227 (±0.00018)	0.01095	0.00173	9	0.757 (±0.028)	0.61044
		Res	40	-	6	2	2	2	0.00230 (±0.00039)	0.01020	0.00176	9	0.676 (±0.074)	0.26373
		Sus	40	-	4	1	2	1	0.00204 (±0.00014)	0.01014	0.00167	6	0.762 (±0.031)	1.10499
KIN12	395	All	80	exon	1	-	1	-	0.00108 (±0.00011)	0.00575	0.00000	2	0.425 (±0.042)	1.32948
		Res	40	-	1	-	1	-	0.00091 (±0.00019)	0.00483	0.00000	2	0.358 (±0.073)	0.74452
		Sus	40	-	1	-	1	-	0.00122 (±0.00011)	0.00650	0	2	0.481 (±0.042)	1.49197
MEE12	384	All	80	5′-UTR	1	1	-	-	0.00130 (±0.00002)	-	-	2	0.506 (±0.008)	1.81156
		Res	40	-	1	1	-	-	0.00124 (±0.00011)	-	-	2	0.481 (±0.042)	1.49197
		Sus	40	-	1	1	-	-	0.00124 (±0.00011)	-	-	2	0.481 (±0.042)	1.49197
PCMP-E91	124	All	68	exon	4	-	-	4	0.00910 (±0.00065)	0.00000	0.01149	5	0.667 (±0.033)	0.74798
		Res	30	-	2	-	-	2	0.00803 (±0.00053)	0.00000	0.01013	4	0.683 (±0.053)	1.99045
		Sus	38	-	4	-	-	4	0.00984 (±0.00102)	0.00000	0.01242	5	0.653 (±0.047)	0.68160
PHR1	486	All	80	3′-UTR	3	3	-	-	0.00216 (±0.00012)	-	-	5	0.578 (±0.036)	1.36955
		Res	40	-	2	2	-	-	0.00206 (±0.00012)	-	-	3	0.549 (±0.041)	2.12756 *
		Sus	40	-	3	3	-	-	0.00230 (±0.00022)	-	-	5	0.614 (±0.059)	1.2714
UGT5	297	All	78	exon	5	-	4	1	0.00845 (±0.00020)	0.03037	0.00216	3	0.558 (±0.027)	3.27745 **
		Res	40	-	5	-	4	1	0.00856 (±0.00036)	0.02959	0.00205	3	0.528 (±0.051)	2.72844 **
		Sus	38	-	5	-	4	1	0.00856 (±0.00036)	0.03053	0.00225	3	0.585 (±0.038)	2.92830 **

Frag. Size − size of the amplified fragment; Noncod − SNPs in noncoding regions; Syn − synonymous SNPs; NonSyn − nonsynonymous SNPs; π − nucleotide diversity; πS − nucleotide diversity in synonymous sites; πN − nucleotide diversity in nonsynonymous sites; Hap. − number of haplotypes; Tajima's *D* neutrality test (Tajima, 1989); SD − standard deviation. * *p*-value < 0.05; ** *p*-value < 0.01.

The neutrality test Tajima's *D* rejected the null neutral model for *PHR1* in the resistant group and for *UGT5* (Table 4.3). In both cases, *D* values were positive, indicating an excess of intermediate frequency alleles consistent with balancing selection or population decline.

4.4.5. Association Analysis

Association analysis between single SNPs and the phenotype was performed after excluding SNPs with a minor allele frequency below 0.05 (six) and SNPs outside of the Hardy–Weinberg equilibrium (two) (Supplementary Table S4.5). The association analysis for each SNP and genetic model is represented in Supplementary Figure S4.3.

MEE12 SNP 197 showed a significant association with the phenotype (Table 4.4), both before ($p = 0.0244$ for the dominant model and $p = 0.0222$ for the additive model) and after adjusting for diameter at the basis of the stem and plant height (Carrasquinho et al., 2018)($p = 0.0168$ for the dominant model and $p = 0.0109$ for the additive model). For this SNP, the genotypes A/C and C/C were associated with a higher chance of being resistant to PWN inoculation, while A/A genotype seems to be associated with susceptibility in both additive and dominant models (Table 4.4, Supplementary Figure S4.4). *PCMP-E91* SNP 178 was also significantly associated with the phenotype for the recessive model, with both non-adjusted ($p = 0.0295$) and adjusted ($p = 0.0074$) statistical tests. For this gene, the genotype G/G was associated with an increased probability of being susceptible (Table 4.4, Supplementary Figure S4.4). These association results were not significant after Bonferroni correction.

Association analyses were also performed between haplotypes and phenotypes for each gene (Table 4.5, Supplementary Table S4.6). Two haplotypes of the gene *HIPP41* were significantly associated with susceptibility (haplotype 3, $p = 0.0263$, and haplotype 4, $p = 0.0441$) (Table 4.5). However, these association results were not significant after Bonferroni correction.

Table 4.4. Significant association results between genotypes and phenotypes. All analyses were performed with SNPassoc using a logistic regression model.

SNP	Genetic Model	Geno	Sus *n* = 20 *n* (%)	Res *n* = 20 *n* (%)	OR (95% CI)	*p*-Value	AIC	OR (95% CI) adj.	*p*-Value adj.	AIC adj.
MEE12 SNP197	Dominant	A/A	8 (40%)	2 (10%)	1.00	0.0244 *	54.4	1.00	0.0168 *	54.7
		A/C-C/C	12 (60%)	18 (90%)	6.00 (1.08–33.27)			7.40 (1.20–45.67)		
	log-Additive	0,1,2	20 (50%)	20 (50%)	3.00 (1.09–8.25)	0.0222 *	54.2	3.69 (1.23–11.09)	0.0109 *	53.9
*PCMP-E91 SNP*178	Recessive	A/A-A/G	10 (52.6%)	13 (86.7%)	1.00	0.0295 *	45.9	1.00	0.0074 **	44.1
		G/G	9 (47.4%)	2 (13.3%)	0.17 (0.03–0.97)			0.07 (0.01–0.69)		

Geno − genotypes; Sus − susceptible; Res − resistant; *n* − number of samples; OR − odds ratio; CI − confidence interval; AIC − akaike information criterion; adj. − results of the statistical analysis adjusted for diameter at the basis of the stem and plant height. * *p*-value < 0.05; ** *p*-value < 0.01.

Table 4.5. Significant results of the haplotype association analysis. All analyses were performed with SNPassoc using a logistic regression model.

Gene	Haplotype		Haplotype Freq.	OR (95% CI)	*p*-Value
HIPP41	1	CAG	0.3868	1.00	-
	2	TAA	0.1018	1.06 (0.19–5.91)	0.9459
	3	TAG	0.2232	0.22 (0.06–0.84)	0.0263 *
	4	TGA	0.2475	0.34 (0.12–0.97)	0.0441 *
		genoH.rare	0.0407	1.00 (0.07–14.61)	0.9995

Freq. − frequency; OR − odds ratio; CI − confidence interval. * *p*-value < 0.05.

4.5. Discussion

In this work, we used previously published transcriptomics data of *P. pinaster* plants inoculated with PWN for SNP detection. This strategy allowed for the identification of SNPs in genes expressed during PWN infection that may be associated with PWD resistance. As *P. pinaster* genome is quite large (24.5 Gb) (Chagné et al., 2002), the detection of SNPs at the genome level can be difficult and expensive. The use of RNA-seq data provided a more targeted and efficient way of detecting SNPs in

candidate genes for the trait of interest (Guo et al., 2018; Liu et al., 2014; Liu et al., 2016). SNPs here detected may not directly affect the phenotype after inoculation, but rather be physically linked to causal variants that are not detectable with the method used, such as variants in regulating regions or structural variants.

Although genomic resources for conifer species are usually limited, an Illumina Infinium SNP array comprising 8,410 SNPs was previously developed for *P. pinaster* (Plomion et al., 2016). However, this array had an extremely limited number of SNPs in candidate genes for biotic stress response (53 transcripts). Furthermore, this SNP array was never tested for the reference population for PWD resistance (Carrasquinho et al., 2018; Ribeiro et al., 2012), from which the half-sib family used in this study originated (Comporta, Portugal). This population may present distinct variants from the ones previously studied with the SNP array (Hurel et al., 2021; Plomion et al., 2016). In fact, only a very low percentage (1.3%) of the 186,506 SNPs detected here was present in the SNP array, corresponding to only 2,297 SNPs (or 2,569 before filtering) in common. None of the SNPs with high F_{ST} values between resistant and susceptible plants identified in our study were included in this set. Therefore, detecting SNPs in genes expressed after PWN inoculation in the samples showing contrasting phenotypes for the trait of interest might be a better approach to identify SNPs that can be used in future selection programs for PWD resistance. Although a larger sample size would increase the statistical power to detect significant SNP associations with phenotype, it was still possible to detect a high number of SNPs (180,506) in the RNA-seq data.

To ensure the quality of the SNP dataset obtained in this work, stringent hard filters were used. Although the final dataset included a large number of SNPs, several true SNPs have been excluded by filtering, as demonstrated by the detection of excluded SNPs in the Sanger sequencing validation results. On the other hand, two samples were wrongly identified as homozygotes for two SNPs in the RNA-seq analysis, when these

samples were in fact heterozygotes. This probably resulted from a low RNA-seq read coverage in these regions leading to the detection of only one of the alleles. Including filters for minimum depth coverage may decrease the number of miscalled genotypes and further improve the SNPs dataset.

Genetic differentiation between resistant and susceptible groups was low, as the samples were all from the same half-sib family, but highly variable probably due to the small sample size. In contrast, a small set of SNPs presented very high levels of differentiation, with one allele being prevalent in the susceptible group while the resistant group presented mostly the other allele, suggesting they might be linked to phenotype. Some of these highly differentiated SNPs were located in transcripts with functions described as relevant for PWN resistance (Modesto et al., 2021). For instance, one SNP was positioned in the 3′UTR of a resistance gene, which can impact the post-transcriptional regulation of this gene. Other SNPs of interest were found in *peroxidase 31* (*PER31*) and *laccase-3* (*LAC3*), which code for proteins involved in the lignin biosynthesis pathway (Vogt, 2010; Xie et al., 2018), and *UGT5*, involved in the synthesis of phenolic compounds in *Picea glauca* (Mageroy et al., 2017). In *PER31* and *UGT5*, the SNPs highly differentiated between resistant and susceptible plants were nonsynonymous, leading to amino acid changes and being consequently more likely to impact protein function, which may in turn affect lignin deposition or accumulation of phenols. This is consistent with the results from a previous work (Modesto et al., 2021), in which resistant plants were shown to have increased cell wall lignification after inoculation when compared to susceptible plants. Future studies addressing the functional effect of these SNPs could be of interest to further elucidate *P. pinaster* resistance to PWD.

When genotyping a set of the candidate genes identified by the genetic differentiation analysis in a sample of 40 individuals, it was possible to confirm the association between two SNPs, located in the genes *MEE12*

and *PCMP-E91*, and the phenotype. These associations were nominally significant, but did not remain significant following stringent correction for multiple testing. These results should therefore be taken with caution. *MEE12* is a transcription initiation factor involved in embryo development (Pagnussat et al., 2005) and pollen tube guidance (Chen et al., 2007; Li et al., 2016) in Arabidopsis. Although a role for *MEE12* in plant defence is unknown, other *MEE* genes have been implicated in defence responses (Huibers et al., 2009; Wang et al., 2010). Alternatively, *MEE12* SNP197 may be in linkage with a polymorphism that has functional relevance in resistance, instead of directly affecting the phenotype.

The protein encoded by *PCMP-E91* is part of the pentatricopeptide repeat (PPR) protein family, a very large family found in higher plants that is involved in RNA modification processes (Qin et al., 2021; Saha et al., 2007), such as RNA editing (Hayes et al., 2015), splicing (Ichinose et al., 2012), and processing (Hao et al., 2019). Although the function of many of these proteins is still unknown, studied PPR proteins have various roles in regulating embryogenesis, fruit growth and ripening, circadian rhythm, among others (Qin et al., 2021; Saha et al., 2007). Several PPR proteins have been also associated with response to abiotic (Koussevitzky et al., 2007; Zsigmond et al., 2008) and biotic stresses (Laluk et al., 2011; Xing et al., 2018). Therefore, *PCMP-E91* may have an important role in *P. pinaster* defence and resistance to PWN. The SNP associated with phenotype is a nonsynonymous SNP, resulting in an amino acid change and may consequently impact protein function. Furthermore, nucleotide diversity at nonsynonymous sites (π_N) in *PCMP-E91* was higher than nucleotide diversity at synonymous sites (π_S), suggesting that this gene may be under positive selection. As PWN was detected in the Iberia Peninsula only in the late 1990s (Mota et al., 1999), *PCMP-E91* may have evolved in response to other selective pressures, such as other pests or pathogens, and now be effective against PWD.

An association was also detected between two haplotypes of the gene *HIPP41* and phenotype, which were not significant after correction for multiple testing. These haplotypes seem to be associated with susceptibility to PWN. HIPPs are a large family of metal-binding metallochaperones that occur only in vascular plants (De Abreu-Neto et al., 2013). They are involved in a variety of functions, including heavy-metal homeostasis and detoxification, plant development, response to abiotic stresses, and response to biotic stresses (De Abreu-Neto et al., 2013; Radakovic et al., 2018; Zhang et al., 2020). In rice, *HIPP41* was associated with response to cadmium and to cold (De Abreu-Neto et al., 2013). In *P. pinaster*, *HIPP41* may be directly involved in response to PWN and have a role in susceptibility to PWD, as described for other *HIPP* genes in response to the beet cyst nematode *Heterodera schachtii* (Radakovic et al., 2018).

Although there was no statistically significant association between the SNPs in *UGT5* and phenotype, the Tajima's *D* test for this gene was significantly positive, indicating that this gene may be under balancing selection. Several genes with known roles in plant defence response have been described as being under balancing selection (Keith and Mitchell-Olds, 2013; Tiffin and Moeller, 2006). The interaction of *P. pinaster* with multiple pests and pathogens during its long lifespan would create a selective pressure to maintain variability in genes relevant for defence response. In accordance, UGT5 seems to be involved in the biosynthesis of the phenolic compounds acetophenones, which have a role in *P. glauca* resistance to spruce budworm (Mageroy et al., 2017). Different contents of these phenolic compounds may also impact *P. pinaster* outcome during PWN infection.

Even though SNP-phenotype associations were confirmed for two SNPs in two candidate genes, no significant association remained after stringent correction for multiple comparisons. The absence of strong associations may be due to the small effect that each SNP likely has on the resistance phenotype, a trait that is most likely polygenic given its quantitative nature

(Carrasquinho et al., 2018; Hurel et al., 2021). Therefore, the sample size used may be too small to have enough statistical power to detect significant results for variants with small effects. Although these results cannot be directly applied, polymorphisms in candidate genes, especially in *MEE12*, *PCMP-E91*, *HIPP41*, and *UGT5*, may be useful in the development of markers for resistance to PWD, and warrant further investigation in genotyping assays of a larger sample representing several families of the reference population for PWD resistance (Carrasquinho et al., 2018; Ribeiro et al., 2012).

4.6. Conclusions

Our results confirmed that using RNA-seq data for SNP discovery is a valuable approach to identify SNPs in candidate genes potentially linked to the trait of interest. These SNPs can be particularly informative as they were identified under the biotic stress in study and in a population showing contrasting phenotypes for the relevant trait. The identified SNPs have the potential to be used in future association studies searching for markers connected to PWD, not only in the half-sib family 440, but also in other families originating from the same population in the South of Portugal. The SNPs here identified can be added to other previously discovered *P. pinaster* SNPs to obtain a high-density SNP array that include interesting SNPs for PWD resistance, increasing the potential for discovery of significant genome wide associations.

4.7. Author Contributions

Conceptualization and Methodology, I.M. and C.M.M.; Formal Analysis and Visualization, I.M.; Validation, I.M. and V.I.; Resources, I.C., C.M.M. and Y.V.d.P.; Writing—Original Draft Preparation, I.M.; Writing—Review and Editing, all authors; Supervision, P.N., C.M.M., I.C. and Y.V.d.P.; Funding Acquisition, C.M.M. All authors have read and agreed to the published version of the manuscript.

4.8. Funding

This work was supported by Fundação para a Ciência e a Tecnologia, I.P. (FCT/MCTES), through Grants GREEN-it (UID/Multi/04551/2013), BioISI (UIDB/04046/2020 and UIDP/04046/2020) and the doctoral fellowship SFRH/BD/111687/2015 (to I.M.). Support was also provided by FCT/MCTES through national funds (PIDDAC) and co-financed by Fundo Europeu de Desenvolvimento Regional (FEDER) of the EU, through Programa Operacional Regional de Lisboa do Portugal 2020 or other programs that may succeed - PTDC/BAA-MOL/28379/2017, LISBOA-01-0145-FEDER-028379.

4.9. Data Availability Statement

The sequencing data presented in this study are openly available in European Nucleotide Archive (ENA) at EMBL EBI under accession number PRJEB51636.

4.10. Acknowledgments

We thank Maria L. Inácio (INIAV) for providing the nematode cultures and Hugo Matias (ITQB NOVA) for all the technical support in the greenhouse.

4.11. Conflicts of Interest

The authors declare no conflict of interest.

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4.13. Supplementary Materials

The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f13060946/s1.

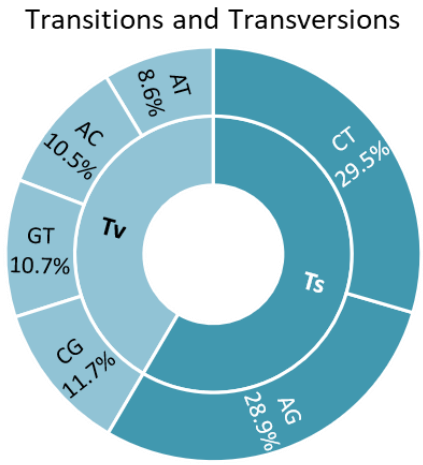
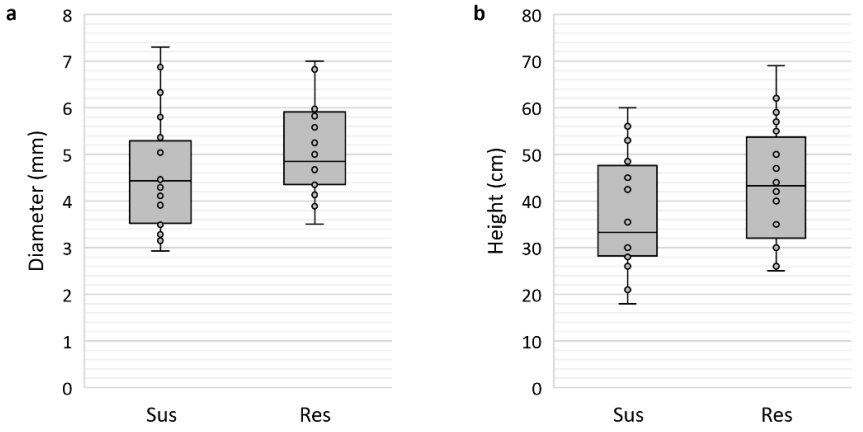


Figure S4.1. Type of SNPs identified in *P. pinaster* RNA-seq analysis. Ts – transitions; Tv – transversions.



c

	N	Mean (mm)	SD	*p* -value
Susceptible	20	4.59	1.20	0.13
Resistant	20	5.16	1.01	

d

	N	Mean (cm)	SD	*p* -value
Susceptible	20	37.3	12.4	0.11
Resistant	20	43.9	12.4	

Figure S4.2. Boxplots of the height and diameter at the base of the stem of inoculated plants (half-sib family 440) and *t*-test results for the comparison of these parameters' means between susceptible and resistant plants. (a) Boxplot of diameter at the base of the stem and **(b)** of height. Both measurements were made before inoculations. **(c)** Results of the two-sample unpaired *t*-test between the mean diameter of susceptible and resistant plants. **(d)** Results of the two-sample unpaired *t*-test between the mean height of susceptible and resistant plants. *N*, number of plants; SD, standard deviation.

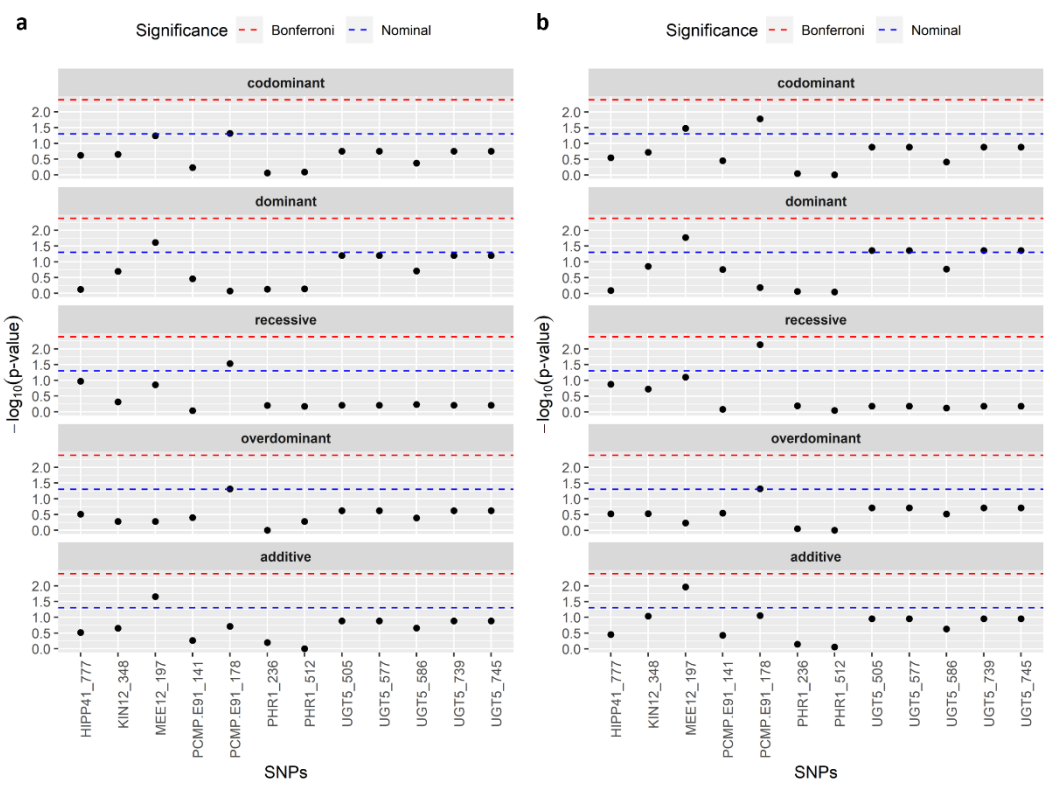


Figure S4.3. Association analysis of the SNPs in the six sequenced gene fragments under different genetic models with resistance to PWN. All analyses were performed with SNPassoc using a logistic regression model **(a)** or a logistic regression model adjusted for diameter at the basis of the stem and plant height **(b).**

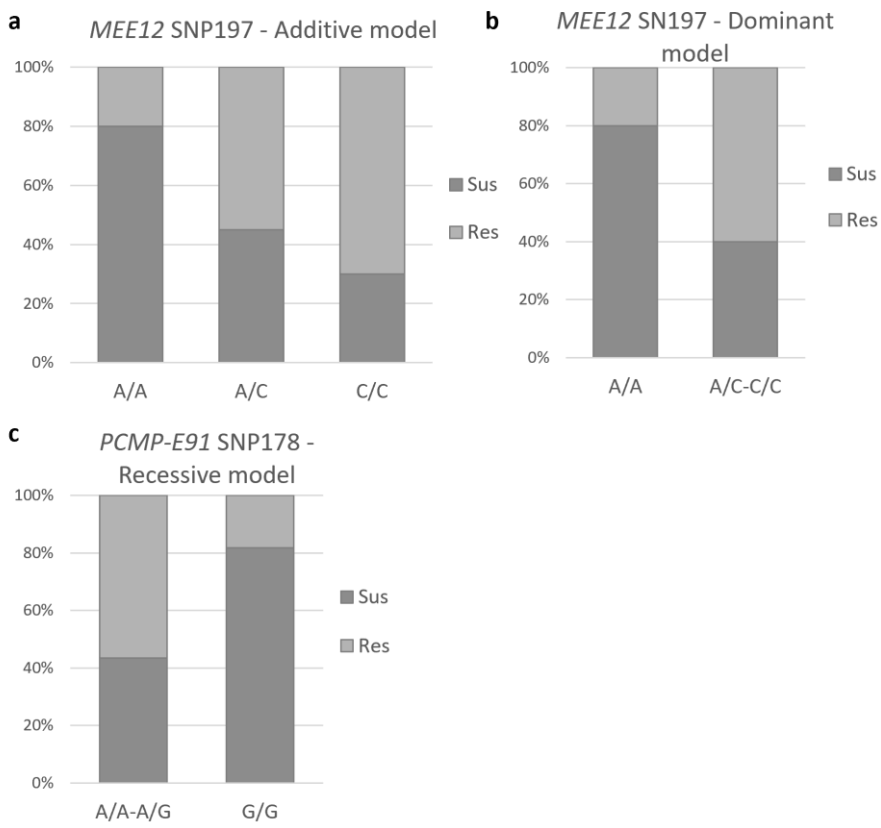


Figure S4.4. Genotypes distribution for SNPs associated with phenotype. (a) *MEE12* SNP 197 genotypes distribution following the additive genetic model and **(b)** following the dominant genetic model. **(c)** *PCMP-E91* SNP 178 genotypes distribution following the recessive genetic model. Susceptible (Sus) plants are represented in dark grey, while resistant (Res) plant are in light grey.

Table S4.1. Summary of PCR conditions and sequencing results of the 26 SNPs selected for validation. Sequenced - information about the success of amplification and sequencing of the samples for validation; Genotyped - information about which genes were later genotyped for 40 additional samples. (available online)

Table S4.2. Summary of mapping statistic per sample and per sequencing lane. Ppi - *Pinus pinaster*; PWN - pinewood nematode; Stdev - standard deviation. (available online)

Table S4.3. SNPs detected in *P. pinaster* RNA-seq data. (available online)

Table S4.4. Details and functional annotation of the SNPs with an Fst ≥ 0.80. (available online)

Table S4.5. Allele frequencies and Hardy-Weinberg Equilibrium significance values calculated by SNPassoc. SNPs with minor allele frequencie ≤ 0.05 (5%) and Hardy-Weinberg Equilibrium with p ≤ 0.001 (***) were excluded from further analysis.

SNPs	Alleles	MAF	Hardy-Weinberg Equilibrium		
			All samples	Susceptible	Resistent
MEE12_197	A/C	50	1.0000	1.0000	0.6473
KIN12_348	G/A	70	0.4481	0.6473	0.5283
PCMP.E91_119	A/T	97.5	-	-	-
PCMP.E91_128	A/T	97.5	-	-	-
PCMP.E91_141	A/G	51.5	0.0432	0.0604	0.6035
PCMP.E91_178	A/G	52.9	0.0180	0.0039	1.0000
PHR1_95	C/T	96.2	-	-	-
PHR1_236	A/G	62.5	1.0000	1.0000	1.0000
PHR1_512	T/C	55	0.7484	1.0000	0.6496
UGT5_505	T/C	52.6	0.3447	0.1762	1.0000
UGT5_577	T/A	52.6	0.3447	0.1762	1.0000
UGT5_586	C/G	59	0.5062	0.3788	1.0000
UGT5_739	G/T	52.6	0.3447	0.1762	1.0000
UGT5_745	C/T	52.6	0.3447	0.1762	1.0000
HIPP41_646	T/C	60	0.0002**	0.0006**	0.20425
HIPP41_649	A/G	72.5	0.0026	0.0006**	1.0000
HIPP41_i496	G/A	95	-	-	-
HIPP41_i516	C/T	98.8	-	-	-
HIPP41_689	C/A	97.5	-	-	-
HIPP41_777	G/A	63.7	0.0837	0.03170	1.0000

MAF – minor allele frequency

Table S4.6. Non-significant results of the haplotype association analysis obtained with SNPassoc. All analyses were performed using a logistics regression model. Freq. - frequency; OR - odds ratio; CI - confidence interval.

Gene	Haplotype	Haplotype Freq.	OR (95% CI)	*p*-value
PCMP-E91	GA	0.4224	1.00	-
	AA	0.1070	3.81 (0.53-27.54)	0.1851
	AG	0.4077	0.70 (0.28-1.78)	0.4535
	GG	0.0629	1.51 (0.16-14.45)	0.7210
PHR1	CCAT	0.550	1.00	-
	CCGC	0.350	1.25 (0.46-3.41)	0.6587
	CGAC	0.063	0.64 (0.09-4.76)	0.6608
	genoH.rare	0.038	4.87E-09 (4.87E-09-4.87E-09)	0.0000
UGT5	TTCGC	0.5256	1.00	-
	CACTT	0.0641	0.40 (0.05-3.06)	0.3781
	CAGTT	0.4103	0.46 (0.16-1.39)	0.1704

Chapter 5

General discussion and future perspectives

5. General discussion and future perspectives

5.1. Plant material and experimental approach

When doing “omics” studies in the context of resistance to pathogens and pests, it is important to use previously genetically characterized plant material. Families with established levels of heritable resistance to a given disease are considered a good starting point for basic research aimed at discovering resistance mechanisms (Sniezko and Koch, 2017). In the absence of characterized plant material within the species of interest, research is usually restricted to the use of commercially available seedlings that are most likely susceptible to the disease, especially when dealing with species highly susceptible to the disease under study. This strategy has been previously used for studying PWD in *Pinus pinaster* (Gaspar et al., 2017; Gaspar et al., 2020; Santos et al., 2012). However, limiting the research to susceptible responses does not increase our knowledge about resistance mechanisms, and comparing susceptible to resistant species can be misguiding. For instance, the comparison of the response of an ash tree species (*Fraxinus mandshurica*) resistant to emerald ash borer (*Agrilus planipennis*) with several susceptible species (e.g., *Fraxinus americana*, *Fraxinus pennsylvanica*, *Fraxinus excelsior*) indicated that certain phenolic compounds were linked to resistance (Eyles et al., 2007; Whitehill et al., 2012). However, when research expanded to other susceptible species this correlation was not found, and initial differences detected were attributed to evolutionary divergence instead of resistance to emerald ash borer (Whitehill et al., 2012). Similarly, high levels of total phenolic compounds were found in three pine species resistant to pine wilt disease (PWD), while low levels were detected in the susceptible species *Pinus sylvestris* (Pimentel et al., 2016; Trindade et al., 2022). When comparing the levels of total phenolic compounds in the also susceptible *Pinus pinaster*, high levels were also measured, showing that in fact there is no correlation with resistance to PWD (Pimentel et al., 2016; Trindade et al., 2022). Therefore, to understand disease resistance mechanisms that may be useful for the

development of biomarkers or to highlight candidate genes for resistance in a susceptible species of interest, resistant and susceptible plants should be compared within this species (Sniezko and Koch, 2017). This strategy has been adopted in our study.

For *P. pinaster*, two breeding programs for resistance to PWD have been initiated in Portugal (Carrasquinho et al., 2018) and Spain (Menéndez-Gutiérrez et al., 2017a). In Spain, 81 families from the Galician *P. pinaster* breeding program (Zas and Merlo, 2008) focused on classical traits such as productivity and stem straightness, were evaluated for their response to artificial inoculations with PWN (Menéndez-Gutiérrez et al., 2017a). In Portugal, trees were chosen from a mass selection program initiated in 2009, in which healthy adult trees in a heavily affected area were identified as potential resistant trees (Ribeiro et al., 2012). The progeny of 96 of these open-pollinated trees was artificially inoculated with PWN and evaluated for survival (Carrasquinho et al., 2018). In these studies, moderate family heritability for survival (0.37; Carrasquinho et al., 2018) and mortality (0.59; Menéndez-Gutiérrez et al., 2017a) after inoculation was obtained. For our study, we selected one of the 15 top-ranked half-sib families characterized by Carrasquinho et al. (2018) to be used for coding and non-coding transcriptomics analysis, as well as SNP detection.

By choosing to analyse samples from a single half-sib family, we expected to obtain less variability in the transcriptomic responses of plants with the same phenotype (Modesto et al., 2021; Modesto et al., 2022a), as clones were not available. To further account for variation in *P. pinaster* individual plant response to PWN inoculation, we selected the plants for which the symptoms progressed more quickly as the susceptible group for RNA sequencing. Although a low level of variation between plants with the same phenotype increases statistical power for transcriptomic analysis, there might be a risk that the detected plant defence mechanisms are specific to the half-sib family studied. However, as mentioned in section 1.3., several of the pathways and genes here implicated in resistance to PWN have been

also reported as relevant for resistance in other pine species (Hirao et al., 2012; Liu et al., 2017; Nose and Shiraishi, 2011). If resistance mechanisms are similar between different susceptible species, it is likely that they are also common among *P. pinaster* families. On the other hand, there is no information available about the expression of small RNAs (sRNA) in the stem of other pine species during PWN infection and it remains unknown if the microRNAs (miRNAs) here identified are involved in the defence response of susceptible pine species, or even of other *P. pinaster* families. Studying the coding and non-coding transcriptional response of more *P. pinaster* families would help elucidate if any family-specific responses have been detected by our analysis. Although the low variation between samples from the same phenotype group is an advantage for coding and non-coding transcriptomics analyses, it would have been more interesting to have high genetic variation for SNPs discovery, by including samples from several families. Nevertheless, it was possible to identify a high number of SNPs (186,506) in our samples, from which some might be associated with PWD resistance (Modesto et al., 2022b).

5.2. Resistance mechanisms highlighted by the transcriptomics analysis

Aiming at discovering the pathways involved in *Pinus pinaster* resistance to PWN, we compared the transcriptional changes after inoculation in resistant and susceptible plants (Modesto et al., 2021). Differential gene expression analysis revealed the activation of phytohormone pathways, namely SA, JA and ABA pathways, secondary metabolism pathways, such as terpene biosynthesis and phenylpropanoid biosynthesis, lignin biosynthesis pathway, oxidative stress response genes, and resistance genes. Based on these results, we quantified several hormones and lignin contents in stem samples. The higher lignification of stem tissues around the inoculation zone in resistant plants suggests that limiting PWN migration and feeding on plant tissues might be crucial for PWD resistance. On the other hand, the detection of higher levels of SA in susceptible plants

suggests that the activation of this hormone pathway is linked to susceptibility to PWN. Although several of the pathways and functions highlighted by our study have been associated to response or resistance to PWN based on differential expression analyses in other pine species (e.g., Gaspar et al., 2020; Hirao et al., 2012; Liu et al., 2017; Nose and Shiraishi, 2011; Shin et al., 2009), the relevance of phytohormones pathways had not yet been reported. For *P. pinaster*, higher levels of SA were also detected in the susceptible plants of another half-sib family (family 152) (Rodrigues et al., 2021). The finding of similar results in different studies and distinct *P. pinaster* families supports the relevance of the SA pathway in PWD susceptibility in this pine species.

Although the transcriptomics results indicated several pathways potentially relevant to PWN resistance, which translated into changes in metabolite levels in the case of lignin and phytohormones (Modesto et al., 2021), future proteomics and metabolomic studies should be performed to confirm their roles. Assays such as quantification of ROS and its correlation with the quantity and activity of ROS detoxifying enzymes, quantification of specific secondary metabolites, and the study of their effects on PWNs and *M. galloprovincialis* feeding, among others, would improve our understanding of how these defence mechanisms influence the plant phenotype after PWN infection. Further functional characterization of candidate genes in model systems could confirm their roles in resistance to PWD (Naidoo 2019).

Other than the nematicidal and repellent effects that terpene compounds may have on PWN (Liu et al., 2020; Suga et al., 1993), they seem to also influence the feeding behaviour of the insect vector *Monochamus alternatus* (Chen et al., 2021). The induction of terpenes biosynthesis by JA application in *P. massoniana* seedlings inhibited *M. alternatus* feeding (Chen et al., 2021). Interestingly, several chemotypes regarding the constitutive composition of terpene compounds have been described for *P. pinaster* (Rodrigues et al., 2017). It would be interesting to investigate if

these chemotypes have an influence on PWD development or on *M. galloprovincialis* feeding choices, which would influence the spread of the disease. In case of positive correlations, specific metabolites or their combination may be potentially used as biomarkers for the selection of *P. pinaster* plants to be planted in the field.

Since PWD was introduced in Portugal, slightly over 20 years ago (Mota et al., 1999), there was not enough time for *P. pinaster*, or any other tree species with such a long lifespan, to evolve resistance to this disease. Therefore, the genetic variation found in the survival of seedlings inoculated with PWN has likely evolved in response to other biotic stresses or through genetic drift. The molecular mechanisms here associated with resistance to PWD are probably part of a basal defence response effective against several pathogens or pests. In fact, several pathways and genes highlighted by the present work have been described in conifer interactions with other pests and pathogens. For instance, the expression of genes related to ROS scavenging, such as *peroxidases*, *glutathione S-transferase* or *thioredoxins*, cell-wall related genes, such as *hydroxyproline-rich glycoprotein* (*HRPG*), *PR* genes, such as *PR-1*, *PR-5* or *chitinases*, and phytohormones signalling, have been reported in conifers response to several pathogenic fungi (e.g., *Pinus monticola* response to *Cronartium ribicola*, *P. pinaster* response to *Fusarium circinatum*, or *Pinus sylvestris* response to *Heterobasidion annosum*) (Hernandez-Escribano et al., 2020; Li and Asiegbu, 2004; Liu et al., 2013; Zamany et al., 2012) and herbivorous insects (e.g., *Pinus sylvestris* response to *Hylobius abietis*, or *Picea sitchensis* response to *Choristoneura occidentalis* and *Pissodes strobi*) (Kovalchuk et al., 2015; Ralph et al., 2006). In conifer response to insects, special focus has been given to the role of secondary metabolites (e.g., Martin et al., 2002; Moreira et al., 2009; Zas et al., 2014; Zeneli et al., 2006; Zhao et al., 2004; Zulak et al., 2009). The expression of genes involved in the phenylpropanoid biosynthesis pathway, such as *PAL*, *4CL* or *chalcone synthases*, as well as several *terpene synthase* genes, were induced by *H.*

abietis feeding on *P. sylvestris* (Kovalchuk et al., 2015), *C. occidentalis* and *P. strobi* feeding on *P. sitchensis* (Ralph et al., 2006), and methyl-jasmonate (MeJA) application in *Picea glauca* (Celedon et al., 2017). The overexpression of *peroxidases* and *laccases*, involved in lignin synthesis, has also been observed in response to herbivorous insects (Kovalchuk et al., 2015; Ralph et al., 2006).

5.3. Pathways post-transcriptionally regulated during *P. pinaster* response to PWN

The role of miRNAs in the post-transcriptional regulation of *P. pinaster* response to PWN inoculation was also investigated (Modesto et al., 2022a). Several conserved miRNA families that were previously described as important for plant defence response were here identified (e.g., miR159, miR166, miR390, miR396). To characterize the putative functions of these miRNAs, we analysed their predicted target genes. However, miRNA target prediction algorithms can identify thousands of targets, with a high degree of false positives (Srivastava et al., 2014; Zhou et al., 2020). As plant miRNAs mostly act by leading to the cleavage of their target mRNAs, the expression levels of a miRNA and its target are expected to negatively correlate, although exceptions are known to occur (Yu et al., 2017). Therefore, the mRNA transcriptomics data was integrated with the miRNA expression data, allowing for a more reliable target prediction and functional analysis. Although integrating miRNA and mRNA expression data increases the accuracy of the predicted targets, experimental validation of the interaction between miRNAs and respective target genes is still necessary. Physical interactions can be observed, for instance, by using a luciferase reporter system (Alves et al., 2022; Riolo et al., 2021; Zhou et al., 2020). In PWN inoculated plants, the pathways and genes putatively regulated by miRNAs included JA response genes, genes involved in ROS detoxification, terpenoid biosynthesis pathways, *RLKs/RLPs* and resistance genes, emphasizing the importance of these mechanisms in *P. pinaster* immune response to PWN. On the other hand, the analysis of the

targets of miRNAs differentially expressed between resistant and susceptible plants highlighted the relevance of JA pathway, *RLKs* and *GDP-L-fucose synthase*, with a possible role in the initiation of PTI and ETI, in resistance to PWD.

Several of the miRNA conserved families identified as responsive to PWN were previously implicated in defence response to root-knot nematodes or cyst nematodes in angiosperms (e.g., Arabidopsis, soybean) (Hewezi et al., 2008; Jaubert-Possamai et al., 2019; Tian et al., 2017), as well as *Pinus taeda* response to fusiform rust (Lu et al., 2007) (e.g., miR159, miR166, miR390, miR396). However, the predicted targets for the miRNAs of these families in *P. pinaster*-PWN interaction were distinct from the ones described in the literature (e.g., Hewezi et al., 2008; Jaubert-Possamai et al., 2019; Tian et al., 2017). The differences in the predicted target genes might be due to differences in the targets' sequences between species. On the other hand, there is a great variety of miRNA isoforms in each conserved family that may be differentially expressed depending on the plant tissues or environmental conditions being studied (Guo and Chen, 2014; Neilsen et al., 2012; Perdiguero et al., 2020). The various isoforms, although belonging to the same miRNA family, may have different target genes. As the defence mechanisms involved in the response to biotrophic pathogens, such as fusiform rust fungus or sedentary nematodes, are mostly described as antagonistic of the response to migratory nematodes or herbivore insects (Caarls et al., 2015), it is plausible that miRNAs regulate distinct genes and pathways in these immune responses, even though the same miRNA families might be involved.

Given that evidence for trans-kingdom gene silencing in plant-pathogen and plant-pest interactions has been accumulating in recent years (Han and Luan, 2015; Karimi and Innes, 2022; Rose et al., 2019), we hypothesized that PWN miRNAs might target *P. pinaster* genes. Several *P. pinaster* genes were predicted as being targeted by the PWN miRNAs identified in the stem samples after inoculation, which were supported by degradome

data. The identified miRNAs may have an important role in PWN pathogenicity by interfering with *P. pinaster* defence response, as it was found in other plant-pathogen interactions (e.g., Wang et al., 2016; Wang et al., 2017; Weiberg et al., 2013). Despite the support of the degradome data for mRNA cleavage guided by these miRNAs, further experimental analyses are necessary to confirm the transfer of miRNAs between species, the miRNA-mRNA physical interactions and the resulting mRNA cleavage. Such analyses can help to better understand the pathogenicity of PWN.

The silencing of pathogen genes by plant sRNAs has also been reported (Cai et al., 2018; Wang et al., 2016; Zhang et al., 2016), showing that trans-kingdom sRNA transference can be bidirectional. Although the transference of naturally occurring sRNAs was never described for plant-nematode interactions, host-induced gene silencing (HIGS) has been shown to be an efficient method to disrupt nematode pathogenicity (Ghag, 2017; Han and Luan, 2015; Karimi and Innes, 2022). HIGS is a method in which plants are genetically engineered to express a double strand RNA (dsRNA) or hairpin RNA (hpRNA) that is processed into a small interfering RNA (siRNA), miRNA or artificial miRNAs (amiRNA), which then targets and silences the pathogen or pest genes, conferring resistance to the transgenic plant. This technique has the potential to be used as an efficient pest management method for nematodes (Dinh et al., 2014; Ibrahim et al., 2011), insects (Zha et al., 2011; Zhang et al., 2015) and pathogens (Koch et al., 2013; Nowara et al., 2010). In *P. pinaster*-PWN interaction, several PWN genes were predicted to be targeted by *P. pinaster* miRNAs. However, this *in silico* analysis was not validated experimentally and more evidence is needed to confirm if the plant miRNAs (or hpRNAs) are naturally transferred to the PWN cells and are able to silence the nematode gene expression. The naturally occurring trans-kingdom gene silencing would open new opportunities for management of PWD. The use of HIGS is, however, not currently possible in several regions of the world, such as Europe, where the plantation or sowing of transgenic plants is not allowed in open fields.

Another technique based on the similar principles as HIGS that does not involve transgenic plants is the spray-induced gene silencing (SIGS), in which dsRNA is sprayed on the plant, causing gene silencing in the pathogen (Koch et al., 2016; Werner et al., 2020) or pest (Biedenkopf et al., 2020; San Miguel and Scott, 2016). SIGS should therefore be explored as method for PWN gene silencing in *P. pinaster* infected plants.

5.4. Discovery of SNPs potentially associated with PWD resistance

Identifying SNPs in a species with a large genome size (28 Gb) and many highly repetitive regions (Sterck et al., 2022) such as *P. pinaster*, can be difficult and expensive. Using transcriptome data instead allows for the identification of SNPs mostly in coding regions, which can be more easily linked to candidate genes with interesting functions, in an affordable way. On the other hand, SNPs here described (Modesto et al., 2022b) may be linked to causal variants not detectable by the used approach, such as variants in non-coding or regulatory regions, as well as structural variants. The identification of SNPs in individuals from a family included in *P. pinaster* breeding program for PWD resistance, and with contrasting phenotypes, can be particularly useful in future association studies. The identified SNPs may be used to build a SNP array specific for PWD studies in *P. pinaster* or be added to other previously discovered *P. pinaster* SNPs to obtain a more complete and high-density SNP array useful for multiple studies (Chancerel et al., 2013; Plomion et al., 2016). The use of a high-density SNP array may give us more information about genetic variability in the families chosen for breeding, ensuring the desirable high levels of variability to be maintained in breeding programs (Sniezko and Koch, 2017), and thus allowing to maintain the adaptability potential of *P. pinaster* populations to environmental changes and new pests and pathogens.

Despite the small number of phenotyped individuals with available data for SNP discovery, it was possible to identify a high number of SNPs (186,506), even after the application of strict filters (Modesto et al., 2022b). From

these, a small set (31 SNPs), located in candidate genes with functions described as relevant for resistance to PWN (e.g., lignin biosynthesis, biosynthesis of phenolic compounds) were highly divergent between resistant and susceptible plants. This set of SNPs can be particularly interesting for association studies aiming at finding molecular markers for PWD resistance. Accordingly, the association of two of these SNPs with plant phenotype after PWN inoculation was confirmed when a larger sample size (40 samples) of the same *P. pinaster* family was genotyped. However, the association results were not significant after correction for multiple testing and should, therefore, be taken with caution and be further explored in future association analysis, as well as functional studies. The genotyping of a larger sample size, including a higher number of *P. pinaster* families, is still needed to elucidate if these SNPs can be used in the future as molecular markers for breeding for PWD resistance.

5.5. Conclusions

Overall, this work largely contributed to a better understanding of *P. pinaster* resistance response to PWD, providing new insights into the molecular mechanisms involved in resistance and the pathways possibly regulated at the post-transcriptional level during *P. pinaster* immune response to PWN. The defence response in susceptible and resistant plants seems to be similar, although more intense and efficient in resistant plants (Figure 5.1). The higher expression of genes encoding for chitinases that may interfere with nematode development and feeding, oxidative stress response genes that are essential to prevent damage caused by ROS, the activation of secondary metabolites synthesis pathways that may directly affect PWN mobility or survival, and the reinforcement of cell walls through lignification that may limit nematode migration, probably act together to achieve resistance. The activation of these genes and pathways may be mediated by the JA signalling pathway in resistant plants, while in susceptible plants the activation of the SA signalling pathway may inhibit the JA response. The regulation of the JA response pathway, oxidative

stress response genes, and terpenoid biosynthesis by miRNAs emphasizes the importance of these processes in *P. pinaster* response to PWN. The detection of SNPs highly divergent between resistant and susceptible plants in genes encoding for enzymes involved in the synthesis of lignin and phenolic compounds further support their relevance in PWN resistance. Interestingly, trans-kingdom gene silencing by *P. pinaster* miRNAs of PWN genes involved in functions such as the detoxification of plant xenobiotic compounds or digestion of plant tissues seems to also have a role in plant resistance.

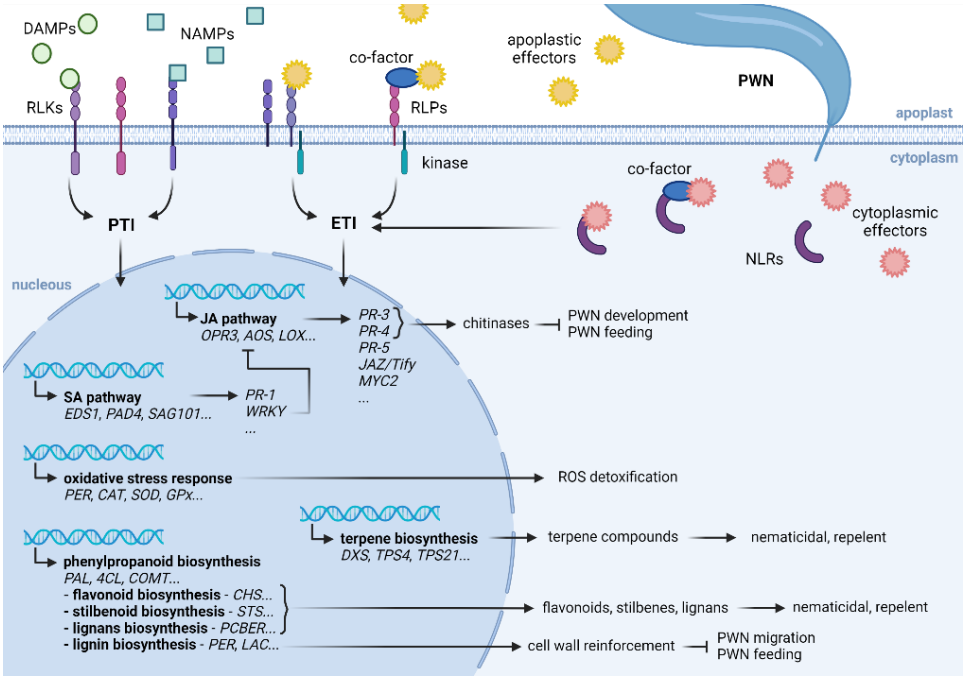


Figure 5.1. Representation of *P. pinaster* defence response to pinewood nematode (PWN). Pattern-triggered immunity (PTI) is initiated after recognition of nematode-associated molecular patterns (NAMPs), such as BxCDP1, or damage-associated molecular patterns (DAMPs) from damaged plant cells, by cell surface receptor-like kinases (RLKs) or receptor-like proteins (RLPs). PTI leads to a transcriptional reprograming of the plant cell, which includes the activation of hormone pathways, such as jasmonic acid (JA) and salicylic acid (SA) pathways, the expression pathogenesis-related genes (PR), genes involved in oxidative stress response, and genes encoding enzymes involved in the biosynthesis of secondary metabolites (terpenes and phenylpropanoids). In susceptible plants, it is possible that the activation of the SA pathway inhibits the JA pathway. PWN releases

effectors, such as the apoplastic VAP1 and BxSapB1, that repress the plant defence response. These effectors may be recognized by resistance genes, whether directly or indirectly through the monitoring of a co-factor protein that is altered by the effector. Effectors can be recognized by cell surface receptors, when they are apoplastic, or internal nucleotide-binding/leucine-rich-repeat (NLR) receptors, when they are injected into the cytoplasm by the PWN. Recognition of effectors by resistance genes initiates a stronger and more sustained defence response, the effector-triggered immunity (ETI).

Based on the conclusions from this study, promising new approaches for PWD management, such as the use of SIGS or the use of biomarkers (e.g., lignin, terpene compounds) for selecting resistant trees in breeding programs, were highlighted for further investigation. Although it was not possible to develop robust molecular markers for PWD resistance, the first steps were given in this direction by identifying a high number of interesting SNPs that can be used for future association studies, as well as several candidate genes with interesting functions that may be relevant to PWD resistance. In the case of quantitative traits such as PWD resistance, the development of plants with durable resistance is a major challenge. However, as supported by our results, the analysis of diverse and complementary data generated by genomics, transcriptomics, and metabolomics, among other approaches, may be key to the design of innovative strategies to mitigate the devastating effects of PWD. The availability of robust genomic variability and phenotypic data will further allow taking advantage of approaches such as GWAS to advance breeding efforts towards increased disease resistance.

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Zha, W., Peng, X., Chen, R., Du, B., Zhu, L. & He, G. (2011) Knockdown of Midgut Genes by dsRNA-Transgenic Plant-Mediated RNA Interference in the Hemipteran Insect *Nilaparvata lugens*. *PLOS ONE*, 6, e20504.

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Curriculum Vitae

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Work Experience

February 2016 - now	**Doctoral Researcher** Bioinformatics and Evolutionary Genomics Lab – VIB, Ghent University Forest Biotech Lab – ITQB/NOVA, Portugal Supervised by: Prof. Célia Miguel (ITQB/NOVA), Prof. Yves Van de Peer (VIB) and Dr. Isabel Carrasquinho (INIAV) Fundamental research on plant disease resistance using bioinformatics (study model: pine wilt disease in maritime pine) • Phenotyping of inoculated plants in several assays • RNA and DNA purification, RT-qPCR, PCR • Maintenance of database with phenotyping data and metadata • Development of transcriptomics, microRNA and SNP analysis pipelines and biological interpretation of data • Running my own project and collaboration with INIAV, Portugal • Guidance of bachelor and master students
June 2015 - December 2015	**Research Fellowship** Forest Biotech Lab – ITQB/NOVA, Portugal Supervised by: Prof. Célia Miguel Fundamental research on plant disease resistance using molecular biology techniques (study model: pine wilt disease in maritime pine) • Drawing up experimental design • Developing phenotyping method • Optimizing protocols

April 2013 - May 2015	**Research Fellowship** CoBiG2 – Faculty of Science, University of Lisbon (FCUL), Portugal CIFC – Instituto Superior de Agronomia, University of Lisbon (ISA), Portugal Forest Biotech Lab – ITQB/NOVA, Portugal Supervised by: Prof. Octávio Paulo (FCUL), Dr. Dora Batista (ISA) and Prof. Célia Miguel Fundamental research on a pathogenic fungus that infects coffee plants (study model: coffee leaf rust) • DNA purification, PCR, Sanger sequencing • Phylogeographic analysis • SNP detection and association with virulence phenotypes
January 2012 - December 2012	**Research Fellowship** Forest Biotech Lab – ITQB, NOVA, Portugal Supervised by: Prof. Célia Miguel and Dr. Dora Batista Fundamental research on plant adaptation to climate (study model: cork oak) • DNA purification, PCR, Sanger sequencing • SNP association analysis with climate variables
November 2008 - July 2009	**Integration in Research Fellowship** CBA, Faculty of Science of the University of Lisbon, Portugal Supervised by: Prof. Manuela Coelho Assisting in the development of a DNA quantification method

Education

2022 (exp.)	**Joint PhD degree in Bioinformatics** Ghent University, Belgium **Joint PhD degree in Molecular Biology** NOVA University of Lisbon, Portugal

2012	**Master degree in Evolutionary and Developmental Biology** University of Lisbon, Portugal
2009	**Bachelor degree in Molecular Biology and Genetics** University of Lisbon, Portugal

Publications

Publications in indexed journals

Modeto I, Mendes A, Carrasquinho I, Miguel CM (2022) Molecular Defense Response of Pine Trees (*Pinus* spp.) to the Parasitic Nematode *Bursaphelenchus xylophilus*. *Cells*, 11, 3208. doi: 10.3390/cells11203208

Modesto I, Inácio V, Novikova P, Carrasquinho I, Van de Peer Y, Miguel CM (2022). SNP Detection in *Pinus pinaster* Transcriptome and Association with Resistance to Pinewood Nematode. *Forests*, 13, 946. doi: 10.3390/f13060946.

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Modesto I, Sterck L, Arbona V, Gómez-Cadenas A, Carrasquinho I, Van de Peer Y, Miguel CM (2021). Insights Into the Mechanisms Implicated in *Pinus pinaster* Resistance to Pinewood Nematode. *Frontiers in Plant Sciences* 12, 1–18. doi:10.3389/fpls.2021.690857.

Modesto IS, Miguel C, Pina-Martins F, Glushkova M, Veloso M, Paulo OS, Batista D (2014). Identifying signatures of natural selection in cork oak (*Quercus suber* L.) genes through SNP analysis. *Tree Genetics and Genomes* 10, 1645–1660. doi:10.1007/s11295-014-0786-1.

Book chapters

Sterck L, de María N, Cañas RA, de Miguel M, Perdiguero P, Raffin A, Budde KB, López-Hinojosa M, Cantón FR, Rodrigues AS, Morcillo M, Hurel A, Vélez MD, de la Torre FN, **Modesto I**, et al. (2022). “Maritime Pine Genomics in Focus” in *The Pine Genomes*, ed. A. R. De La Torre (Cham:

Springer International Publishing), 67–123. doi:10.1007/978-3-030-93390-6_5.

Publications in proceedings with referee

Batista D, **Modesto I**, Loureiro A, Martins R, Pereira AP, Talhinhas P, Paulo OS, Várzea V (2014). Highly divergent alleles in a candidate gene of Hemileia vastatrix suggest a putative role of adaptive significance. In: "Proceedings of the 25th International Conference on Coffee Science". (Eds.) Association for Science and Information on Coffee (ASIC), Armenia, Colombia.

Paulo OS, Costa JS, Pina-Martins F, **Modesto I**, Simões F, Matos J, Almeida H, Miguel C, Veloso M, Oliveira MM, Ricardo C, Batista D (2012). Cork Oak genetic differentiation and diversity. In: "Present and Future of Cork Oak in Portugal". Oliveira MM, Matos JA, Saibo NJ, Miguel C, Gil L (Eds.) Soc. Portuguesa de Fisiologia Vegetal, Oeiras, Portugal, pp: 31-32.

Paulo OS, Pina-Martins F, **Modesto I**, Batista D (2012) Cork Oak ESTs Consortium - Polymorphism detection and validation. In: "Present and Future of Cork Oak in Portugal". Oliveira MM, Matos JA, Saibo NJ, Miguel C, Gil L (Eds.) Soc. Portuguesa de Fisiologia Vegetal, Oeiras, Portugal, pp: 129-130.

Communications in conferences

Oral presentations

Modesto I, Mendes A, Inácia V, Novikova P, Carrasquinho I, Van de Peer Y, Miguel C (2022). *Pinus pinaster* resistance to pine wilt disease: uncovering early molecular responses and mechanisms involved. 20th IUFRO Tree Biotech and the 2nd FTMB Conference, 7-9 July, Harbin, China (*online*). (oral presentation by Miguel C)

Modesto I, Sterck L, Novikova P, Inácio V, Arbona V, Gómez-Cadenas A, Carrasquinho I, Van de Peer Y, Miguel CM (2022). Defense mechanisms highlighted by the transcriptional response of *Pinus pinaster* to pinewood nematode. Webinar "Pine Wilt Disease: recent advances in molecular analyses and breeding approaches", 30 June, FCUL, Lisbon, Portugal (*online*) (oral presentation by Modesto I)

Modesto I, Mendes A, Milhinhos A, Inácio V, Silva AC, Van de Peer Y, Miguel CM (2022). MicroRNAs as post-transcriptional regulators of response to pinewood nematode infection. Webinar “Pine Wilt Disease: recent advances in molecular analyses and breeding approaches”, 30 June, FCUL, Lisbon, Portugal (*online*) (oral presentation by Mendes A)

Inácio V, **Modesto I**, Milhinhos A, Silva C, Carrasquinho I, Cervera MT, Cabezas JA, Miguel CM (2022). Genomic variability associated with *Pinus pinaster* resistance to Pinewood Nematode. Webinar “Pine Wilt Disease: recent advances in molecular analyses and breeding approaches”, 30 June, FCUL, Lisbon, Portugal (*online*). (oral presentation by Inácio V)

Modesto I, Sterck L, Arbona V, Gómez-Cadenas A, Carrasquinho I, Van de Peer Y, Miguel CM (2022). Uncovering the mechanisms involved in *Pinus pinaster* resistance to pine wilt disease by analysis of coding and non-coding transcriptomes. 16th Congress of the Mediterranean Phytopathological Union, 4-8 April, Limassol, Cyprus. (oral presentation by Miguel CM)

Modesto I, Sterck L, Milhinhos A, Inácio V, Carrasquinho I, Van de Peer Y, Miguel CM (2020). Genomic and genetic approaches to identify molecular markers of resistance to pine wilt disease in *Pinus pinaster*. Workshop “Biotechnology/Synthetic Biology Approaches to Forestry Management”, 25 September, organized by ITQB NOVA (iGEM), Oeiras, Portugal (*online*). (oral presentation by Miguel CM)

Modesto I, Sterck L, Carrasquinho I, Van de Peer Y, Miguel CM (2020). Resistance to pine wilt disease in *Pinus pinaster*: molecular response and regulation. 10th ITQB NOVA PhD Students Meeting, 22-24 January, ITQB NOVA, Oeiras, Portugal. (oral presentation by Modesto I)

Modesto I, Sterck L, Carrasquinho I, Van de Peer Y, Miguel CM (2019) Transcriptomic analysis reveals pathways associated with resistance to pinewood nematode in *Pinus pinaster*. IUFRO Tree Biotechnology Meeting, 23-28 June, Raleigh, NC USA. (lightning talk presented by Miguel CM and poster)

Modesto I, Sterck L, Carrasquinho I, Van de Peer Y, Miguel CM (2018). Identification of genes involved in the resistance response of *Pinus pinaster* to the pinewood nematode infection. 6th International Workshop on the Genetics of Tree-Parasite Interactions: Tree Resistance to Insects and Diseases: Putting Promise into Practice, 5-10 August, Mt. Sterling, OH, EUA. (oral presentation by Modesto I)

Modesto I, Sterck L, Carrasquinho I, Van de Peer Y, Miguel CM (2017). Differential gene expression analysis reveals different responses to pine wilt disease between susceptible and tolerant maritime pine seedlings. Proteção das Plantas 2017 - 2º Simpósio SCAP de Proteção das Plantas; 8º Congresso da Sociedade Portuguesa de Fitopatologia e 11º Encontro Nacional de Proteção Integrada, 26-27 October Santarém, Portugal. (oral presentation by Miguel CM)

Poster presentations

Inácio V, **Modesto I**, Milhinhos A, Silva C, Carrasquinho I, Cervera MT, Cabezas JA, Miguel CM (2022). Genomic variability associated to *Pinus pinaster* resistance to pinewood nematode. 20th IUFRO Tree Biotech and the 2nd FTMB Conference, 7-9 July, Harbin, China (*online*).

Mendes A, **Modesto I**, Milhinhos A, Inácio V, Miguel CM (2022) Characterization of miRNAs implicated in maritime pine resistance to pinewood nematode. III International Meeting of the Portuguese Society of Genetics, 27-28 June, Universidade de Évora, Portugal.

Modesto I, Sterck L, Arbona V, Gómez-Cadenas A, Carrasquinho I, Van de Peer Y, Miguel CM (2021). Transcriptomic and biochemical analyses highlight the molecular mechanisms involved in *Pinus pinaster* resistance to pine wilt disease. Plant Biology Europe 2021, 28 June - 1 July (*online*).

Modesto I, Sterck L, Carrasquinho I, Van de Peer Y, Miguel CM (2017). Transcriptome analysis reveals different responses to pine wood nematode infection in tolerant and susceptible *Pinus pinaster* seedlings. 8th ITQB NOVA PhD Students Meeting, 15-17 November, ITQB NOVA, Oeiras, Portugal.

Modesto IS, Loureiro A, Martins R, Pereira AP, Várzea V, Paulo OS, Batista D, Miguel C (2015). Analysis of allele-specific expression in a putative pathogenesis-related gene of coffee leaf rust. XVth Congress of the European Society of Evolutionary Biology (ESEB2015), 9-14 August, University of Lausanne, Lausanne, Switzerland.

Batista D, **Modesto I**, Loureiro A, Martins R, Pereira AP, Talhinhas P, Paulo OS, Várzea V (2014). Highly divergent alleles in a candidate gene of *Hemileia vastatrix* suggest a putative role of adaptive significance. 25th International Conference on Coffee Science – ASIC, 8-13 September, Centro Cultural Metropolitano de Convenciones del Quindío, Armenia, Colombia.

Modesto IS, Miguel C, Pina-Martins F, Glushkova M, Veloso M, Batista D, Paulo OS (2013). Identifying signatures of natural selection in cork oak (*Quercus suber* L.) genes. XIV Congress of the European Society of Evolutionary Biology (ESEB2013), 19-24 August, Faculty of Sciences of the University of Lisbon, Lisbon, Portugal.

Modesto IS, Miguel C, Pina-Martins F, Glushkova M, Veloso M, Paulo OS, Batista D (2013). Assessing adaptive genetic variation in cork oak (*Quercus suber* L.) through SNP analysis. XIII Luso-Spanish Congress of Plant Physiology (FV2013), 24-27 July, Faculty of Sciences of the University of Lisbon, Lisbon, Portugal.

Modesto IS, Miguel C, Pina-Martins F, Glushkova M, Veloso M, Paulo OS, Batista D (2013). Testing associations between genetic and environmental variation in cork oak (*Quercus suber* L.). IUFRO Tree Biotechnology Conference 2013, 26 May - 1 June, Convention Center of the Marriott Renaissance Hotel, Asheville, NC, USA.

Modesto IS, Miguel C, Pina-Martins F, Glushkova M, Veloso M, Paulo OS, Batista D (2012). Population frequency data of SNP markers in cork oak. IUFRO 2012 conference “Genetics of Fagaceae and Nothofagaceae”, 9-12 October, INRA, University of Bordeaux, France.

Modesto IS, Pina-Martins F, Batista D, Paulo OS (2011). Single Nucleotide Polymorphisms validation and preliminary application as phylogeographical markers in cork oak. VII National Meeting of Evolutionary Biology, 21 December, University of Coimbra, Portugal.

Paulo OS, Pina-Martins F, **Modesto I**, Batista D (2011). Cork Oak ESTs Consortium - Polymorphism detection and validation. Workshop "Present and Future of Cork Oak in Portugal", 21 October, ITQB-UNL, Portugal.

Additional training

2019	**Whole transcriptome data analysis** EMBL (June 3-7), Heidelberg, Germany
2018	**Basic statistics in R, part II** VIB BITS (May 25 and June 1), Ghent, Belgium
	Basic statistics in R, part I VIB BITS (May 15, 22 and 28), Ghent, Belgium

	Introduction to Biopython VIB BITS (March 29-30) Ghent, Belgium
2017	**Python for data processing** VIB BITS (October 5-6), Leuven, Belgium
	Gentle hands-on introduction to Python programming VIB BITS (September 18 and 25), Leuven, Belgium
	Functional Plant Bioinformatics using PLAZA VIB PSP and ELIXIR Belgium (September 14 and 15), Gent, Belgium
	RNA-Seq analysis for differential expression in GenePattern VIB BITS (May 8), Leuven, Belgium
2016	**Bioinformatics of Plants and Plant Pathogens 2016** EBI-EMBL (May 23-25), Hinxton, UK
2015	**GACT15 - Genomic Architecture of Complex Traits** GTPB, IGC (September 28-October 1), Oeiras, Portugal
2014	**Phython 101** CoBiG^2, CBA, FCUL (January 6-10), Lisbon, Portugal
2012	**BFB12 - Biostatistical Foundations in Bioinformatics** GTPB, IGC (December 3-7), Oeiras, Portugal
2010	**Practical Course on Phylogeography** CoBiG^2, CBA, FCUL (September 13-17), Lisbon, Portugal
	Techniques for communicating and presenting results ("Técnicas de Comunicação e Apresentação de Resultados") WeValue, ICAT (November 27-28), Lisbon, Portugal

Teaching and mentoring

2020	**Bachelor course "Project" ("Genotipagem de SNPs por PCR e sequenciação Sanger para associação com a resistência ao nemátodo da madeira em pinheiro bravo")** Bachelor in Cellular and Molecular Biology, FCT-NOVA University of Lisbon 21 February-31 July Student: Madalena Matos
2015-2016	**Master thesis ("Molecular analysis of the response of *Pinus pinaster* plants with differential behavior towards the Pine Wood Nematode infection")** School of Sciences, University of Minho 1 September 2015-12 December 2016 Student: Ana Sofia Gonçalves
2011	**Master course "Research practice"** Masters in Developmental and Evolutionary Biology, Faculty of Sciences, University of Lisbon, Portugal 1 July-22 July Student: João Fradique **Bachelor course "Laboratory Practice"** Bachelor in Biology – Branch Developmental and Evolutionary Biology, Faculty of Sciences, University of Lisbon, Portugal 1 March-5 April Students: Lara Broom, Sofia Fernandes, Ana Lopes, Ana Santos

Languages

Portuguese	Native
English	Proficient
Dutch	Elementary (A2)

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Oeiras, **August 2022**

Exploring genetic variability of *Pinus pinaster* in the molecular response pathways to the pinewood nematode as a source of markers for assisted selection

Inês Modesto