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Evaluating modes of resistance to *Plasmodiophora brassicae* in *Arabidopsis thaliana*

by

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Statement of the Ph.D. supervisor and auxiliary supervisor

We declare that this work has been performed under our direction, and state that it meets the conditions for presenting it in the procedure for granting the degree of Doctor of Agricultural Sciences in the discipline of agriculture and horticulture.

20/06/2022

Date and signature of the supervisor

20/6/2022 William Turnen

Date and signature of the auxiliary supervisor

Statement of the author

Aware of the legal responsibility, I declare that this doctoral thesis was written by me under the direction of the supervisor and auxiliary supervisor and does not contain information obtained in a manner inconsistent with applicable regulations. I also declare that the presented manuscript has not previously been submitted to obtain a scientific degree in any other institution and is identical to the attached electronic version.

Date and Signature of the author

Soham Mukhopadhyay
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Title

"Evaluating modes of resistance to *Plasmodiophora brassicae* in *Arabidopsis thaliana"*

Abstract

Clubroot disease caused by the obligate biotroph *Plasmodiophora brassicae* (*P. brassicae*) is one of the most economically important diseases of brassica crops. The characteristic symptom of the disease is the development of galls on roots and hypocotyls of infected plants, this leads to serious water and nutrient imbalance and results in massive yield loss. A limited set of resistant cultivars have been developed to manage the disease. As pathotypes capable of breaking resistance against the resistant cultivars spread to wider geography, there is an urgent need to find out new sources of resistance against the disease.

To tackle the problem, 142 natural accessions of *Arabidopsis thaliana* were screened with a P1+ *P. brassicae* pathotype collected from the West Pomerania region of Poland. Arabidopsis, being a member of the Brassicaceae group of plants, is prone to clubroot infection. Due to the availability of a wide variety of genomic data for Arabidopsis, it was possible to carry out a Genome Wide Association Study (GWAS) which resulted in identification of a significant SNP on chromosome 1, linked with resistance to clubroot disease. This SNP coincided with a previously identified locus named *RPB1* (*Resistance to Plasmodiophora brassicae 1*) absent in the Col-0 reference genome. *RPB1* codes for a small protein of 16 kDa (148 aa) with no known domains present. The role of *RPB1* was characterized by transferring the gene into susceptible Col-0 under various expression conditions. No transgenic Arabidopsis plant could be recovered following repeated transformation with *RPB1* under control of a 35S promoter. *Agrobacterium* mediated transient expression in tobacco leaves with this *35S::RPB1* construct resulted in localized cell death (but not for a GFP-tagged *RPB1* version) prompting the hypothesis that overexpression of *RPB1* in Arabidopsis results in embryo lethality. Three transgenic lines overexpressing a GFP-tagged *RPB1* under control of the *35S* promoter

gave rise to T3 transgenic plants which displayed a wide range of phenotypical variety in terms of plant size and symptoms of autoimmune lesions. Measurement of *GFP-RPB1* expression in transgenic plants inversely correlated with rosette size, *P. brassicae* pathogen load and clubroot gall diameter, while positively correlating with expression of the salicylic acid signalling marker *PR1* in aerial tissue. Thus, it can be assumed that attaching a GFP tag on RPB1 results in interference with its immune signalling capability, but the overexpressing plants still exhibit stunting due to some degree of defense signalling activation. Finally, the *RPB1* gene from resistance accession Est-1 along with its 1 kb upstream promoter region was transferred to the susceptible Col-0 accession. Clubroot infection resulted in activation of the promoter which indicates that *RPB1* is not involved in direct recognition of pathogen virulence factors. Col-0 transgenic plants harboring *RPB1* under its native promoter did not exhibit resistance comparable to Est-1, though pathogen titer was significantly reduced in three of five lines examined. While *RPB1* is involved in clubroot disease resistance, there seems to be additional components missing from susceptible Col-0 to confer full resistance.

Early changes in the transcriptome of susceptible Col-0 and resistant Est-1 accessions were profiled by mRNA-Seq analysis. The jasmonic acid mediated signalling pathway dominated the Col-0 transcriptome, while salicylic acid mediated signalling was the prominent signature of the Est-1 transcriptome. Genes upregulated in Est-1 in response to *P. brassicae* included a wide variety of immune responsive genes showing an ETI response upon pathogen infection. The Col-0 transcriptome carries changes in a few development related pathways that have been previously identified in other gall forming pathogens, showing how diverse pathogens have evolved to hijack similar pathways.

Apart from characterizing resistance responses from the host perspective, the pathogen's ability to mask its surface chitin moieties was also investigated. By comparing transcriptome datasets at various timepoints of the infection, it was noted that chitin responses in the host are substantially repressed at later stages of infection. A putative chitin binding virulence factor, *PBRA_005081*, was cloned from the pathogen and ectopically expressed in Arabidopsis. Transgenic lines exhibited increased

pathogen load and reduced water content upon infection, indicating enhanced disease progression.

In summary, exploitation of Arabidopsis natural accessions resulted in identification of the *RPB1* gene involved in clubroot resistance and its immunogenic properties when transferred to susceptible accession Col-0. A putative virulence factor was also identified which might be used by the pathogen for protection and production of its spores.

Tytuł

„Ocena mechanizmów odporności *Arabidopsis thaliana* (L.) na *Plasmodiophora brassicae* (Woronin)"

Streszczenie

Kiła kapusty wywoływana przez obligatoryjny, glebowy, patogeniczny mikroorganizm biotroficzny - *Plasmodiophora brassicae* zalicza się do chorób prowadzących do największych strat ekonomicznych w uprawie roślin z rodziny kapustowatych. Do najbardziej charakterystycznych objawów choroby należy pojawianie się narośli na części podziemnej rośliny, które to skutkuje poważną utratą plonu. Ochrona roślin oparta jest na stosunkowo ograniczonym zestawie odpornych gatunków uprawnych. Ze względu na to, że patogen łatwo przełamuje istniejące odporności oraz rozprzestrzenia się globalnie istnieje olbrzymia potrzeba identyfikacji nowych źródeł odporności.

W tym właśnie celu przeszukaliśmy populację 142 genotypów *Arabidopsis thaliana* pod kątem oznak odporności na patogen *P. brassicae* P1+ pochodzący z rejonu zachodniego pomorza w Polsce. Jako, że rzodkiewnik należy do rodziny kapustowatych jest on podatny na infekcję przez *P. brassicae*. To oraz dostępność obszernych danych genomowych dla rzodkiewnika pozwoliło na przeprowadzenie badań asocjacyjnych całego genomu (*ang. Genome Wide Association Study, GWAS*), których rezultatem była identyfikacja w obrębie chromosomu nr 1 polimorfizmu (SNP) powiązanego z

odpornością roślin na kiłę kapusty. Ten rejon SNP pokrywał się z nieobecnym w genomie Col-0, wcześniej zidentyfikowanym loci *RPB1* (*Resistance to Plasmodiophora brassicae 1*). Gen *RPB1* koduje niewielkie białko (16kDa, 148 aminokwasów) nie zawierające żadnych dotychczas scharakteryzowanych domen. W niniejszej pracy badano rolę tego czynnika u roślin genotypu wrażliwego Col-0 z wprowadzonym genem *RPB1*. Pomimo wielu prób nie udało się uzyskać roślin transgenicznych z wprowadzonym genem *RPB1* pod kontrolą sekwencji promotorowej 35S pozwalającej na konstytutywną ekspresję. We wcześniejszych eksperymentach wykazano, że na skutek ekspresji przejściowej genu *RPB1* uzyskanej na drodze infiltracji liści tytoniu bakteriami *Agrobacterium tumefaciens* niosącymi konstrukcję genową 35S::RPB1 następowało lokalne zamieranie komórek. To sugeruje, że niemożność uzyskania stabilnych roślin transgenicznych może mieć związek z potencjalnym efektem embrioletalnym. Zjawiska tego nie obserwowano w przypadku wprowadzania konstruktu, w którym stworzono fuzję pomiędzy sekwencją kodująca genu *RPB1* oraz genu reporterowego *GFP*. U otrzymanych trzech transgenicznych linii z wprowadzonym konstruktem 35S::RPB1-GFP stwierdzono szeroki wachlarz zmian fenotypowych dotyczących wielkości roślin oraz powstawanie, charakterystycznych dla odpowiedzi obronnych, nekroz na liściach. Stwierdzono, że poziom ekspresji genu *RPB1-GFP* był w odwrotny sposób skorelowany z ilością patogenu w porażonych roślinach oraz obwodem narośli, podczas gdy akumulacja w części nadziemnej transkryptu genu *PR1* będącego markerem przekazu sygnału kwasu salicylowego była skorelowana pozytywnie. Na podstawie tego można przypuszczać, że dołączenie t.zw. znacznika GFP do sekwencji kodującej *RPB1* skutkuje zaburzeniem zdolności tego ostatniego do pośreniczenia w odpowiedziach obronnych. Nie mniej jednak rośliny transgeniczne produkujące większą ilość RPB1-GFP nadal wykazywały redukcję wzrostu wynikającą z częściowej aktywacji odpowiedzi obronnych.

W pracy do genotypu Col-0 wprowadzono również gen *RPB1* będący pod kontrolą swojej natywnej sekwencji promotorowej (fragment 1 kb) z genomu Est-1. Infekcja uzyskanych roślin przez *P. brassicae* skutkowała aktywacją promotora, co wskazuje na to, że RPB1 nie jest bezpośrednio zaangażowany w rozpoznanie czynników wirulencji

patogenu. Rośliny genotypu Col-0 z wprowadzonym genem *RPB1* pod kontrolą natywnej sekwencji promotorowej nie przejawiały cech odporności na kiłę kapusty obserwowanych u genotypu Est-1, jednakże ilość patogenu akumulowana w tych roślinach była znacznie zredukowana u trzech z pięciu badanych niezależnych linii transgenicznych. Na tej podstawie stwierdzono, że pomimo tego, iż czynnik RPB1 jest zaangażowany w odpowiedzi obronne na kiłę kapusty w podatnym na tę chorobę genomie Col-0 brak również innych komponentów zapewniających pełną reakcję odporności.

W pracy wykonano również profilowanie zmian transkryptomicznych metodą sekwencjonowania mRNA u form podatnych (Col-0) i odpornych (Est-1) na wczesnych etapach rozwoju infekcji. Stwierdzono, że indukcja szlaków sygnałowych kwasu jasmonowego dominowała u formy Col-0, podczas gdy u Est-1 przeważająca była aktywacja szlaków sygnałowych odpowiedzi na kwas salicylowy. Wśród genów, których ekspresja była nasilona po infekcji P. brassicae u genotypu Est-1 było wiele czynników zaangażowanych w reakcje obronne typu ETI (*ang. effector triggered immunity*). W transkryptomach zainfekowanych roślin Col-0 zaobserwowano zmiany w ścieżkach odpowiedzialnych za procesy rozwojowe wcześniej opisywane u roślin tworzących narośle indukowane przez organizmy patogeniczne. To pokazuje jak różne organizmy wytworzyły podobne wzorce pozwalające na przejęcie i przeprogramowanie programów rośliny żywicielskiej.

W niniejszej pracy oprócz charakterystyki odpowiedzi obronnej rośliny przeprowadzono również badania mające na celu określenie zdolności patogenu do maskowania powierzchniowego chityny. Na podstawie Porównania zmian transkryptomicznych następujących w różnych odstępach czasu po infekcji stwierdzono, że odpowiedzi na chitynę u rośliny żywicielskiej były obniżone na późnych etapach rozwoju choroby. Wytypowano i sklonowano z genomu *P. brassicae* gen *PbChiBD2* przypuszczalnie wiążący czynnik wirulencji a następnie uzyskano jego ektopiczną ekspresję w roślinach rzodkiewnika. Otrzymane rośliny transgeniczne wykazywały akumulację większej ilości patogenu oraz spadek zawartości wody po

infekcji *P. brassicae* w stosunku do roślin kontrolnych, co wskazuje na nasilenie przebiegu choroby.

Podsumowując; wykorzystanie zakresu zmienności w naturalnych ekotypach rzodkiewnika przyczyniło się do identyfikacji genu *RPB1* a dalsze badania, w których wprowadzono ten gen do wrażliwego genotypu Col-0 wykazały rolę tego czynnika w reakcjach odporności na kiłę kapusty. Zidentyfikowano również prawdopodobny czynnik wirulencji, który może być wykorzystywany przez patogen w celu zapewnienia rozwoju zarodników przetrwalnikowych.

Abbreviations

ADP	Adenosine diphosphate
AMM	Accelerated mixed model
ATP	Adenosine triphosphate
BH	Benjamini Hochberg
CC	Coiled coil domain
cDNA	Complementary DNA
CDS	Coding sequence
CR	Clubroot resistance
DI	Disease index
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dpi	days post inoculation
EDTA	Ethylenediaminetetraacetic acid
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
GFP	Green Fluorescent Protein
GO	Gene Ontology
GWAS	Genome wide association study
HR	Hypersensitive response
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria-Bertani
LRR	Leucine rich repeats
MAMP	Microbe-associated molecular pattern
MS	Murashige and Skoog
NLR	Nucleotide-binding leucine-rich repeat receptor
OD	Optical density
PAMP	Pathogen associated molecular pattern
PCA	Principal component analysis

PCD	Programmed cell death
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
PTI	Pattern triggered immunity
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
RFP	Red fluorescent protein
RIL	Recombinant inbred lines
RLK	Receptor like kinase
RNA	Ribonucleic acid
RNAse	Ribonuclease
RPB1	Resistance to Plasmodiophora brassicae 1
SA	Salicylic acid
SAR	Systemic acquired resistance
SNP	Single nucleotide polymorphism
T-DNA	Transfer DNA
TAIR	The Arabidopsis Information Resource
TE	TRIS-EDTA
TIR	Toll-Interleukin receptor domain
TRIS	Tris(hydroxymethyl)aminomethane

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1. Introduction

Plasmodiophora brassicae is an obligate biotrophic protist that infects the Brassicaceae family of plants. It enters the plant root via root hairs and subsequently establishes a metabolic sink to divert nutrients from the host to multiply (Hwang et al., 2012). The pathogen-induced proliferation and expansion of host cells leads to the characteristic swelling of the roots known as 'clubs', thus the disease is named clubroot. Economically important crop plants such as oilseed rape, cauliflower, kale and cabbage belonging to the Brassicaceae family are potential hosts for *P. brassicae* infection (Dixon, 2014). Clubroot infection can lead to a massive loss of agricultural output (Dixon, 2009). The disease lifecycle leads to the release of mature spores into the soil which triggers further disease outbreaks in subsequent planting seasons (Tommerup and Ingram, 1971). Clubroot disease is mostly prevalent in the colder climates of Europe and North America, though has been detected in all continents of the world (Dixon, 2009). Clubroot disease is primarily managed by crop rotation, soil amendment strategies and by employing a limited number of resistant cultivars (Diederichsen et al., 2014). Recent reports of emerging *P. brassicae* strains capable of breaking resistance have become a cause of concern (Hejna et al., 2019). In Poland, almost 0.91 million hectares of land is under oilseed rape cultivation (Woźniak et al., 2019). Therefore, protection of such crops by studying clubroot disease is of national interest. This work addresses the molecular interaction between the model plant *Arabidopsis thaliana* and a Polish *P. brassicae* pathotype (P1+). Arabidopsis being a Brassicaceae family member and the *de-facto* model plant of plant science research, provides several advantages with respect to the availability of existing resources in order to gain deeper insight into the interaction with *P. brassicae* and the different facets of resistance mounted by the host. This study adopts the use of population genetics analysis and transcriptome profiling to study resistance and susceptibility pathways in this pathosystem. Furthermore, a putative pathogen secreted protein that may be used to mask certain chemical moieties to escape host detection has been characterized.

1.1 Economic importance of the disease

Plasmodiophora brassicae infects all members of Brassicaceae family (Karling, 1968). *Brassica oleracea* crops include cauliflower, cabbage, kale, kohlrabi, brussels sprouts and broccoli; *Brassica rapa* crops include vegetables such as Chinese cabbage and turnip. The most significant *Brassica napus* crop is oilseed rape but rutabaga is also susceptible and *Brassica juncea* crops includes mustard seed, an important seed oil in some markets. Brassica crops are important sources of macro and micronutrients, regular consumption has been linked to lower levels of diabetes, arthritis, cancer and other autoimmune diseases (Saban, 2018). Thus, clubroot disease is a cause of concern as 15% of the worldwide brassica cultivation area is under risk of infection. In heavily infested fields yield losses can reach up to 90% (Dixon, 2009). In Canada and E.U. countries, oilseed rape cultivation has come under serious threat of clubroot disease (Diederichsen et al., 2009). In Poland 20% of all cultivated vegetables are brassicas and 15% of arable land is used for oilseed rape production (Czubatka-Bieńkowska et al., 2020). Poland ranks 4th in the E.U. in terms of oilseed rape production and 7th worldwide. *P. brassicae* spores have been found present in more than 250k hectares of land used for oilseed rape production (Czubatka-Bieńkowska et al., 2020) . The 'Mendel' cultivar of oilseed rape was the first commercially available source of resistance to clubroot disease in the Polish market. In recent years, strains capable of overcoming the resistance conferred by Mendel have been increasing in prevalence and spreading in Poland and Germany (Zamani-Noor, 2017).

1.2 Clubroot disease management strategies

The economic damage caused by clubroot disease has led to the development of various strategies to control the spread of spores and mitigate the effects of infection.

1. **Monitoring and sanitation:** Farmers in regions at risk of clubroot disease are encouraged to monitor fields for symptoms and remove infected plants. Protocols for the cleaning of clean farm equipment have also been developed to limit the spread of clubroot spores from field to field.
2. **Crop Rotation and bait crop:** It is recommended to keep a two year-long break between brassica crops. This is especially important to maintain genetic resistance of crops. Chronic use of monoculture has been linked to the rapid spread of clubroot disease in Poland. While not growing brassicas, bait crops can also be planted to stimulate germination of the spores. Those motile zoospores can infect bait crops and within 4-5 weeks of sowing removal of the bait, before the pathogen is able to complete its lifecycle, will remove the spores from the land (Peng et al., 2015).
3. **Liming:** Increasing the pH of the soil to above 7.2 has been linked to a reduction in clubroot severity. Calcium carbonate or calcium hydroxide can be used to increase the pH of the soil, this is a popular method and widely used by farmers (Bélec et al., 2004).
4. **Controlling host weeds:** Fast growing weeds belonging to the Brassicaceae family can lead to the spread of clubroot disease and an increase in the spore density in fields. Weed hosts include flixweed, stinkweed, wild mustard and camelina. These weeds should be immediately removed upon detection (Donald & Porter, 2009).
5. **Fungicide:** There is no fungicide specifically developed for clubroot prevention. However, calcium cyanamide has been demonstrated to be somewhat effective. The high costs of application and potential negative effects on human health have limited its use (Tremblay et al., 2005).

6. **Biocontrol agents:** *Bacillus subtilis* strain NCD-2 has been demonstrated to be effective against clubroot disease (Guo et al., 2019). Serenade which is a registered trademark for *Bacillus amyloliquefaciens* strain QST713 widely used in Canada as a popular biocontrol agent is also useful in limiting clubroot symptoms (Lahlali et al., 2011). Endophytic actinobacteria like *Microbispora rosea* and *Streptomyces olivochromogenes* can be used too against *P. brassicae* growth (Lee et al., 2008).
7. **Genetic Resistance:** Clubroot resistant crops are the cheapest and most environment friendly way to manage the disease. According to the Canola Council of Canada, resistant varieties are designated as having less than 30% infection compared to the susceptible varieties. 'Mendel' variety was the most widely used resistant varieties over the years but pathotypes which overcome this form of resistance are increasingly prevalent. Newer varieties which include additional/new resistance loci are- DKTF 98 CR from Bayer and 45CM39 from Pioneer (https://www.canolacouncil.org/).

1.3 A brief history of clubroot discovery

From the medieval times people have been aware of the presence of clubroot disease. Below is a concise historical timeline of development of clubroot study:

- Romans noted spongy fungus like root swellings (Khalid et al., 2022).
- In the 11th century Albert the Great of Cologne recorded clubroot like root deformations while travelling through Italy, Netherlands, Spain, and Belgium (Dixon, 2014).
- Clubroot disease had already gained attention in 17th and 18th century England and Scotland during the agricultural revolution (Dixon, 2014).
- Karling described widespread records of clubroot disease emergence throughout Europe in the 19th and 20th century and also noted the development of vernacular names (Karling, 1968), some examples are given below (Table 1).

Table 1: List of clubroot vernacular names in different countries.

Country	Common name
Poland	Kiła kapusty
Germany	Fingerkrankheit, galle
France	Hernie du chou, gros pied
Denmark	Kaolbrok, Kaalbrok
United Kingdom	Finger-and-toe, Club root

- During the middle of 19th century, losses due to clubroot became severe in parts of Europe and western Asia which prompted deeper investigations. Michael Woronin, a microbiologist working at St. Petersburg Russia identified the causal agent of the clubroot disease and named the pathogen as *Plasmodiophora brassicae* and classified it as a protist (Woronin, 1878). He also described the primary hosts of the disease and identified key steps of the pathogen lifecycle.
- Breeding of first clubroot resistant brassica varieties- 'Bruce' (swede) in Scotland and 'Badger Shipper' (cabbage) in USA, 1930 (Walker, 1960) .
- In the 1980s the ECD (European Clubroot Differential) panel of cultivars was developed which streamlined the pathogen classification procedure and rapidly accelerated the search for resistance against the disease (Toxopeus et al., 1986).

1.4 Taxonomy and lifecycle of *Plasmodiophora brassicae*

Plasmodiophora brassicae was initially classified as a protist but due to the immense heterogeneity between the species belonging to this kingdom, the classification system has been refined. *P. brassicae* now belongs to Rhizaria supergroup which mostly consists of unicellular eukaryotes (Nikolaev et al., 2004). Moving down the taxonomic scale, *P. brassicae* falls into the Spongospora which is a class of unicellular, gall/scab forming plant parasites. *P. brassicae* is an obligate biotrophic pathogen that completes

its life cycle in the root hairs and root cortex of the host. Due to its biotrophic nature, it is resistant to axenic culture in non-living media.

As illustrated in Figure 1, the *P. brassicae* infection process can be broken up into two stages - primary and secondary infection. During primary infection motile zoospores move towards root hairs, encyst onto them and enter to become intracellular parasites (Liu et al., 2020). Inside the root hairs they develop into primary plasmodium which subsequently form sporangia via repeated divisions. Sporangia develop into secondary zoospores which are subsequently released into the soil. The released zoospores go on to re-infect the root cortex of host plants by 7 days post infection initiating the secondary phase of the disease. Subsequently secondary plasmodia are formed and lead to hypertrophy of the host cells. By 10-14 days post infection, symptoms of clubroot begin to appear which is characterized by reddening and swelling of the hypocotyl. By 24-28 days, the roots and hypocotyl become radically enlarged and starts to disintegrate releasing the resting spores (Liu et al., 2020). Resting spores can survive in the soil for up to 17 years, ready to infect the next batch of plants (Wallenhammar, 1996).

Much effort has been put into characterizing the developmental changes in the host tissue that follows the infection process. By using promoter::GUS fusion lines of auxin and cytokinin markers, it has been shown that growth regulator levels are modulated as early as 4-5 days post infection (Devos et al., 2006). This is followed by increased meristematic activity in the vascular cambium region (Malinowski et al., 2012). This intense proliferative phase lasts from 7-18 days and the dividing cells are locked into the G2/M phase of the cell cycle (Olszak et al., 2019). Next comes an expansive phase when endoreduplication processes are stimulated in the host cells leading to cell enlargement via the stimulation of expansive growth as well as the endocycling process (Olszak et al., 2019). Both cellular processes play a vital role in the establishment of the physiological niche for the pathogen – proliferation leads to increases in host cell numbers that can be colonized by the pathogen, whereas endoreduplication boosts cell size and cellular metabolism and helps to establish a strong sink for nutrients.

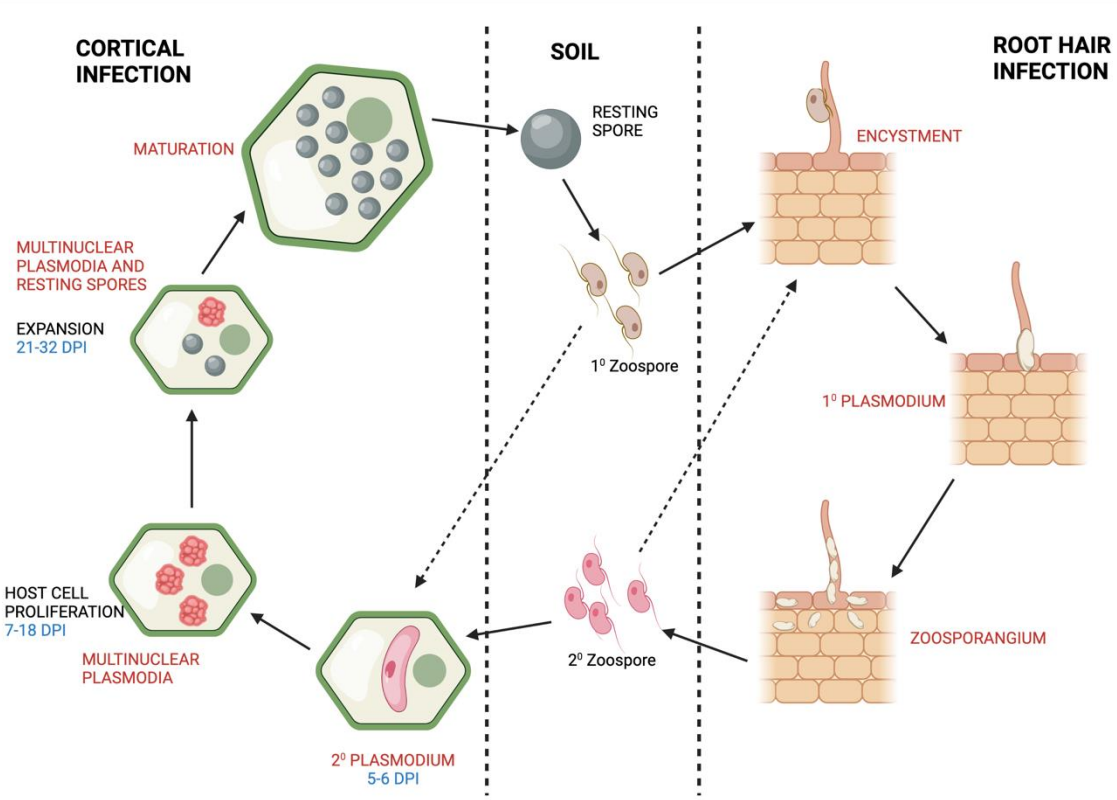


Figure 1: A schematic representation of the *P. brassicae* lifecycle representing all key steps.

The steps include spore germination in soil, root hair encystment, zoospore formation, cortical invasion of spores, multinuclear plasmodia formation and subsequent resting spore production.

1.5 Evolutionary arms race between plants and pathogens

Plants are sessile organisms incapable of physically escaping from herbivores or pathogens in the environment. Plants are continuously exposed to countless microscopic organisms over their entire outer surfaces. Some of those are harmful pathogens, while others may be potential beneficial symbionts. Therefore, plants are constantly surveilling for the presence of non-self cells, differentiating between the threats posed and subsequently mounting appropriate signaling cascades to either initiate immune responses or establish symbiosis. Animals generally have antibody based adaptive immunity which can develop from every infection throughout its lifetime and generate pathogen specific antibodies to counter subsequent infection with better efficacy. Many animals can also control their core temperatures thereby directly inhibiting the growth of temperature sensitive pathogens such as fungi. Plants do not have either of those capabilities. They only have innate immunity at their disposal to fight off invaders. Thus, over millennia, plants have developed very sophisticated innate immune systems capable of resisting the majority of pathogen infections (Han, 2019). Plants employ physical barriers as the first line of defense. Waxy cuticles, thick epidermal layers, tightly regulated stomatal openings and trichomes act as a protective layer for the internal tissues (Zhang et al., 2020). In the apoplast, on the plasma membrane and in the cytosol surveillance receptors are deployed that monitor for the signature molecules of pathogens and initiate defense signalling pathways (Yu et al., 2017). These immune receptors are deployed at the major sites of plant-pathogen interaction: stomatal pores, apoplastic spaces, rhizosphere, intracellular spaces etc. (Zhang et al., 2020). Depending on the location, structure and function of those receptors, plant immunity can be classified into two tiers: Pattern Triggered Immunity (PTI) and Effector Triggered Immunity (ETI) (Jones & Dangl, 2006). PTI is involved with the recognition of Microbe Associated Molecular Patterns (MAMPs). MAMPs are conserved molecules of the pathogen that are essential to their survival and therefore are not prone to rapid change in their structure. Thus, PTI targets those molecules to monitor for the presence of non-self. Examples of MAMPs include chitin, bacterial flagellin, cold-shock proteins, oomycete peptides, peptidoglycans and

lipopolysaccharides (Yu et al., 2017). Besides MAMPs, plants can also sense self-made molecules released upon infection. These are termed Damage Associated Molecular Patterns (DAMPs) and are detected by PTI receptors as well. DAMPs include extracellular ATP, oligogalacturonides and endogenous peptides (Hou et al., 2019). To date over 21 MAMPs and 5 DAMPs have been identified in various model plants.

PTI receptors broadly fall into two classes – Receptor-Like Kinases (RLKs) and Receptor-Like Proteins (RLPs). RLKs have an ectodomain that crosses the plasma membrane into the apoplastic space, it remains attached to the transmembrane domain which is followed by a cytosolic kinase domain. RLPs have structures similar to RLKs but lack the kinase activity in the cytosolic domain. Types of ectodomain of RLKs and RLPs vary depending on the type of ligand they bind to. Leucine Rich Repeat (LRR) domain containing RLKs recognize peptides, LysM domains recognize chitin, lectin containing domains recognize carbohydrates and epidermal-growth factor like domains bind to DAMPs such as oligogalacturonides (Böhm et al., 2014; Couto & Zipfel, 2016). Examples of PTI receptors include FLS2 for flg22 (a fragment of flagellin protein) (Chinchilla et al., 2007), LYK5 for chitin (Cao et al., 2014), EFR for EF-TU bacterial protein (Zipfel et al., 2006), LORE for medium chain fatty acids (Ranf et al., 2015). Upon binding to incoming MAMPs or DAMPs, RLPs and RLKs form heteromeric complexes with other co-receptors. These upstream receptors in association with their partner co-receptors form core receptors. Pathogen produced ligands usually act as a glue between the components of the core receptor and initiate the transphosphorylation of kinase domains to promote downstream signalling cascades (Lu & Tsuda, 2021).

1.6 PTI signalling cascades

The signalling events triggered in response to the MAMP chitin have been well elaborated. Clubroot resting spores contain chitin as a major structural building block (25%) and during its complex lifecycle in the host there are multiple times where chitin is presented to the host as a potential MAMP to initiate PTI signalling (Moxham & Buczacki, 1983). Therefore, chitin detection and masking strategies during clubroot infection are an interesting consideration for *P. brassicae* virulence. Chitin is an

oligomer of N-acetylglucosamine molecules which is the second most abundant natural biopolymer after cellulose (Elieh-Ali-Komi & Hamblin, 2016). Chitin is so crucial to some of the outer layer structures of various pathogens that it incurs a heavy fitness penalty to eliminate or change the structure of the polymer. Plants have evolved LysM domain containing receptors to recognize chitin and chitin derived molecules such as chitosan. In Arabidopsis, chitin is primarily recognized by the LYK5 (LYSIN MOTIF RECEPTOR KINASE 5) receptor which recruits CERK1 (LysM-RLK CHITIN ELICITOR RECEPTOR KINASE 1) as a co-receptor (Cao et al., 2014). LYK4 is another RLK which has lower affinity for binding but is thought to act as a scaffolding protein for the LYK5 and CERK1 complex (Xue et al., 2019). In rice, the primary receptor for chitin is CEBiP (LysM-RLP CHITIN-ELICITOR BINDING PROTEIN) which also recruits a CERK1 ortholog as a co-receptor (Shinya et al., 2012). It has been shown that the kinase activity of these co-receptors is indispensable for further downstream signalling. Next to the core receptors, there are Receptor-like cytoplasmic kinases (RLCKs) which are the direct phosphorylation targets of the core-complex (Figure 2). Many RLCK VII family proteins have been characterized to be suitable targets of various core receptor complexes. In the case of chitin signalling in Arabidopsis, the LYK5-CERK1 complex phosphorylates BIK1 (BOTRYTIS INDUCED KINASE 1), PBL27 (PBS-LIKE 27) and PBL19 (PBS-LIKE 19) (Bi et al., 2018; Shinya et al., 2014; Zhang et al., 2010). These RLCKs then act on various downstream substrates to result in characteristic PTI immune responses including ROS (Reactive Oxygen Species) burst, calcium influx, MAP Kinase activity, stomatal closing and hormone biosynthesis (Yu et al., 2017).

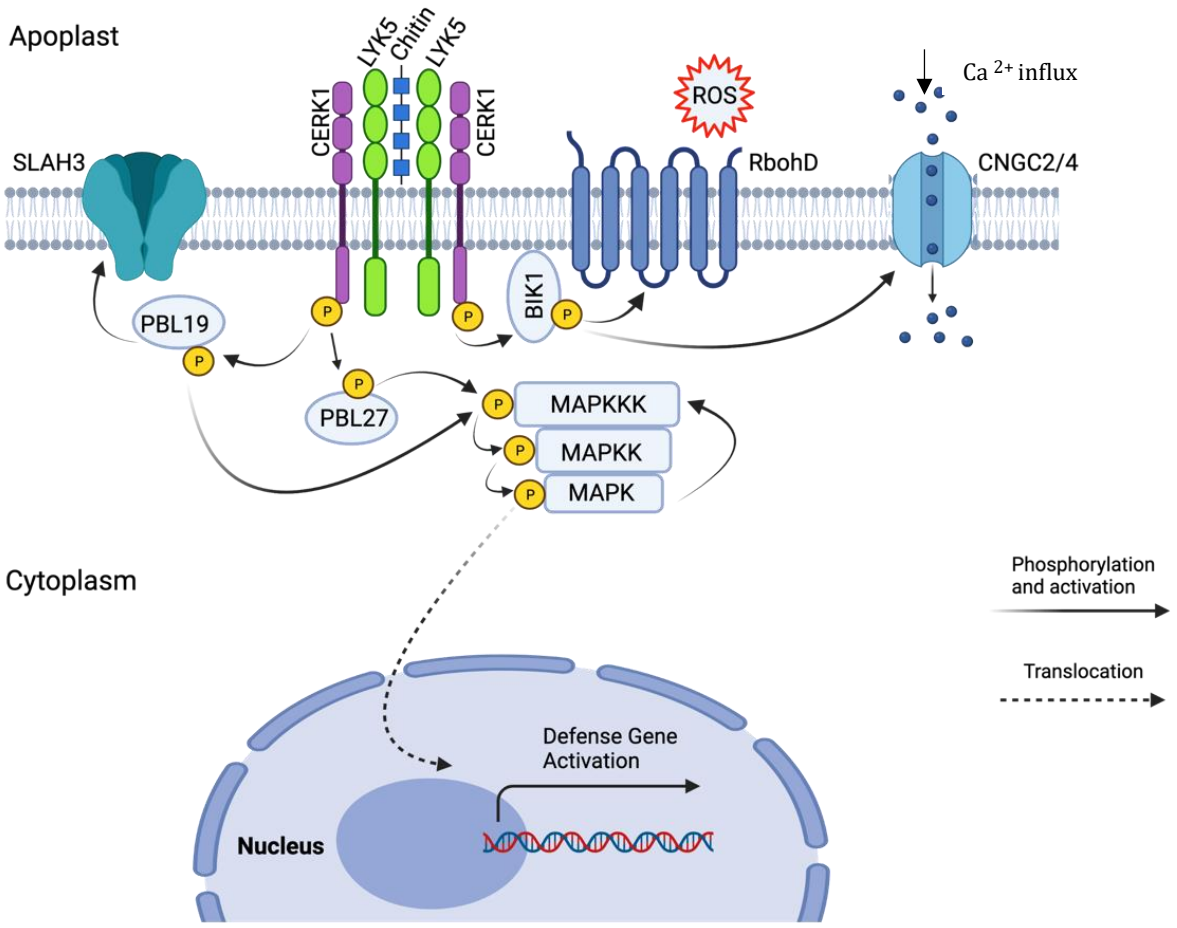


Figure 2: Key interactions during chitin perception and downstream signalling.

Chitin perception leads to the association of CERK1 and LYK5 which initiates a phosphorylation cascade via several cytoplasmic kinases. Cytoplasmic kinases further phosphorylate RBOHD (for ROS generation), CNGC2/4 ion channels (for calcium influx), several MAP Kinases for nuclear translocation and defense gene activation.

1.6.1 Calcium influx

Calcium acts as an important secondary messenger in both plants and animals (Carafoli & Krebs, 2016). The presence of MAMPs such as flagellin and chitin activates calcium channels within minutes of detection. In Arabidopsis, BIK1 phosphorylates and activates the CNGC2 and CNGC4 calcium channels (Wang et al., 2019). Mutants of *cngc2*

and *cngc4* fail to generate the characteristic calcium influx pattern and are deficient in producing local cell death (HR). Those mutants are also known as *defense no death 1* and *2* (*dnd1 & dnd2*) (Jurkowski et al., 2004). Ca^{2+} influx signals are interpreted up by CPKs (Calcium induced Protein Kinases) which further targets downstream substrates (Ullrich et al., 2013).

1.6.2 ROS burst

ROS is a direct weapon against pathogens as oxidative environments have the potential to be antimicrobial. ROS also acts as a signalling molecule for PTI response and is involved in cell wall modification (Torres, 2010). In Arabidopsis, RBOHD plays the leading role of generating the ROS burst upon infection (Kadota et al., 2014). RBOHD can be directly phosphorylated by BIK1 and by CPKs. CPK4, 5, 6 and 11 can all phosphorylate RBOHD to positively regulate ROS production (Ullrich et al., 2013). Subsequently, CPK28 can degrade BIK1 to regulate the uncontrolled ROS burst (Wang et al., 2018). Calcium influx and the ROS burst are not strictly independent pathways, both can positively regulate each other in spreading the defense signalling to neighbouring cells. Arabidopsis RLK HYDROGEN-PEROXIDE-INDUCED CA^{2+}INCREASES 1 (HPCA1) has been shown to be a sensor of H_2O_2. In the *hpca1* mutant, extracellular ROS signal fails to generate internal calcium flux (Wu et al., 2020).

1.6.3 MAP kinase signalling

Mitogen Activated Protein Kinases are universal kinases in both plants and animals that regulate various signalling cascades. In Arabidopsis chitin (and other MAMPS as well) primarily activate two parallel MAPK signalling cascades - MAPKKK3/5-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4 (Gong et al., 2020). The chitin responsive RLCK VII family protein PBL27 phosphorylates MAPKKK5 and its homolog MAPKKK3 to activate the cascade (Yamada et al., 2016). Another RLCK, PBL19 can phosphorylate both MAPKKK5 and MEKK1 to initiate both the cascades (Bi et al., 2018). MAPK3/6 and MAPK4 in turn re-phosphorylate MAPKKK5 and MEKK1 in a positive feedback loop to sustain signalling (Bi et al., 2018). This illustrates the redundancy in different

components of PTI signalling which confers robustness of defense responses, even if one sector fails to perform or is subverted by the pathogen. Activated MAPK3/6 and MAPK4 directly regulate expression of various defense responsive genes and salicylic acid signalling pathways (Tsuda et al., 2013). Salicylic acid in turn plays a crucial role in the ROS burst and generation of Systemic Acquired Resistance (SAR).

1.6.4 Stomatal closure

Plants require stomata to regulate gas exchange and transpiration. These openings serve as excellent entry points for pathogens. Thus, closure of stomata upon invasion is a crucial defense response. Out of the 3 RLCKs involved in chitin signalling, only PBL27 has been shown to phosphorylate S-type anion channel SLAH3 which in turn results in stomatal closure (Zhang et al., 2016). Though in our pathosystem entry via stomata is not a concern, pathogens like *Pseudomonas* and other fungi/oomycetes have evolved ways to bypass the tight regulation.

1.6.5 PTI responses in Arabidopsis upon *P. brassicae* infection

Successful PTI signalling results in callose deposition at the site of infection, cell wall modification, and phytoalexin production (Yu et al., 2017). It has been shown that camalexin (a potent phytoalexin in Arabidopsis) is produced in the clubroot tolerant accession Bur-0 significantly more than the clubroot susceptible Col-0 accession (Lemarié et al., 2015). Accordingly, camalexin biosynthetic genes including *CYP79B2*, *CYP71A13* and *CYP71A12* are more strongly induced in Bur-0. The *pad3* mutant (camalexin deficient) in Col-0 produced larger galls, pointing to the fact that camalexin plays a role in partial resistance even in a susceptible background. The same group also found arginase to be induced in the susceptible Col-0 (Gravot et al., 2012). The *argah2* mutant deficient in arginine biosynthesis resulted in increased disease severity. A jasmonic acid biosynthesis mutant *jar1* showing decreased accumulation of arginase resulted in similar elevated disease symptoms. This points to a jasmonic acid dependent arginase activity in response to clubroot.

1.7 MAMP masking strategies

Pathogens have evolved various strategies to mask MAMPS from being detected by PTI receptors. Fungi are the most devastating pathogens attacking plants and they contain a significant amount of chitin as a structural component of their walls. Thus, most information on chitin masking comes from research on fungal pathogens. As previously described, chitin receptors in plants contain LysM domains to bind chitin chains. Fungal pathogens have evolved LysM domain (CBM50, Carbohydrate-Binding Module family 50) containing secreted proteins that are capable of out-competing the plant's PTI receptors in binding chitin and thus inhibiting further downstream responses (Gong et al., 2020). Examples include, Slp1 from *Magnaporthe oryza*e (Mentlak et al., 2012), Ecp6 from *Cladosporium fulvum* (Jonge et al., 2010) which bind to chitin oligomers directly and prevent detection. *Magnaporthe oryzae* has also evolved chitinase like proteins (MoChia1) that degrade free chitin oligomers preventing them from reaching the plant receptors (Yang et al., 2019). Other LysM containing fungal proteins such as Mg3LysM from *Mycosphaerella graminicola* (Marshall et al., 2011) and Vd2LysM from *Verticillium dahlia* (Kombrink et al., 2017) can bind to both fungal hyphae and free oligomers to both prevent digestion of the hyphae and inhibit plant perception. Fungal pathogens also carry non-LysM CBM14 and CBM18 domain containing proteins to interfere with chitin recognition. *Cladosporium fulvum* deploys Avr4 (CBM14) and closely related homologues to prevent cell wall degradation by plant's endogenous chitinases (Burg et al., 2007). *Verticillium nonalfalfae* secreted VnaChtBP is a CBM18 domain containing protein with the same function (Volk et al., 2019). Besides directly masking chitin and its receptors, pathogens can also convert chitin to a potentially weaker immunogenic form chitosan to achieve similar goals. *Verticillium dahlia* POLYSACCHARIDE DEACETYLASE 1 (PDA1) has been shown to bind and depotentiate chitin oligomers by deacetylating them into chitosan (Gao et al., 2019). Another soil-borne pathogen, *Fusarium oxysporum* also carries a PDA1 homologue which is essential for its capacity to infect cotton (Gao et al., 2019). *P. brassicae* is a soil-borne plant pathogen carrying chitin as part of its cell wall. Genome sequencing of *P. brassicae* has shown that its genome contains several CAZys (Carbohydrate Active enZymes) (Schwelm et al., 2015).

Among those are one CBM50 domain containing protein, 10 CBM18 domain containing proteins, and 9 CE4 class of proteins which can both bind to and deacetylate chitin. In a recent publication, two secreted *P. brassicae* proteins, PbChiB2 and PbChiB4 have been characterized, both were shown to bind to resting spores and chitin oligomers (Muirhead and Pérez-López, 2021). Both proteins contain CBM18 domains and are transcriptionally active during primary plasmodia formation and spore formation. Incubating chitin solution with the purified proteins prevented MAP Kinase signalling in *Brassica napus*. This indicates the potential of *P. brassicae'*s suite of putative chitin binding proteins to bind to resting spores to prevent chitinase mediated degradation and parallelly convert chitin to chitosan to dampen its immunogenic ability (Sánchez-Vallet et al., 2015).

1.8 *P. brassicae* effectors

The above-mentioned examples fall into a broad class of strategies evolved by pathogens to counter plant immunity. One function of secreted pathogen proteins it to mask chitin recognition. There are many other different types of secreted proteins which target various other aspects of PTI signalling to inhibit defense responses. Pathogen secreted proteins which act as to promote virulence are called 'effectors'. Apart from the above-mentioned chitin binding proteins, only a handful of other clubroot effectors have been characterized to date.

1.8.1 Salicylic acid methyltransferase from *P. brassicae*

Salicylic acid (SA) mediated defense signalling is the dominant response to biotrophic pathogens such as *P. brassicae*. Elevated SA accumulation leads to downstream immune responses that could be detrimental for survival of the pathogen. Ludwig-Müller et al., have shown that, upon infection, a *P. brassicae* salicylic acid methyltransferase gene (*PbBSMT*) is massively upregulated (Ludwig-Müller et al., 2015). The gene sequence is predicted to carry a secretory signal as well. This gene has sequence similarity with the Arabidopsis endogenous methyltransferase *AtBSMT1*. *In vitro* enzymatic assays have shown that PbBSMT can methylate SA and convert it into methyl salicylate (MeSA), thus

rendering it less potent and increasing its diffusion away from the site of infection. *In planta* overexpression leads to lower accumulation of SA upon *P. brassicae* infection and results in larger galls.

1.8.2 SnRK1.1 interacting effector

SnRK1.1 is involved in shifting energy resources from growth to defense upon pathogen perception. Wang et al., have shown that the Arabidopsis *snrk1.1* mutant is more susceptible to clubroot infection (Chen et al., 2021). They have also identified a *P. brassicae* an effector termed PBZF1 carrying an RXLR sequence motif, a hallmark of plant pathogen proteins (Grouffaud et al., 2010), which was shown to interact with SnRK1.1 in a yeast two-hybrid assay. Plants ectopically expressing *PBZF1* are more susceptible to clubroot disease and display altered expression levels of downstream SnRK1.1 targets.

1.8.3 Endomembrane targeting effectors

P. brassicae has been shown to carry a suit of endomembrane targeting proteins capable of shutting down programmed cell death (Hossain et al., 2021). Hossain *et al.* characterized 14 putative effectors with predicted secretory peptides, which are capable of being secreted in a yeast secretion system assay. When transiently expressed in onion epidermal leaf cells, 12 out of those 14 candidates localized in the endomembrane of the endoplasmic reticulum and Golgi bodies. The authors further co-expressed these candidates with PCD (Programmed Cell Death) inducing PiINF1 (PHYTOPHTHORA INFESTANS INFESTIN 1) and PiNPP1 (*P. infestans* NECROSIS-INDUCING PHYTOPTHORA PROTEIN 1) in tobacco leaves. 7 out of the 12 candidate effectors were capable of suppressing HR (Hypersensitive response) upon infiltration. PbPE15 was shown to have the highest PCD suppressing effect and was also expressed at the later stages of disease progression, prompting the authors to hypothesize it plays a role in both ETI suppression and tumorigenesis.

1.9 Effector triggered immunity

To counter the negative impacts of pathogen effectors, plants have developed a diverse set of cytoplasmic receptors which can recognize those effectors and re-initiate defense responses. This second branch of immunity is called Effector Triggered Immunity (ETI) (Jones & Dangl, 2006). Unlike MAMPs, effectors are numerous and are not constrained from evolving new forms to evade ETI. Plants respond accordingly by rapidly evolving ETI receptors. Co-evolution of effectors and corresponding ETI receptors is a classic example of the evolutionary arms-race between plant and pathogens. It perfectly fits the Red Queen Hypothesis, which states that competing species must quickly evolve and adapt to the strategies of the enemy to survive (Langerhans, 2008). In a recounting of his life's work, Pierre J.G.M. de Wit from Wageningen University wrote - "Eventually, the arms race between *C. fulvum* and tomato, making use of 100 effectors and 100 corresponding Cf immune receptors, becomes true trench warfare." (Wit, 2016).

Plants have evolved specialized cytoplasmic ETI receptors which are known as NBS-LRRs (NLRs). NLRs are the most diverse and fastest evolving class of protein family in plants (Tamborski & Krasileva, 2020). Nucleotide binding leucine rich repeat proteins are universal across several kingdoms of life. They mainly consist of proteins participating in non-self molecule detection and subsequent immune responses. Plant Resistance (R) genes broadly fall into this category as well. NLRs across various kingdoms possess two key domains: Nucleotide Binding and Oligomerization Domains (NOD) and Super-Structure Forming Repeat (SSFR) domains. In the case of plants, the NOD domain is mainly of the NB-ARC (nucleotide-binding adaptor shared by APAF-1, certain R gene products and CED-4) type while the SSFR domain primarily consists of LRRs (Leucine Rich Repeats) (Monteiro & Nishimura, 2018). Based on the architecture of the N terminal domain, plant NLRs can be broadly classified into three monophyletic groups: the TIR (Toll interleukin-1 receptor) type, CC (Coiled-Coil) type and RPW8 (CCR) type (Adachi et al., 2019). NLRs perceive incoming effectors in various ways:

1.9.1 Direct effector recognition

In this model, intracellular NLRs directly bind to secreted effectors proteins and initiate immune signalling. For example, ARABIDOPSIS THALIANA RECOGNIZED 1 (ATR1) is an effector from the oomycyte *Hyaloperonospora arabidopsidis* that is recognized by Arabidopsis NLR RECOGNITION OF PERONOSPORA PARASITICA1 (RPP1) (Krasileva et al., 2010). Different varieties of RPP1 have evolved to recognize polymorphisms in ATR1 and such specificity is usually mediated by the LRR domain sequence (Steinbrenner et al., 2015).

1.9.2 Indirect Recognition: Decoys and Guardees

Pathogens target essential host components to interfere with host immune signalling. A subset of NLRs have evolved to monitor those host components targeted by effectors. When such host components play a role in immune responses, they can be termed guardees. Plants have also evolved proteins which mimic those guardees but have no defined immune functions, thereby only acting as baits for the effector recognition, these are known as decoys (Hoorn & Kamoun, 2008). NLRs can monitor and respond to chemical modification of both classes of host protein. HOPZ-ACTIVATED RESISTANCE1 (ZAR1) is an Arabidopsis NLR capable of recognizing modification on receptor like RLCK XII family members. ZAR1 recognizes ZED1 modification by *P. syringae* Type-III effectors HopZ1a (Lewis et al., 2013) and also recognizes PBL2 modification by *Xanthomonas campestris* Type-III effector AvrAC (Wang et al., 2015). ZED1 has no defined immune role, thus acts as a decoy, whereas PBL2 is involved in PTI signalling, thus it is considered a guardee.

1.9.3 NLRs with integrated sensors (NLR-IDs)

Some NLRs carry an integrated domain (ID) that can be targeted by pathogen effectors to dampen immunity (Kroj et al., 2016). Those unrelated domains might still possess their original function and therefore are different from decoys. Such close integration leads to rapid sensing of effector activity by modification of the NLR structure. The rice NLR RGA5 is one such example of the handful of NLR-IDs characterized. RGA5 carries a

HMA domain which is targeted by *Magnaportha oryzae* effectors Avr-Pia and Avr-CO39 (Cesari et al., 2013). Such interactions lead to rapid triggering of RGA4 dependent immunity.

1.10 Recent advances in the understanding of direct NLR mediated immunity

Besides classifying NLRs based on modes of effector recognition, they could also be classified into 'sensor' and 'helper' classes. Sensor NLRs usually recognize the effector and then act alone (called 'Singleton') or in combination with Helper NLRs to facilitate downstream signalling (Adachi et al., 2019). Three classes of helper NLRs have been reported: CCR-type ACTIVATED DISEASE RESISTANCE 1 (ADR1), N REQUIRED GENE 1 (NRG1) and Solanaceae-specific NLR required for cell death (NRC) family (Wu et al., 2017). ADR1 and NRG have co-evolved with a crucial ETI component, Enhanced Disease Susceptibility 1 (EDS1) (Lapin et al., 2020; Wang et al., 2019). A clear hallmark of the ETI response is localized cell death, also known as the Hypersensitive Response. HR acts in restricting pathogen progression to surrounding tissues. Structural and biochemical studies on the CC type 'Singleton' NLR ZAR1 (Wang et al., 2019) have shed some light upon the mechanisms by which effector perception leads to localized cell death. Upon effector modification of decoy PBL2, the ZAR1-RKS1-PBL2 complex is formed which assembles into a pentameric structure termed a 'Resistosome' (Bi et al., 2021; Hu et al., 2020). The crystal structure of another TIR type NLR Roq1 from *Nicotiana* has also been recently published. Roq1 when bound to the effector XopQ1 also forms a resistosome like tetramer structure (Martin et al., 2020). Biochemical analysis has shown that these resistosome structures are funnel shaped and plasma membrane associated. It has been hypothesized that such structures directly form pores in the cell membrane causing immediate cell death. A conserved 21 amino acid 'MADA' motif found in the N-terminal domain of the above discussed NLRs are conserved in 20% of all known angiosperm CC type NLRs, indicating similar mode of action (Adachi et al., 2019). While most CC type NLRs can themselves induce HR, most TIR type NLRs are dependent on CC type helper NLRs. When Arabidopsis TIR type NLRs signal through EDS1/PAD4, they recruit the helper NLR ADR1 for transcriptional

reprogramming required for immunity. When the signalling is elaborated through EDS1/SAG101, NRG1 helpers are recruited which primarily results in cell death(Thordal-Christensen, 2020). The outcome of ETI responses is multifaceted, the detailed signalling mechanisms by which those different classes of NLRs co-operate in the network to initiate various levels of defense response remains largely unknown.

1.11 Known clubroot resistance locus/genes

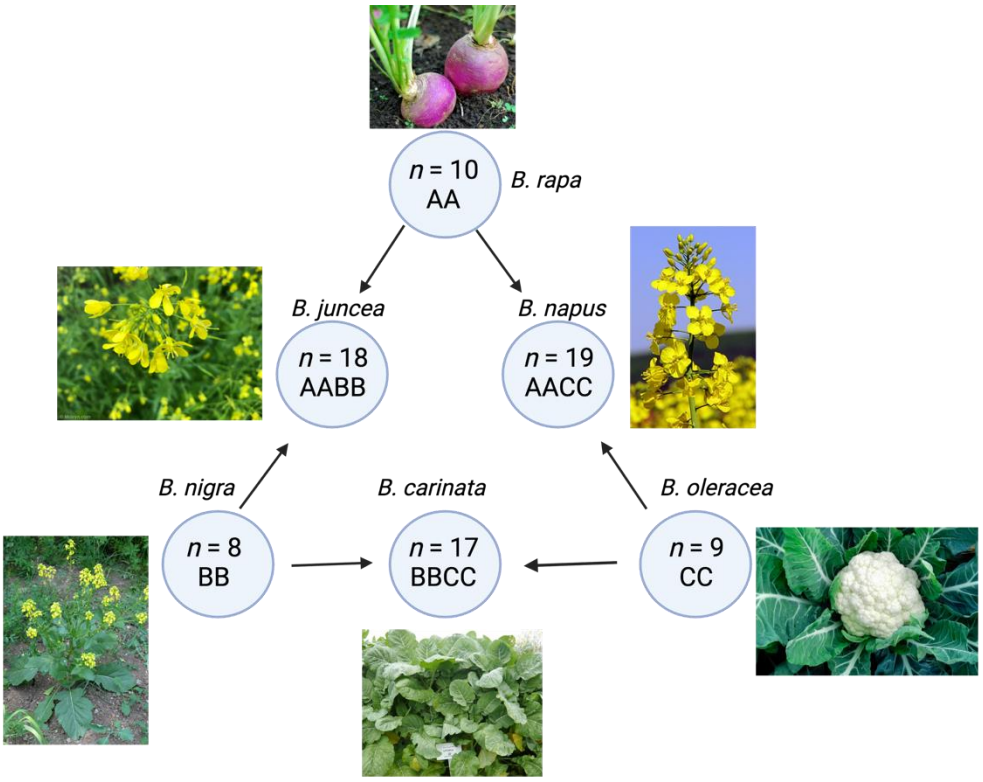


Figure 3: The Triangle of U depicts how three diploid ancestral brassica species hybridized to form three new tetraploid crop species.

As previously mentioned, Brassica group of crops are essential components of our daily food habits. Figure 3 shows the 'Triangle of U' which depicts the genetic relationship between the 6 most prominent brassica crops. Various genetic mapping techniques have helped uncover several resistance loci in brassicas acting against clubroot disease.

Until now the majority of the resistance loci have come from *B. rapa* and most identified resistance loci are QTLs (Mehraj et al., 2020).

Traditionally, Recombinant Inbred Line (RIL) based linkage mapping has been used to identify the genetic causation underlying the phenotypic variations of plant and pathogens. The two main drawbacks of this method are that it results in coarse mapping, i.e. not resolving the associated locus at a fine genetic level and that it is almost impossible to differentiate between pleiotropic or closely situated genes. The advent of Next Generation Sequencing has ushered in a new era of genomics and transcriptomics that gave birth to new methods to solve these problems. One method is Genome Wide Association (GWA) mapping which takes advantage of whole genome or SNP arrays from a large number of accessions to identify the polymorphisms that are linked with the phenotypic variation. GWA mapping exploits the natural Linkage Disequilibrium (LD) to fine map the locus.

In *B. rapa* the *CRa/CRb* loci has been the most extensively used in breeding programmes. The discovery of the loci took over 20 years (Kuginuki et al., 1997). The loci is the main source of resistance in the commercially important resistant cultivar 'Mendel' of oilseed rape (Diederichsen et al., 2006). The *CRa* loci is present on chromosome A03 and encodes a TIR-NBS-LRR. By conducting mutation studies, it was shown that the non-functional allele of the NLR leads to loss of clubroot resistance (Ueno et al., 2012). Another important discovery was the *Crr1* locus in turnip by Hatakeyama et al., fine mapping revealed that the *Crr1a* gene is a TIR-NBS-LRR which was expressed in cortex and stele of the resistant plants (Hatakeyama et al., 2016). Complementing susceptible Arabidopsis and *B. rapa* lines with a functional allele of *Crr1a* led to resistance (Hatakeyama et al., 2013). A bulked segregant RNA-Seq method was employed to explore the *Rcr1* locus and two candidate NLRs- *Bra019409* and *Bra019410* were identified (Chu et al., 2014). Huang et al. identified the *Rcr2* locus and fine mapped it using the Kompetitive Allele Specific PCR method (Huang et al., 2017). Out of the four candidate NLRs present in the locus, *Bra019410* was common with the

previously mentioned *Rcr1* locus. There have been more than 18 CR loci identified in *B. rapa* and more continue to be uncovered (Mehraj et al., 2020).

Clubroot resistance in *B. oleracea* appears to be polygenic in nature (Tomita et al., 2013) as over 50 QTLs have been identified till date (Mehraj et al., 2020). Nine QTLs involved in resistance against 5 pathotypes tested were found (Rocherieux et al., 2004). Between two and five QTLs were involved per pathotype and the *Pb-B01* QTL was common for all pathotypes. Voorips *et al.* identified two QTLs, *Pb3* and *Pb4* in double haploid lines of cabbage (Voorrips et al., 1997). *Rcr7* remains the only fine mapped resistant locus in *B. oleracea* (Dakouri et al., 2018). Fine mapping revealed 9 candidate NLRs out of which only 2 are expressed upon infection.

B. rapa being the major source of clubroot resistance, has been used in various breeding programmes to introgress the resistance to *B. oleracea* and *B. napus* (Dakouri *et al.*, 2018). In *B. napus* only one strong resistance locus has been identified but more than 30 QTLs have been reported with additive effects (Mehraj *et al.*, 2020). Manzanares et al. have identified a resistant locus *Pb-Bn1* on chromosome A03 in double haploid lines of the cross between resistant and susceptible cultivars (Manzanares-Dauleux et al., 2000). The authors also identified two QTLs having additive effects. In another study, 19 QTLs showing resistance to 7 pathotypes were discovered but no single QTL was sufficient to confer full resistance (Werner et al., 2007). The 60k Brassica Infinium SNP array was used to conduct a GWAS study using 472 *B. napus* accessions against pathotype 4 prevalent in China (Peng et al., 2018). The authors found nine QTLs having additive effects towards resistance. Recently, Kopec et al. have used 60k SNP arrays and SCAR markers to identify a resistant locus on chromosome A03 which overlaps with the previously described *Crr3* (Kopec et al., 2021). Fine mapping of the region revealed a tandemly duplicated TIR-NBS-LRR pair as the promising candidate.

Many of the discussed resistance loci has been used to develop markers for MAS (Marker Assisted Breeding). Continuous discovery of resistance sources is required as the pathogen population continues evolving based on the selection pressure applied by the introgressed genetic fragments conferring resistance. In contrast to economically

important brassicas, the model plant *Arabidopsis thaliana* has not been extensively surveyed to identify resistant genes. So far only one resistant locus has been mapped to chromosome 1 of the resistant Arabidopsis ecotypes Tsu-0 and Ze-0 (Fuchs & Sacristán, 1996). Fuchs et al. termed the loci *RPB1 (Resistance to Plasmodiophora brassicae 1)* and have shown it to be monogenetically inherited. Resistant accessions have been shown to display localized cell death (HR) upon infection (Kobelt et al., 2000), pointing to activation of ETI pathways. Though two Genbank submission are available annotating a 12 kb long putative *RPB1* loci from two resistant accessions, no publication exists describing the characterization of the particular gene involved.

1.12 How hormones amplify immunity

Initial activation of defense responses by PTI and ETI pathways are further amplified by the involvement of defense associated phytohormones. Salicylic acid (SA) and jasmonic acid (JA) are two key players in regulating immunity in land plants. Ethylene, auxin, ABA, cytokinins and brassinosteroids are also important components of the hormonal cross-talk that regulates immune responses (Shigenaga & Argueso, 2016). Usually, SA plays a positive role in mediating defense against biotrophic/hemibiotrophic pathogens, while JA acts against necrotrophs (Liu et al., 2016b) but there are certain overlaps (Mur et al., 2006). Arabidopsis mutants lacking specific biosynthetic or signalling genes in SA/JA pathways display increased susceptibility and it has been found that various pathogen secreted effectors target those pathway genes (Kourelis & van der Hoorn, 2018). It has also been shown that these hormones work in a coordinated network, where the loss of one sector is buffered by the function of other hormones, thereby maintaining the robustness of immunity (Hillmer et al., 2017).

In Arabidopsis the isochorismate (IC) pathway plays the pivotal role in pathogen triggered SA synthesis which takes place in chloroplast. ISOCHORISMATE SYNTHASE 1 (ICS1) is a key enzyme which converts chorismate (a shikimate pathway end product) into isochorismate (Strawn et al., 2007). Arabidopsis mutants lacking a functional ICS1 fail to initiate pathogen triggered SA production (Wildermuth et al., 2001). The enzyme

that converts IC into SA is still unknown. In Arabidopsis, the primary hub of SA perception goes through NPR1 (Seyfferth & Tsuda, 2014) and its homologues, NPR3 and NPR4 (Fu et al., 2012). SA binding to NPR1 leads to the nuclear translocation of NPR1 from the cytosol, NPR1 then interacts with TGA transcription factors to initiate defense gene expression. NPR3/4 on the other hand are transcriptional co-repressors which inhibit gene expression by interacting with TGA factors. At higher SA concentrations the effect of NPR3/4 repression is inhibited. This is how the SA receptor NPR1 and NPR3/4 pair fine tune transcriptional control of SA responses (Ding et al., 2018; Withers & Dong, 2016). In Arabidopsis, it has been shown that NPR1 can suppress ABA and JA responses through the downstream target WRKY70 (Li et al., 2004). ABA treatments can in turn cause proteasomal degradation of NPR1 to suppress SA responses (Ding et al., 2016).

Jasmonic acid biosynthesis takes place in the chloroplast membrane where lipids are converted to 13-hydroperoxyoctadecatrienoicacid (13-HPOT) by the action of lipoxygenases (LOXs) and subsequently to 12-oxophytodienoic acid (OPDA) by the action of Allene Oxide Synthase (AOS) and Allene Oxide Cyclase (AOC) (Ruan et al., 2019; Wasternack & Hause, 2013). OPDA is then converted to jasmonic acid in the peroxisome and is then isoleucine-conjugated by Jasmonate Resistant 1 (JAR1) to form JA-Ile, the most active form of JA. JA-Ile is detected by the receptor Coronatine-Insensitive 1 (COI1) which induces proteasomal degradation of JAZ family proteins which are transcriptional repressors of JA signalling. JAZ degradation unleashes MYC transcription factors (Zhang et al., 2015). MYC2 is the core player of JA signalling and can suppress SA signalling by controlling ICS1 activity (Boter et al., 2004) and inducing SA metabolizing genes with the help of SNAC-A transcription factors (Takasaki et al., 2015). In parallel, MYC2 can further suppress SA accumulation by directly inhibiting Phytoalexin Deficient 4 (PAD4) (Mine et al., 2017). Interestingly, MYC2 induces EDS5 which is a positive regulator of SA accumulation. It has been theorized that there is an incoherent feed-forward loop which ensures SA mediated defense response in times where the PAD4 sector stops functioning (either due to higher temperature or

pathogen attack) (Mine et al., 2017). This shows the complexity and fine tuning of the immune network which remains robust even when one sectors fails.

Lovelock et al. have tested Arabidopsis SA related mutant lines against *P. brassicae* isolates (Lovelock et al., 2016). They selected two mutants (*dnd1* and *cpr1*) that display constitutive defense activation and 3 other mutants (*npr1, NahG* and *sid2*) that lack key components of SA signalling and are thus deficient in immune responses. Exogenous SA application resulted in reduced gall size in all the SA deficient mutants. The authors noted reduced disease severity in *dnd1* and *cpr1* compared to susceptible Col-0, but exogenous SA application could only enhance disease resistance in *dnd1*, not in *cpr1*. *PR1* expression is very high in those mutants compared to Col-0, the authors concluded that disease resistance against *P. brassicae* is also controlled by other factors in parallel to SA. Lemarie et al. tested the role of SA and JA in clubroot susceptible (Col-0) and clubroot tolerant (Bur-0) accessions (Lemarié et al., 2015). They found strong induction of SA signalling in Bur-0 but not in Col-0. In contrast Col-0 had an elevated JA response and Bur-0 showed only slightly enhanced JA responses. Exogenous SA application reduced gall size consistent with the previous experiments described by (Lovelock et al., 2016). It has been shown that the *jar1* mutant (deficient in JA signalling) was more susceptible to clubroot infection. Another mutant, in a JA responsive gene, *nata1* displayed enhanced disease severity. The authors concluded that although SA plays the major role in disease resistance against *P. brassicae*, basal immunity mediated by JA is also important.

1.13 Update of PTI/ETI model

Recent studies on the individual effects of PTI and ETI signalling has blurred the line between these two tiers of immune regulation. It has been shown that effector triggered activation of the Arabidopsis NLRs RPM1 and RPS2 results in transcript and protein accumulation of various PTI regulators such as *BIK1, RBOHD, MPK3 and SOBIR1* (Ngou et al., 2021; Yuan et al., 2021). Consistently, production of macroscopic HR is inhibited in *fls2, bak1/bak1/cerk1* mutants when *RPS2* is activated by inducible expression of *Pseudomonas* effector AvrRpt2 in the absence of any PTI signalling (Ngou et al., 2021).

ROS production is a hallmark of PTI signalling; ROS production is rapid and transient in the case of PTI, while ETI triggered (dependent on functioning PRRs) ROS generation is stronger in amplitude (Yuan et al., 2021). New evidence suggests that ETI is a magnified version of PTI (Figure 4). Therefore, when researchers observe an equal pattern of PRR gene expression in resistant and susceptible cultivars upon clubroot infection and state "PTI does not play a role in the interaction between CR BJN 3-2 and *P. brassicae*." (Chen et al., 2016) this may represent a misinterpretation of current knowledge and the potential for interactions between ETI and PTI. More research is necessary to figure out the complex interdependent regulation of PTI and ETI signaling and how this may be applicable to clubroot resistance.

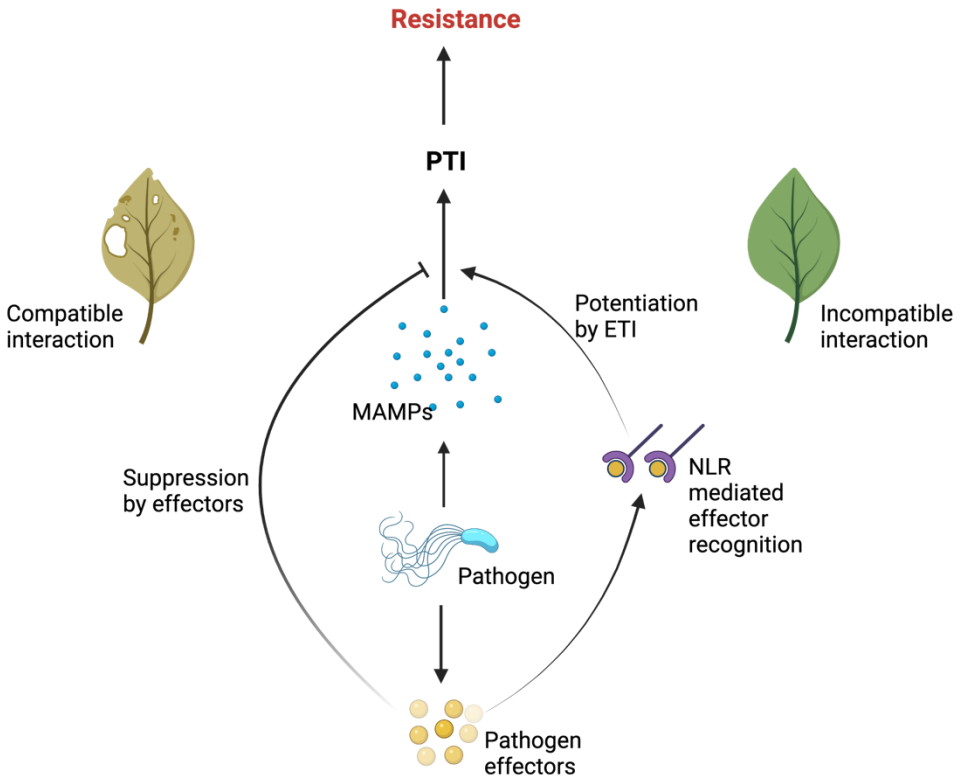


Figure 4: Current model of the PTI-ETI interplay. Initial PTI responses are further potentiated upon effector recognition.

1.15 Use of transcriptomics to probe molecular plant-pathogen interactions

Screening of various accessions of the Brassica genus has led to identification of both resistant and susceptible genotypes. Researchers have attempted to decipher the biochemical/physiological pathways that contribute to resistance/susceptibility. Comparative transcriptomics is one way of comparing the state of gene expression between contrasting genotypes that has the potential to identify the key metabolic and regulatory pathways involved.

Technologies such microarray analysis and next-generation sequencing has enabled the quantification of mRNA in a high-throughput manner (Wolf, 2013). Clubroot infection leads to early and strong immune response from resistant hosts which restricts pathogen growth (Ludwig-Müller, 2022). The pathogen can manipulate those defense responses in susceptible accessions and subsequently stimulate host pathways to acquire nutrients (Walerowski et al., 2018). By 7 days post inoculation, both primary and secondary infections by the pathogen are underway (Liu et al., 2020) and this window determines resistance/susceptibility. Therefore, it is crucial to profile gene expression changes in early timepoints of the infection.

1.15.1 Responses in *Brassica oleracea*

Responses to clubroot infection in *Brassica oleracea* have been characterized at 7, 14 and 28 dpi timepoints (Ning et al., 2019; Peng et al., 2018). Researchers have also collected field samples of *B. oleracea* to find infected and symptomless roots from the same plots to identify intra-plant heterogeneity in clubroot responses (Ciaghi et al., 2019). A common feature of the findings has been the upregulation of salicylic acid mediated signalling genes, genes involved cell wall modification and secondary metabolite biosynthesis and various immune related genes in the resistant backgrounds. It was reported that cell wall modification enzymes, glucosinolate biosynthesis and plant hormone signal transduction genes were more prominently

upregulated at 7 dpi compared to the 14 dpi timepoint, indicating an early defense signalling cascade determining the outcome (Ning et al., 2019). Another interesting finding has been the involvement of plant cytoskeleton/ microtubule-based processes at 7 dpi in the resistant background that might be associated with reinforcement of host cell walls at the pathogen entry site and the transport of antimicrobial compounds. Findings from the analysis of galls and symptomless roots from the same field might be associated with non-uniform distribution of pathogen spores in the field resulting in dose dependent PTI activation.

1.15.2 Responses in *Brassica rapa*

The *P. brassicae* interaction with B. rapa has been investigated with detailed timecourse studies. Timepoints included in the various studies are: 12, 72, 96 hours post infection (hpi) (Chen et al., 2016); 2, 5, 8, 13, 22 dpi (Yuan et al., 2021) and 3, 9, 20 dpi (Wei et al., 2021). As with *B. oleracea*, the common findings included activation of SA mediated response in resistant lines, upregulation of various PTI related defense genes and oxidative stress responsive genes in resistant backgrounds. The genes *ARF2, EDR1, LOX4, NHL3, NHL13, NAC29, AOP1, EARLI1*, and *POD56* were identified by gene co-expression network studies as key genes regulating defense against clubroot disease (Yuan et al., 2021). Another important finding has been the significant downregulation of cell division and expansion related genes in the resistant lines, indicating how restriction of developmental changes might interfere with disease progression. The up-regulation of various NLRs has been highlighted though this may represent a general increase in pathogen surveillance as part of SAR rather than implicating specific effector-receptor interactions.

1.15.3 Responses *in Brassica napus*

Resistance responses in *B. napus* at 7, 14 and 21 dpi have been profiled revealing a key role for ethylene and salicylic acid mediated responses (Zhou et al., 2020). The authors

have proposed a role of *WRKY, ERF* and *bZIP* transcription factors in orchestrating the defense signalling cascade. Furthermore, they have noted the contrasting finding of ethylene response in resistance mechanism against *P. brassicae* which was hypothesized to be the result of pathotype specific response. While more publications have described host transcriptome changes at various timepoints of disease progression in *B. napus*, mostly echoing similar findings of previous articles (Chen et al., 2016; Mei et al., 2019), recent studies are going beyond that and highlighting the role of environmental factors such as the soil microbiome (Daval et al., 2020) or nitrate input (Aigu et al., 2022) in modulation of disease symptoms. It was found that soil microbial diversity had early effect on plant's transcriptional reprogramming, while its impact on the pathogen's transcriptome was revealed at later stages of the disease. Furthermore, the resistant *B. na*pus cultivar could respond better to infection when growing in an environment with higher microbial diversity. Changes in pathogen gene expression were most pronounced when infecting the susceptible accession. This shows the advantages of such complex studies that might be useful in optimizing new methods of biological control of the disease. Low nitrate input was found to be correlated with lower disease symptoms but did not have a significant effect on the modulation of host defense responses. It was hypothesized that induction of various nitrate transporters (*NRT2.2*) could be mediating disease tolerance. Limitation of nitrogen input could be detrimental for a biotrophic pathogen which has to compete with the host for securing nutrients.

1.15.4 Responses in Arabidopsis

The first publication on Arabidopsis transcriptome changes upon clubroot disease highlighted the importance of auxin and cytokinin in development of the disease (Siemens et al., 2006). *Nitrilase1, Nitrilase2* and the *GH3* group of auxin responsive elements were upregulated upon infection. Cytokinin dehydrogenases (*AtCKXs*) were downregulated which was hypothesized to be involved in the maintenance of tissue cytokinin levels to facilitate developmental changes. *AtCKX1* and *ATCKX3* overexpressing lines had reduced gall development, further supporting the hypothesis.

In another study laser dissection microscopy assisted extraction of infected cells revealing the involvement of brassinosteroid (BR) responsive genes in the enlarged cells (Schuller et al., 2014). Reduced disease symptoms were observed when wild type plants were treated with BR inhibitor propiconazole, and *bri1-6* mutant plants, defective in brassinosteroid perception produced smaller galls as well. Responses to clubroot infection have also profiled in shoot tissue in parallel to the root tissue, showing that the number of genes upregulated in aerial tissue is higher compared to the root (Irani et al., 2018). While pathways differentially regulated in root tissue involved carbohydrate metabolism, cell wall modification, and various transporters; downregulation of photosynthesis and starch metabolism was the most prominent feature in the shoots. Most of the transcriptomics studies on Arabidopsis have focused on timepoints after 10 dpi as the characteristics of gall development takes time and many researchers are focused on the events leading to reprogramming of host tissue for pathogen colonization (Olszak et al., 2019; Rolfe et al., 2016; Walerowski et al., 2018). Zhao et al. have analyzed transcriptomics response in Col-0 24 and 48 hours post infection with *P. brassicae* (Zhao et al., 2017). Lignin biosynthesis genes including *CCR1, CCoAOMT, CAD6* and *CAD9* were induced, pointing to a primary defense response toward pathogen invasion. Consistent with the previous observation that auxin inhibitor treatment reduced clubroot symptoms in *B. rapa* (Ludwig-Müller, 2014), the authors noted induction of 13 IAA responsive and 3 cytokinin pathway genes. The signature of increased tryptophan metabolism also points to elevated IAA biosynthesis. Though the authors report induction of a few SA responsive defense genes, the defense pathways were not significantly enriched. A study comparing Arabidopsis accession Bur-0's tolerant and intolerant responses to different *P. brassicae* pathotypes revealed a key role of host primary metabolism and the host's ability to suppress cell expansion as primary drivers of tolerance (Jubault et al., 2013) . Most studies in Arabidopsis have focused on later stages of the disease, while the ones conducted at the early timepoints did not investigate a strong resistance response mediated by ETI. It could be partially attributed to the lack of accessions showing strong resistance against virulent *P. brassicae* isolates, thus leaving a scope for research community to investigate it further for identification of key pathways leading to strong defense responses.

2. Objective and Hypothesis

2.1 Objectives of the research work

The primary aim of the work was to screen the natural germplasm diversity of Arabidopsis accessions against a virulent strain of *P. brassicae* (P1+), collected from the west Pomerania region of Poland, and identify the genetic factors underpinning resistance and susceptibility via Genome Wide Association Study (GWAS). Having identified clubroot resistant Arabidopsis accessions, transcriptome profiling was adopted to characterize the differences in compatible and incompatible interactions.

A secondary objective was to identify and characterize putative chitin binding proteins from *P. brassicae* that might be involved in the masking of chitin moieties from host detection.

2.2 Hypothesis

- Exploration of natural variation in Arabidopsis accessions would be useful in identifying genetic factors underlying clubroot resistance.
- Comparative transcriptomics analysis would shed light on potential immune receptors, enzymes, transcription factors etc. that might be involved in establishing resistance in certain accessions.
- *P. brassicae* secretome may carry chitin binding/modifying enzymes used to prevent host recognition.

3. Materials and Method

3.1 Plant material used for the experiments

142 *Arabidopsis thaliana* accessions for the GWAS study were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The core of these accessions came from the Nordborg collection (Nordborg et al., 2005), parental accessions of the MAGIC line collection (Kover et al., 2009) and 118 of the lines belonged to 1001 Genome Project (Weigel & Mott, 2009). Figure 5 shows the geographical distribution of the accessions used. Table 3 lists the GWA codes used for the GWAS study. All the chitin signalling associated T-DNA knock out lines were also ordered from NASC (Table 2).

Figure 5: Geographical distribution of the Arabidopsis accessions used in this study.

Table 2: Information on the chitin-perception related T-DNA knock-out mutants assessed in this study.

Gene name	TAIR ID	Insert	NASC code
LYK4	*AT2G23770*	WiscDsLox297300_01C	CS850683
LYK5	*AT2G33580*	SALK_131911	N667354
CERK1	*AT3G21630*	GABI_96F09	N409189
CHIA	*AT5G24090*	SALK_095362	N676276
(chitinase)	*AT2G43620*	SALK_056680	N657631
(chitinase)	*AT1G02360*	SALK_116178	N616178
B-CHI	*AT3G12500*	SALK_005725	N859510
CHIC	*AT4G19810*	SAIL_798_D06	N835709
(chitinase)	*AT2G43610*	SAIL_559_F11	N823666
(chitinase)	*AT4G01700*	SALK_090662	N590662
(chitinase)	*AT2G43570*	GABI_600G05	N457581

Table 3: List of 142 Arabidopsis accessions used along with corresponding GWA codes.

no.	GWA code	Accession	no.	GWA code	Accession
1	7514	RRS-7	72	6972	Tsu-1
2	7515	RRS-10	73	6939	Mt-0
3	6927	Knox-10	74	6945	Nok-3
4	6928	Knox-18	75	7394	Wa-1
5	7524	Rmx-A02	76	9941	Fei-0
6	7525	Rmx-A180	77	6970	Ts-1
7	7523	Pna-17	78	6971	Ts-5
8	7526	Pna-10	79	8213	Pro-0
9	6913	Eden-2	80	6933	LL-0
10	6043	Lov-1	81	6929	Kondara
11	6046	Lov-5	82	6962	Sakhdara
12	6917	Fab-2	83	6963	Sorbo
13	6918	Fab-4	84	6938	Ms-0
14	6901	Bil-7	85	7058	Bur-0
15	7516	Var2-1	86	7288	Oy-0
16	7517	Var2-6	87	6981	Ws-2
17	6964	Spr1-2	88	6986	Abd-0
18	6965	Spr1-6	89	9609	Adam-1
19	7518	Omo2-1	90	6987	Ak-1
20	7519	Omo2-3	91	428	Borky1
21	6974	Ull2-5	92	7063	Can-0
22	6973	Ull2-3	93	7064	Cnt-1
23	6984	Zdr-1	94	8233	Dem-4
24	6985	Zdr-6	95	9711	Dolna-1-39
25	5837	Bor-1	96	5907	DraIV 2-9
26	6903	Bor-4	97	7107	Durh-1
27	6956	Pu2-7	98	7125	Er-0
28	6951	Pu2-23	99	7127	Est
29	7520	Lp2-2	100	7133	Fr-2
30	6924	HR-5	101	7143	Gel-1
31	6923	HR-10	102	430	Gr-1
32	6944	NFA-8	103	9732	Halca-1
33	6943	NFA-10	104	8304	Hi-0
34	6966	Sq-1	105	8312	Is-0
35	6967	Sq-8	106	7186	Kn-0
36	6907	CIBC-17	107	7209	La-0
37	6968	Tamm-2	108	7213	Ler-0
38	6969	Tamm-27	109	7223	Li-2:1
39	6930	Kz-1	110	7255	Mh-0
40	6931	Kz-9	111	8343	Na-1
41	6920	Got-22	112	7273	No-0
42	6959	Ren-1	113	15593	OOE3-2
43	6960	Ren-11	114	7298	Pi-0
44	6975	Uod-1	115	7308	Po-0
45	6976	Uod-7	116	7306	Pog-0
46	6911	Cvi-0	117	9949	Qui-0
47	6936	Lz-0	118	8365	Rak-2
48	6915	Ei-2	119	7322	Rsch-4
49	6922	Gu-0	120	7328	Sf-2
50	6932	Ler-1	121	9718	Smolj-1
51	6942	Nd-1	122	9728	Stiav-1
52	6906	C24	123	7349	Ta-0
53	7438	CS22491(N13)	124	7373	Tsu 0
54	6979	Wei-0	125	6296	Udul 1-11
55	6980	Ws-0	126	10022	Uk-3
56	7416	Yo-0	127	7413	Wil-2
57	6909	Col-0	128	7415	Wu-0
58	6898	An-1	129	7417	Zu-0
59	7383	Van-0	130	9906	IP-Mah-6
60	6904	Br-0	131	9869	IP-Moj-0
61	6916	Est-1	132	9513	IP-Adc-5
62	8214	Gy-0	133	9830	IP-Bus-0
63	6958	Ra-0	134	9565	IP-Orb-10
64	6899	Bay-0	135	9883	IP-Piq-0
65	6919	Ga-0	136	9890	IP-Rib-1
66	6937	Mrk-0	137	7207	Kyoto
67	6940	Mz-0	138	9758	Altai-5
68	6982	Wt-5	139	7521	Lp2-6
69	7183	Kas-1	140	6908	CIBC-5
70	7067	Ct-1	141	6961	Se-0
71	7522	Mr-0	142	7111	Edi-0

3.2 Plant growth conditions

Plants for experiments were grown in environmentally controlled growth chambers under 9 hours light and 15 hours dark conditions with 22/20 °C ambient temperatures and 60% relative humidity. Osram Fluora T5 fluorescent tubes were used to maintain 120 µmol m^{-2} s^{-1} PAR at the pot level. Long day growth conditions (15 hours light, 9 hours darkness) were used to induce early flowering for transformation. The soil substrate was prepared as a 5:1 vol/vol mix of Klasmann no. 11 soil and perlite. A sand-soil mix was prepared using 1:1 vol of Klasmann no. 11 soil and sand for experiments where roots needed to be rapidly cleaned for subsequent RNA extraction. Soil preparations were sterilized by autoclaving for 1 hour at 121 °C. For plants germinated on agar plates, seeds were sterilized by mixing with 50% commercial bleach (4% NaClO) for 8 mins and then rinsed with sterilized water at least 6 times. MS plates (pH 5.7) were prepared using 0.5 X Murashige-Skoog salts (Duchefa), 1% sucrose and 0.7% agar (Bioshop). Sterilized seeds were stratified in water at 4 °C for 2-4 days in the dark before being spread on the plates. 10 days old seedlings from the plates were transferred to soil for further experiments

3.3 Plant genotyping methods

T-DNA knock out lines ordered from NASC were genotyped using primers designed from the SIGnAL website (http://signal.salk.edu/cgi-bin/tdnaexpress). To extract DNA, first leaves were ground in 400 µl extraction buffer (200 mM Tris-HCL pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The solution was spun at 16000 rpm for 2 mins. 300 µl was transferred to a new tube and precipitated with 300 µl of isopropanol. The pellet was washed with 70% and resuspended in 100 ul of water. Homozygous lines were selected following genotyping by PCR with appropriate T-DNA and insert flanking primers. For rapid genotyping of transgenic lines, the Phire Plant Direct PCR kit (Thermo Scientific, USA) was used. A small amount (~4 mm^2) of Arabidopsis leaf tissue was crushed with a bead beater in 20 µl of extraction buffer, this was used as the template for genotyping PCR following the manufacturer's protocol.

3.4 *P. brassicae* spore extraction

6-7 grams of gall tissue was blended in 200 ml of water for 1.5 minutes. The homogenate was passed through 3 layers of medical gauze and centrifuged at 14500 rpm for 15 mins at 4 °C. Soil and starchy tissue were removed using a spatula. Residual spores were again suspended in a water following centrifugation. The centrifugation and resuspension steps were repeated a few times to remove as much debris as possible. Clear dark spores were resuspended in 50 ml of distilled water. Spores were counted using a Neubauer chamber. The spore solution was kept at 4 °C for up to one month.

A P1+ strain of *P. brassicae* collected from West Pomerania was used in these experiments. The designation of P1 is based on the pathotype classification system of (Some et al., 1996). The "+" indicates its virulence on the Mendel oilseed rape cultivar frequently used in European soils at risk of clubroot. This P1+ pathotype is a strain of *P. brassicae* that is prevalent in Poland and is particularly aggressive (Řičařová et al., 2016; Zamani-Noor et al., 2022). The spores were multiplied in susceptible host *Brassica rapa* var. pekinensis (Granat).

3.5 DNA extraction from Arabidopsis galls

Hypocotyls plus the upper 1 cm of root was harvested to an Eppendorf tube containing 700 ml of extraction buffer (100 mM Tris, 50 mM EDTA, 0.5 M NaCl, 1.3% SDS) and 3 metal balls. Plant material was macerated using a TissueLyser II (Qiagen, Germany) and incubated at 70 °C. The solution was spun briefly and 535 µl was recovered in a fresh tube. Next, 130 µl of cold 5M potassium acetate was added and centrifuged for 5 mins. The supernatant was transferred to a fresh tube containing 60 µl 3M sodium acetate and 640 µl of isopropanol and centrifuged for 5 mins. The dried pellet was dissolved in 200 µl of BTE (2 M Tris-HCL, 0.5 M EDTA) solution. 297 µl of BTE and 3 µl of RNAse (0.6 µg μl^{-1}) (Qiagen) mixture was added and incubated at 37 °C for 30 mins. 500 µl of 24:1 chloroform: isoamyl mix was added and vortexed. The solution was centrifuged and

400 µl of the upper aqueous layer was removed to a fresh tube. The DNA was precipitated by the addition of 800 µl of 96% ethanol and 40 µl 3 M sodium acetate. The pellet was washed with 70% ethanol and resuspended in 200 µl of water. The DNA concentration was quantified on a Nanodrop (Thermo Scientific, USA) and a final solution of 20 ng μl^{-1} was used as a template for qPCR.

3.6 Pathogen assay- qPCR and Disease Index Scoring

17 days old seedlings were inoculated with 2 ml of a 10^6 spores ml^{-1} preparation. Plants were harvested 19 dpi and the gall plus 1 cm of root was collected for DNA extraction. Relative pathogen load was quantified by qPCR using *AtSK11* as a single-copy host gene for relative normalisation and *Pb18S* as the pathogen target. 20 ng μl^{-1} concentration template was used for the qPCR reaction using Luna Universal qPCR Master Mix (NEB, USA) and the LightCycler 480 System (Roche, Switzerland). 10 µl of total reaction volume was used for individual qPCR runs: 5.5 µl of master mix and 4.5 µl of DNA template (90 ng). The qPCR reaction cycle was as follows: Initial denaturation at 98 °C for 2 mins. 40 amplification steps with 15 seconds at 95 °C and 30 seconds at 60 °C. A melting curve analysis of the products was run from 50 °C to 95 °C. CP values were obtained using the Second Derivative Max analysis in the Roche LightCycler software. Two technical replicates were used for every sample run. If the difference in CP between technical reps was more than 0.5 cycles, the sample was repeated. Technical replicates were then averaged. The normalized pathogen DNA level was obtained by subtracting the CP of *Pb18*S from the CP of *AtSK11*.

The galls for the GWAS analysis was also scored using the Disease Index (DI) method according to the method of (Ludwig-Müller et al., 2017).

Each gall was given a score between 0-4 according to the following scale:

0 for no visible symptoms of swelling in the main root and lateral roots.

1 for gall formation on lateral roots but main root remains unaffected.

2 for significant gall formation on lateral root leading to loss of parts and thickening of the main root.

3 for significant loss of lateral roots and medium size gall formation in the main root and hypocotyl.

4 for complete loss of lateral root and formation of a large gall around the hypocotyl, often pushing above the soil.

DI was calculated using the following equation:

$$\mathrm{DI} = \frac{1 * n_1 + 2 * n_2 + 3 * n_3 + 4 * n_4}{4N}$$

N= Total number of plants tested, n_1 to n_4 denote the number of plants belonging to each particular disease severity scale.

3.7 RNA extraction and quality control (QC)

Harvested tissue was pulverized in liquid nitrogen and the RNA was extracted using the Invitrap Spin Plant RNA Kit (Invitek-Molecular, Germany) using the manufacturer's protocol. The RNA was quantified using the Nanodrop (Thermo Scientific, USA) and the concentration was adjusted with the elution buffer for different downstream applications. Purified RNA was run on agarose gels to check for integrity. For the RNASeq experiment, the RNA samples were further checked via Experion RNA Stdsens Analysis Kit (Bio-Rad, USA) which assesses the RNA integrity according to the RQI (RNA Quality Indicator) metric.

3.8 cDNA synthesis

Extracted RNA was DNase treated using the Ambion DNase I (Thermo Fisher, USA) kit. Then the RNA samples were checked with PCR using ACTIN2 or SK11 primers to detect any residual DNA contamination. 1 µg of RNA was used to carry-out first strand cDNA synthesis using M-MLV Reverse Transcriptase, RNase H Minus (Promega, USA). The cDNA was then diluted 5 times with nuclease free water to be used as qPCR template. 10 µl of (4 µl of cDNA template, 6 µl of master mix) qPCR reaction volume was applied

in each well. Luna Universal qPCR Master Mix (NEB) and SensiFAST Real Time PCR kit (Bioline) were used as qPCR mixes.

3.9 Gel electrophoresis

1-1.5% agarose (Duchefa, Netherlands) gels were prepared with TE (Tris-EDTA) buffer for DNA electrophoresis runs. 3 µl of SYBR Safe (Thermo Fisher) visualization dye was used per 100 ml of agarose gel. Gels were run between 100-120 V. 3-5 µl of Generuler 1Kb/1Kb+ was used as a marker ladder. Gels were visualized and photographed using the Gel Doc XR+ system (Bio rad).

3.10 Plasmid isolation

Overnight LB (Luria Bertani) (Sezonov et al., 2007) cultures of *E. coli* or 6-hour cultures of *Agrobacterium* were used for plasmid extraction. 2-3 ml of culture were centrifuged at 5000 g for 5 mins. Plasmids were extracted using the Syngen Plasmid Mini kit (Syngen Biotech, Taiwan).

3.11 Gene cloning steps

Targeted DNA fragments were amplified using Phusion High Fidelity DNA polymerase (NEB, USA). The amplified fragments were TA-ligated into entry vectors pCR8/GW/TOPO or pENTR/SD/D-TOPO (Thermo Fisher, USA). Ampicilin (100 µg/mL) was used for screening of transformed colonies. The cloned fragments were checked by Sanger sequencing. LR Gateway recombination was performed for transfer into destination binary vectors pJCV53, pK7FWGF2 and pKGWFS7 using Gateway LR Clonase II Enzyme mix, (Thermo Fisher, USA) The binary vectors were transformed into *E. coli* One Shot TOP10 Chemically Competent Cells (Invitrogen, USA) for propagation and into *Agrobacterium tumefaciens* EHA105 strain for transformation of Arabidopsis.

3.12 Floral dip and transgenic selection

Agrobacterium tumefaciens electrocompetent EHA105 cells were transformed with binary vectors carrying desired genetic fragments. Electroporation was done at 2.5 kV in an electroporation cuvette with a 0.1 cm gap. The cells were recovered in LB medium and incubated at 27 °C for 2 hours. LB plates were prepared with Spectinomycin (50 µg ml^{-1}) / Kanamycin (50 µg ml^{-1}) for vector selection and Rifampicin (25 µg ml^{-1}) for *Agrobacterium* selection. Incubated cells were spread on the plate and incubated at 26 °C for 3 days to obtain transformed colonies. A single agrobacterium colony containing the insert was inoculated in a 5 ml LB culture and incubated at 26 °C for 2 days. The starter culture was added to a 400 ml LB culture at incubated at 26 °C for 16-20 hours to reach OD_{600} between 1.5 and 2. The Agrobacterium cells were collected by centrifuging the culture at 4000g for 10 mins and the pellet was dissolved in 1 volume of 5% sucrose solution. 0.02% Silwet L-77 was added to the solution just before the floral dipping. Arabidopsis plants were grown at long days (15 hours light/ 9 hours dark) conditions to induce early flowering. Initial inflorescences were clipped off to encourage denser inflorescence emergence. The final inflorescence clipping was done 5-7 days before the floral dip in *Agrobacterium* solution. Arabidopsis aerial parts were dipped in freshly prepared *Agrobacterium* solution for 10 seconds with gentle agitation. Excess liquid was drained off for 2-5 seconds and plants were covered in aluminium foil to avoid excess heat/light and moisture loss. Plants were uncovered after 24 hours and were grown in long day conditions until seed collection. The floral dip was repeated once more after 7 days to increase the number of transformed seeds. Harvested seeds were plated on MS plates with Kanamycin (50 µg ml^{-1}) to select for transgenic plants. Putative transgenic plants were genotyped with insert specific primers. If the plants carried pJCV53 vector with *RFP* insert, then an AxioZoom V16 monoscope was used to check for red fluorescence protein signal to verify transgenic status.

3.13 Protein extraction and chitinase assay

For chitinase activity assays, entire Arabidopsis root systems were harvested and pulverized in liquid nitrogen. The powder was suspended in an extraction buffer

consisting of 50 mM Tris-HCL (pH 8), 5mM NaCl, 1mM EDTA (pH 8) and protease inhibitor cocktail (cOmplete mini, Roche). The solution was centrifuged and the supernatant containing the soluble proteins was carefully removed to a fresh tube. Protein concentration was measured in each sample using the Qubit Broad Range (BR) Assay Kit (Thermo Fisher, USA). Then the protein concentration was equalized among samples with extraction buffer. The endo and exo-chitinase enzymatic assay was performed using a fluorometric Chitinase Assay Kit (Sigma-Aldrich, USA) and a Synergy HTX plate reader (BioTek, USA).

3.14 Transcriptome sequencing

The experimental setup included Col-0 and Est-1 plants with two treatments: mock and *P. brassicae* infection at 7 dpi. Five biological replicates (each sample consisting of 12 plants) were included in the experimental setup, out of which 3 were chosen for sequencing based on the expression level of immune markers. 2 μg of extracted RNA was sent to be sequenced by Genomed (Poland) using the BGISeq-500 platform. The methodology for library preparation was based on oligo-dT priming of mRNA transcripts and the generation of strand specific data with paired-end reads of 100 bp.

3.15 RNAseq data analysis pipeline

Upon completion of the sequencing, paired-end FASTQ files containing sequencing reads were received along with FastQC report files containing various sequencing quality metrics for each sample. Mapping of those reads to a reference genome and further analysis to obtain differentially expressed genes/enrichment analysis was performed as follows:

Trimmomatic (Bolger et al., 2014) was used to trim contaminating adapter sequences from the ends of the reads. Reads were mapped to TAIR 10 Arabidopsis genome using the HISAT2 (Kim et al., 2019) package. The HTSeq Count (Anders et al., 2015) package was used to generate a read count file for each sample. The DESeq2 (Love et al., 2014) package was used to identify significantly differentially expressed genes between the

various conditions. MultiQC (Ewels et al., 2016) was used to generate graphical reports for these steps. KEGG and GO graphs were generated using ShinyGO (http://bioinformatics.sdstate.edu/go/) online tools (Ge et al., 2020). Bingo (Maere et al., 2005) GO term analysis was performed in Cytoscape (Shannon et al., 2003). Cluster 3 was used to generate complete linkage hierarchical clusters of expression data (de Hoon et al., 2004). Galaxy online bioinformatics suite (Afgan et al., 2018) , CLC Genomics (Qiagen, USA) and R (https://www.R-project.org/) were used as platforms for the above-mentioned analyses. PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw/) was used for promoter analysis.

3.16 Statistical analysis and other bioinformatics software

Statistical analysis was performed in GraphPad Prism (GraphPad Software Inc., USA), R and Microsoft Excel. Linear mixed modelling was done using the lme4 package in R (Bates et al., 2015). Normalisation genes used for qRT PCR were selected using NormFinder (Andersen et al., 2004). The Geneious (BioMatters, Australia) bioinformatics suite was used to perform analysis of Sanger sequencing data, perform sequence alignments, primer design, and cloning strategies.

3.17 List of Primers

Table 4: Table of primer used in the study.

Primer name	Sequence 5'-3'	Function of the primer
AtSK11_F	CTTATCGGATTTCTCTATGTTTGGC	qPCR forward primer of *AT5G26751*
AtSK11_R	GAGCTCCTGTTTATTTAACTTGTACATACC	qPCR reverse primer of *AT5G26751*
Pb18S_F	AAACAACGAGTCAGCTTGAATGC	qPCR forward primer of *P. brassicae 18S* gene.
Pb18S_R	AGGACTTGGCTGCGGATCAC	qPCR reverse primer of *P. brassicae 18S* gene.
RPB1a-F	ATGGAGACTGTCTCCGCCG	Forward primer for cloning CDS of Est-1 *RPB1* gene.

RPB1a-R	TTAAACTGGTGGTAACTGAGG	Reverse primer for cloning CDS of Est-1 *RPB1* gene.
RPB1a-prom(new)-F	CACCGGAAACCCAAATATAAAACCA	Forward primer for cloning 1kb promoter + CDS of Est-1 *RPB1* gene.
RPB1a_qPCR_F	GGTTTAGTCCCAAGGCTCATT	qPCR forward primer of Est-1 *RPB1* gene.
RPB1a_qPCR_R	GAGAGCCAGATAAACCAGAGAAG	qPCR forward primer of Est-1 *RPB1* gene.
qRT_3G48140_F	TTGTTCGCTGCTACCGGAGTTG	qPCR forward primer of *AT3G48140*
qRT_3G48140_R	CCTGTTCTCCTTGGTGCATCTGAC	qPCR reverse primer of *AT3G48140*
VAB1_fwd	TGGACATTGCTC CGTATCTTC	qPCR forward primer of *AT1G76030 (V-ATPASE B subunit 1*)
VAB1_rev	TCGATAAGATAA CCTCCATTACCTC	qPCR reverse primer of *AT1G76030 (V-ATPASE B subunit 1*)
AT5G1076_qPCR_F	CGGTGACATACC CGACGATT	qPCR forward primer of *AT5G10760 (AED1)*
AT5G1076_qPCR_R	CATTCCCTGCAA ACGCCAAA	qPCR reverse primer of *AT5G10760 (AED1)*
AT2G30770_qPCR_F	ACGATAAAGCGG ATTTCGTGGA	qPCR forward primer of *AT2G30770 (CYP71A13)*
AT2G30770_qPCR_R	GAAGTTGTTGAC GTTCCTCCG	qPCR reverse primer of *AT2G30770 (CYP71A13)*
PbChitD_F	CTCGATGGATTGGACGGGAG	qPCR forward primer of *PbChiBD2*
PbChitD_R	TCTCCACCGTCACAAACTCG	qPCR reverse primer of *PbChiBD2*
TriCHIT_F	ACCGTGAGAACATTCGGCAT	qPCR forward primer of Trichoderma chitsonase *AY571342*
TriCHIT_R	CGGATGACTGGGGGTTGAAA	qPCR reverse primer of Trichoderma chitosanase *AY571342*
5081_No Sig_F	CACCATGTATGGGCCTGTCTGCCAC	Forward primer for cloning CDS of *PbChiBD2* without signal peptide.
5081_Stop_R	TTATGTCTCATCGAACGCGCC	Reverse primer for cloning CDS of *PbChiBD2*.
PR1_F	CGGAGCTACGCAGAACAACT	qPCR forward primer of *AT2G14610 (PR1)*

PR1_R	CTCGCTAACCCACATGTTCA	qPCR reverse primer of *AT2G14610 (PR1)*
JAZ10_F	AACCAACAACGCTCCTAAGC	qPCR forward primer of *AT5G13220(JAZ10)*
JAZ10_R	ACTAGACCTGGCGAGAGACG	qPCR reverse primer of *AT5G13220(JAZ10)*
PAD3_F	TCCAAAACTCTGGGAAAACG	qPCR forward primer of *AT3G26830 (PAD3)*
PAD3_R	CCCCAATCGAAGAAGTAAAGC	qPCR reverse primer of *AT3G26830 (PAD3)*
AT2G43620_F	ACACTCGCCAGGCTTTCATTGC	qPCR forward primer of *AT2G43620* (chitinase)
AT2G43620_R	ATCTCACGCTTAGCGACGGTTC	qPCR reverse primer of *AT2G43620* (chitinase)
At3g54420_F	GAGATTGCAGCGTTCTTTGC	qPCR forward primer of *AT3G54420* (chitinase)
At3g54420_R	GATCCGTGGCTACTGTTTCC	qPCR reverse primer of *AT3G54420* (chitinase)
At1g02360_F	AATGGCGGTTTAGAGTGTGG	qPCR forward primer of *AT1G02360* (chitinase)
At1g02360_R	TTTTATGGCTCCATGTGTGG	qPCR reverse primer of *AT1G02360* (chitinase)

4. Results

4.1 Genome Wide Association Study (GWAS) of 142 Natural Arabidopsis accessions against a Polish *Plasmodiophora brassicae* pathotype reveals the role of *RESISTANCE TO PLASMODIOPHORA BRASSICAE 1 (RPB1)* gene in mediating resistance against clubroot disease.

4.1.1 *In planta* density of *P. brassicae* spores reaches a plateau 19 days post infection

To select a timepoint suitable for quantifying the growth of *P. brassicae* in Arabidopsis, a time-course experiment on the highly susceptible Arabidopsis accession Col-0 was set up. 17 days old seedlings were inoculated with the P1+ pathotype and galls were harvested at 10, 13, 16, 19, 22, 25, 28 and 31 dpi. The plant tissue collected included the hypocotyl plus the upper 1 cm of root. DNA was extracted from the harvested material and relative pathogen DNA levels was determined by qPCR. 19 dpi represented a timepoint when the pathogen DNA concentration reached a plateau (Figure 6). Thus, 19 dpi was chosen as the timepoint to score relative pathogen growth among 142 diverse accessions. Later timepoints were not chosen due to the increased amount of starchy material in galls that prevents optimal DNA extraction. Additionally, galls become brittle at later timepoints making it difficult to collect the entire tissue without losing parts of it.

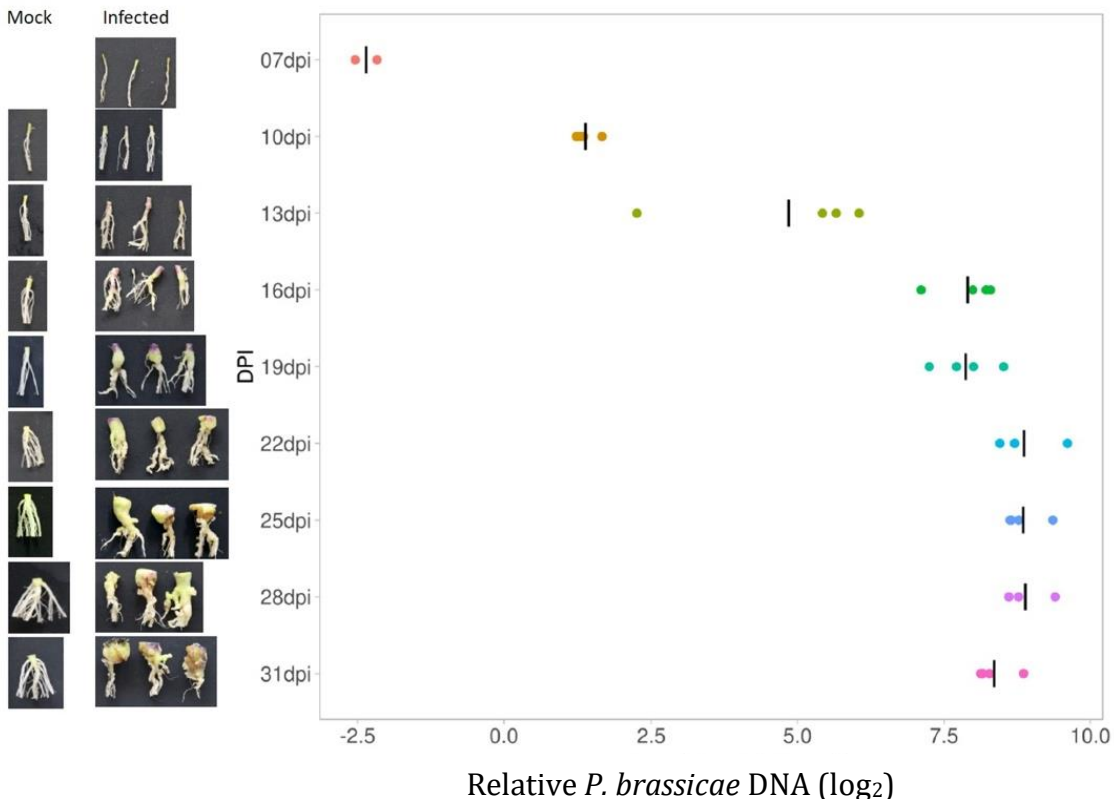


Figure 6: *P. brassicae* growth curve from 7 to 31 days post infection.

Black bars for each day represent the mean of 4 data points, each consisting of 3 plants.

4.1.2 Arabidopsis accessions show a wide spectrum of disease susceptibility

142 natural inbred lines of Arabidopsis plants were screened against the P1+ pathotype of *P. brassicae*. The plants showed a range of root deformities 19 days post infection. 11 accessions showed little to no gall formation (resistant), most had large, expanded galls (susceptible), the rest had intermediate degrees of hypocotyl and root swelling (Figure 7). Disease severity was scored using two methods: 1) Relative pathogen load by performing qPCR on the DNA extracted from galls, 2) Assigning a Disease Index score by visual inspection of gall symptom development (Figure 8). Based on the two methods of quantifying disease severity, 11 accessions were considered resistant. Pro-

0 was an outlier which possessed the highest amount of pathogen DNA despite average gall size.

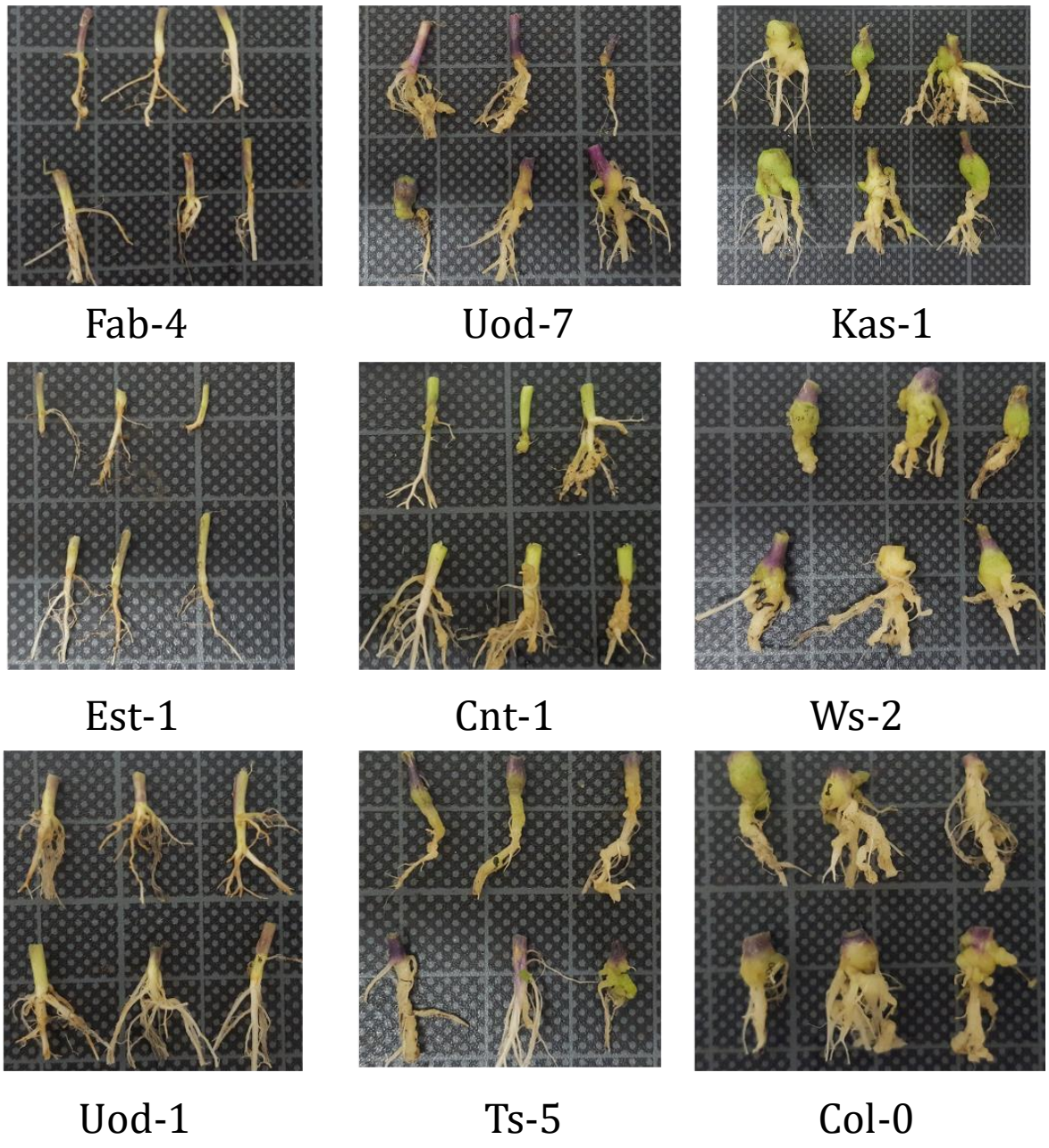


Figure 7: Exemplary pictures showing the severity of root system deformation in *P. brassicae* - infected accessions at 19 dpi.

Galls were harvested 19 days post infection. Fab-4, Est-1 and Uod-1 roots have been shown as examples of resistant lines. Uod-7, Cnt-1 and Ts-5 have been shown as intermediate lines. Kas-1, Ws-2 and Col-0 are examples of fully susceptible accessions.

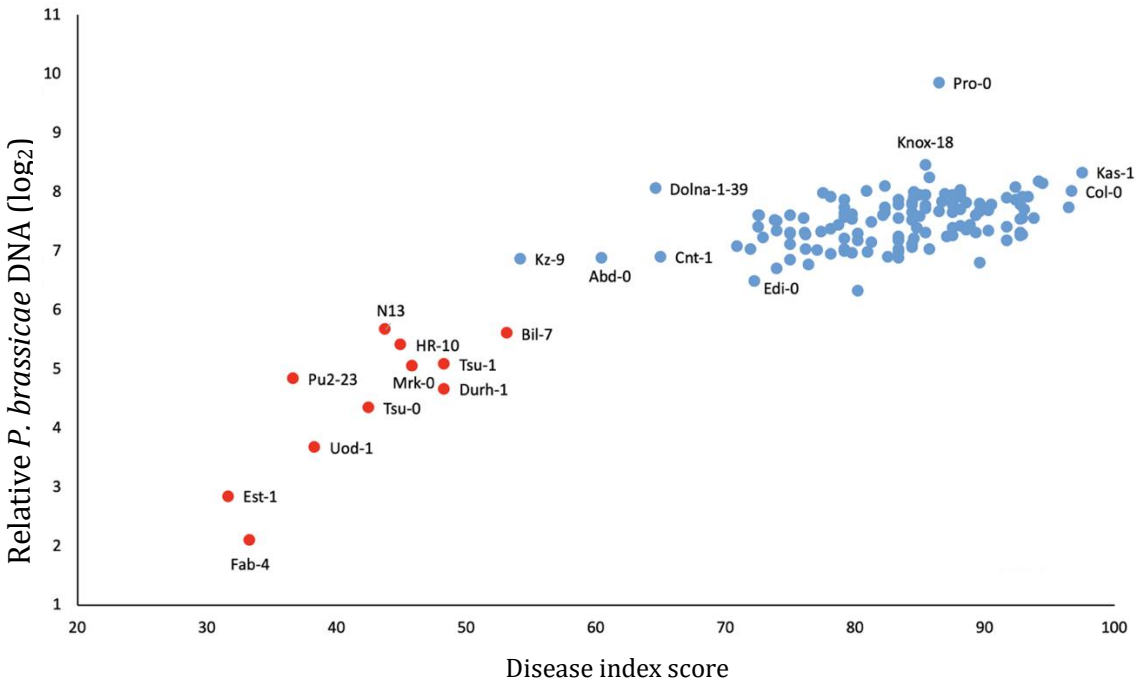


Figure 8: Susceptibility of 142 accessions to *P. brassicae* with relative pathogen load and disease index scores.

Accessions considered resistant have been marked in red.

4.1.3 Identification of a significant SNP by GWAS analysis

GWAS analysis with the input of relative pathogen load was performed using the GWA-Portal online toolset (https://gwas.gmi.oeaw.ac.at/#/home). Genotype-phenotype data for 141 accessions was uploaded to the portal for analysis. Pro-0 being an outlier, was excluded from the dataset to avoid skewness. The data was BOX-COX transformed to provide a normal distribution (Shapiro Wilk *p*-value 0.71) (Figure 9). GWA-Portal provides four genotype datasets for Arabidopsis accessions: 1) 250K SNP dataset from 1386 accessions consisting of 214,000 SNPs, 2) Swedish Genome dataset with 259 samples and 6 million SNPs, 3) 1001 genomes dataset with 1135 accessions and 10 million SNPs and 4) Imputed full sequence dataset that combines 250K SNP and 1001 genomes by imputation (2029 samples and 10 million SNPs) (Seren et al., 2018). For our analysis the imputed full sequence dataset was chosen as it covered 140 out of 141 accessions uploaded (except Po-0). The Accelerated Mixed Model (AMM) method for GWA analysis was chosen as this method accounts for population structure. A

Bonferroni multiple testing correction threshold of < 0.05 was considered significant for SNP calls.

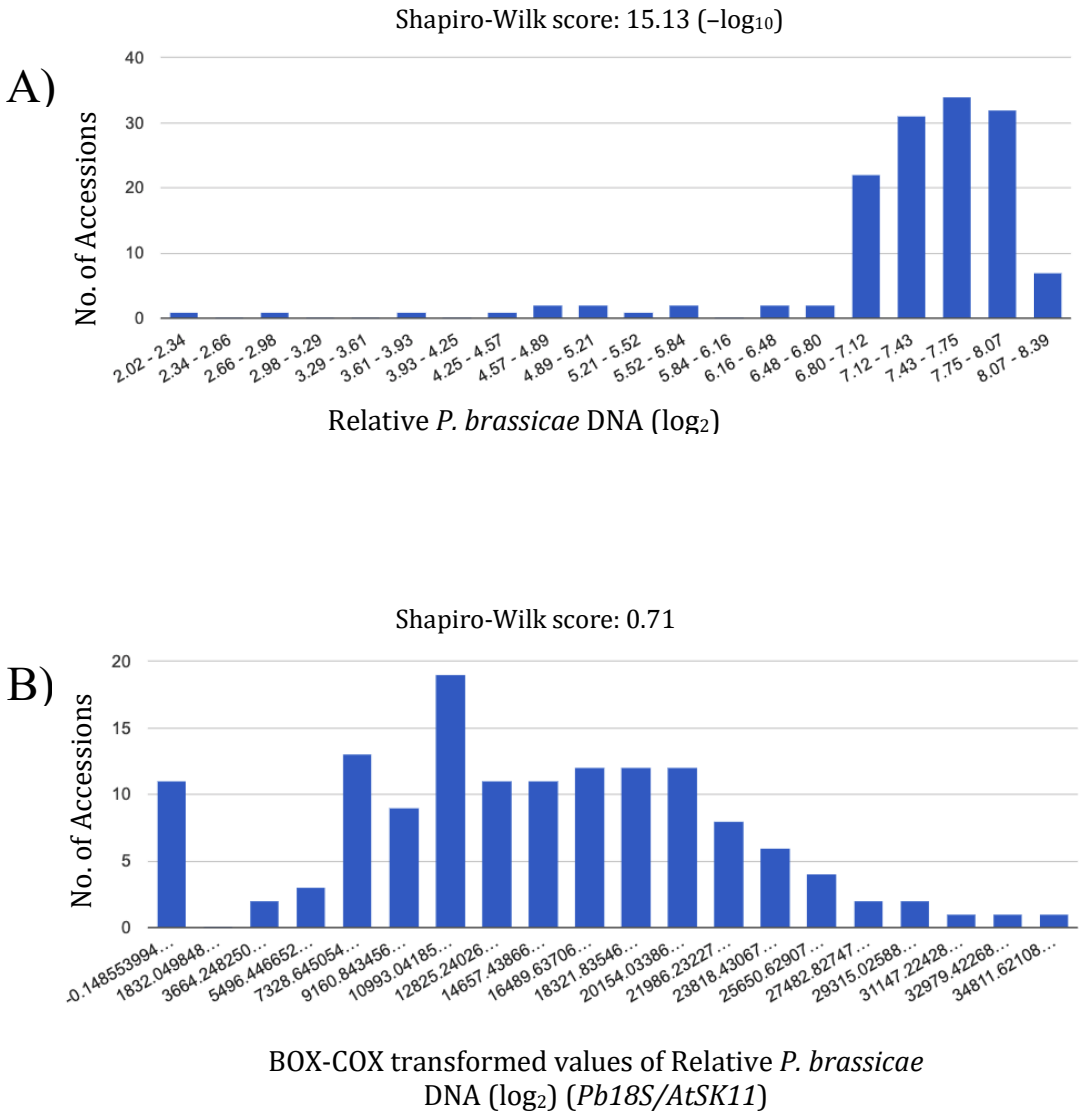


Figure 9: Histogram of raw and transformed phenotype data used for GWAS.

A) The histogram of raw phenotype data distribution. The distribution is heavily right-skewed. B) Box-Cox transformed distribution of the raw phenotype data.

GWAS analysis identified one SNP with a highly statistically significant association with the relative pathogen quantification in accessions ($-\log_{10}$ *p* value 9.19) situated on chromosome 1 (Figure 10). The SNP is located in the gene *AT1G32030* and corresponds to a mis-sense mutation (A to T transition). This substitution is present in 13 accessions out of which 10 are resistant (Figure 11).

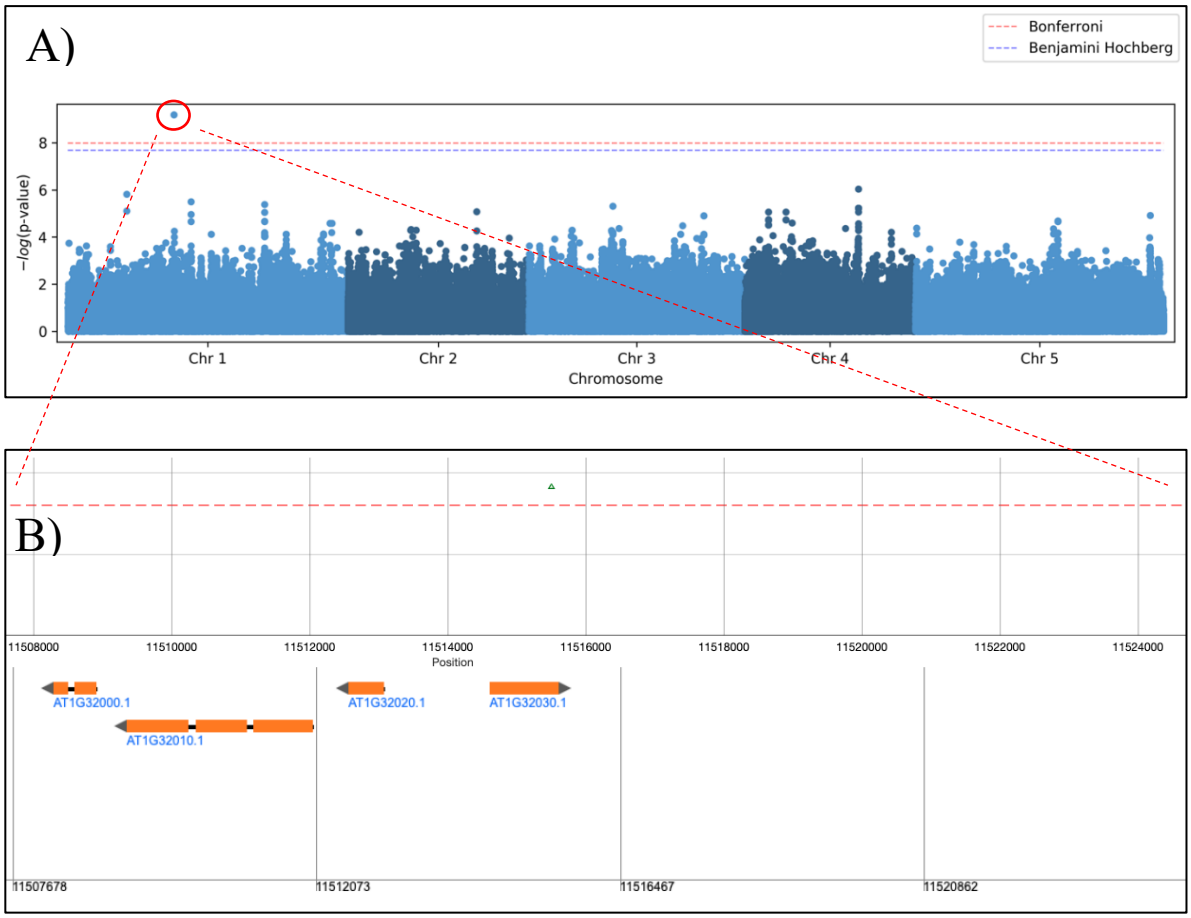


Figure 10: Manhattan plot generated by the GWAS analysis.

A) The SNP with a p-value below the Bonferroni threshold for significance is situated in Chromosome 1. B) Shows the zoomed-in view of the significant SNP. The SNP is located in *AT1G32030*.

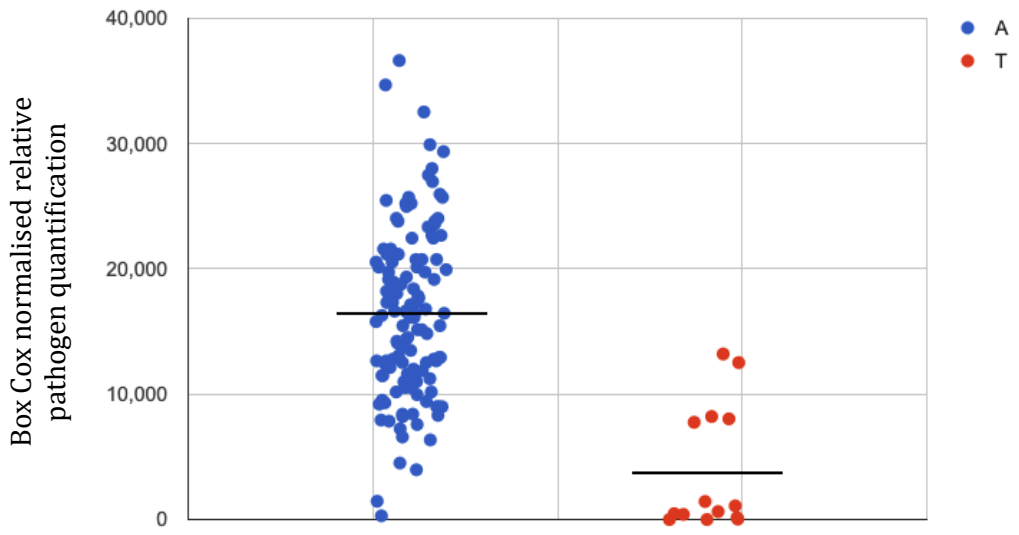


Figure 11: The allele distribution of the Chr1: 11515504 SNP

4.1.4 The significant SNP is located near the previously discovered *Resistance to Plasmodiophora brassicae 1 (RPB1)* locus

The *RPB1* locus was previously identified in a Ze-0 x Tsu-0 mapping population against the 'e3' strain of *P. brassicae* (Fuchs & Sacristán, 1996). The locus identified in Tsu-0 confers complete resistance against clubroot infection with signs of hypersensitive response. This resistance is monogenetically inherited. The sequence of the resistance loci from two resistant backgrounds Tsu-0 and RLD has been deposited in the GenBank database, however, no publication describes the gene responsible for conferring resistance. Comparing the *RPB1* locus in Tsu-0 and RLD with the reference Col-0 genome sequence, it is clear that a sizeable portion of the locus is missing from Col-0 genome (Figure 12). In Col-0, the area lacking the sequences from the resistance accessions contains transposable elements (*AT1G32040, AT1G32045*) which might explain such a deletion. The *RPB1* ORF is around 2 kb downstream of *AT1G32030* which carries the SNP identified by GWAS. The sequenced region available for Tsu-0 is around 13 kb long (GenBank ID FJ807885) and carries two ORFs with identical sequences

named *RESISTANCE TO PLASMODIOPHORA BRASSICAE 1* (*RPB1a* and *RPB1b*). Tsu-0 also carries an ORF with high sequence similarity to *RPB1*, named *RPB1-like 1.* In the region sequenced in RLD fragment (GenBank ID FN400762), there is only one copy of *RPB1* and three other RPB1-like sequences: *RPB1-like 2, RPB1-like 3, RPB1-like 4*. Col-0 has no functional copy of *RPB1* but carries a pseudogene with 54% similarity at the DNA sequence level but no open reading frame (*AT1G32049*).

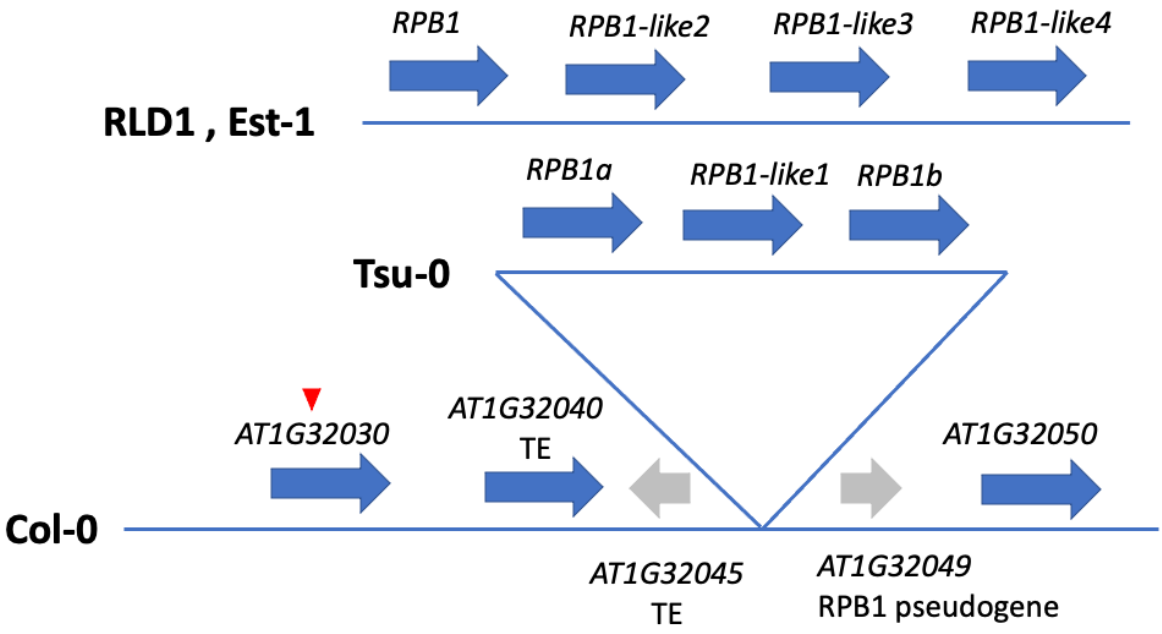


Figure 12: Schematic showing the *RPB1* region in Col-0 and three resistant lines.

RPB1a, RPB1b and *RPB1-like* genes are absent in Col-0 accession. The red arrows indicate the location of GWAS SNP.

4.1.5 *RPB1a* is a potential candidate to be involved in the resistance response

Tsu-0 carries 2 copies of *RPB1* identical at the amino acid level (148 aa length). There are 3 SNPs present between *RPB1a* and *RPB1b* which are synonymous polymorphisms (Figure 13). RLD carries only *RPB1a*. Est-1 from our set of resistant accessions also carries the *RPB1* gene as determined by Sanger sequencing. The similarity between the 1000 base pair upstream promoter sequence between *RPB1a* of Tsu-0, Est-1 and RLD

is above 99%. *RPB1* and *RPB1-like* genes were investigated to determine if they were expressed in Est-1 upon infection. 17 days old plants were infected with the P1+ pathotype and 7 days post infection, roots from mock and inoculated plants were harvested for gene expression studies. *RPB1* was strongly upregulated upon infection (Figure 14) while *RPB1-like 4* also had slight but statistically significant induction. Other *RPB1-like* genes were not expressed. Considering the facts that *RPB1a* being the only common factor between the Tsu-0 and RLD loci, *RPB1a* promoter region being highly similar between resistant accessions and it being the strongly upregulated *RPB1* locus associated gene upon infection, *RPB1a* was considered as a promising candidate for further exploration. As Est-1 has only one copy of *RPB1,* from now on all the results related to *RPB1a* will be denoted as *RPB1*.

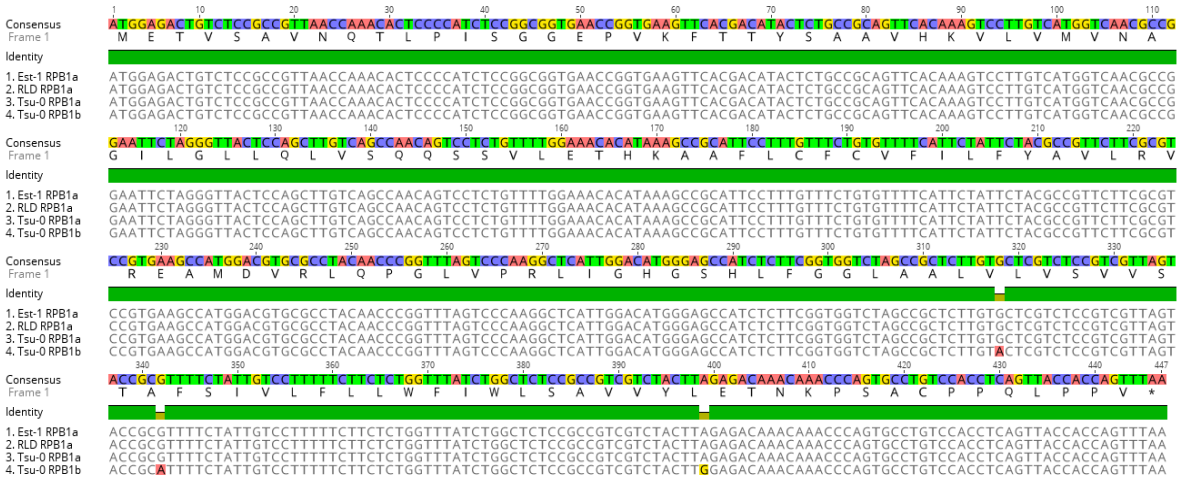


Figure 13: Multiple alignment between Est-1 *RPB1a* , RLD *RPB1a* ,Tsu-0 *RPB1a* and *RPB1b* coding sequences.

The presence of 3 SNPs do not affect the protein sequence.

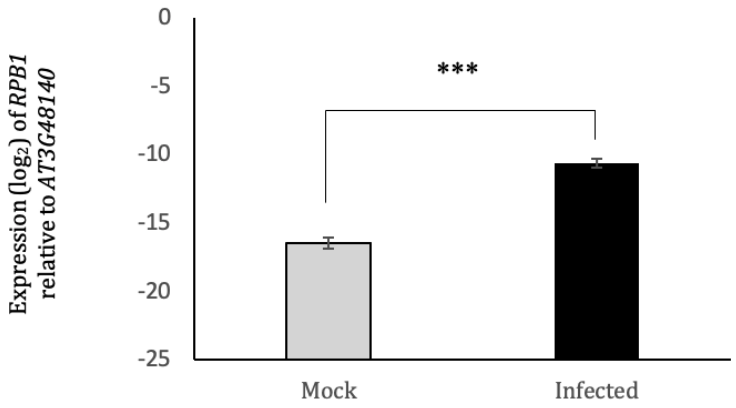


Figure 14: Relative expression of *RPB1* gene in Est-1 upon infection.

The graph represents 5 replicates each consisting of 12 plants. Entire root tissue was harvested 7 days post infection. Asterisks (***) indicate a significant difference ($p < 0.0005$) calculated by Student's T-Test.

To experimentally characterize the role of *RPB1*, it was knocked by CRISPR-Cas9 mediated techniques by another researcher in the group, Juan Camilo Ochoa. The deletion of *RPB1* resulted in total loss of resistance in Est-1 and Uod-1 accessions (Ochoa, 2022). To further characterize the role of *RPB1* it was planned to ectopically express *RPB1* in susceptible Col-0 in three different ways. The first approach involved over-expressing *RPB1* under the control of a 35S constitutive promoter, the second approach was to use the same promoter but with a *GFP* tagged version of the gene and the third strategy involved cloning the entire native promoter plus *RPB1* from Est-1 and transferring the entire fragment to Col-0.

4.1.6 Overexpression of *RPB1* under 35S constitutive promoter is non-viable in Col-0 and elicits PCD in tobacco

Repeated attempts were made to transform Col-0 with the 35S-*RPB1* construct by the floral dip method. Figure 15 depicts the pJCV53 vector that was used for the construct preparation with the the ccdb sequence replaced by the *RPB1* coding sequence including the STOP codon. Multiple transformation attempts were made and several thousand seeds were screened but no transformants could be obtained. *RPB1* is

potentially an immune response mediator gene, thus, there is always the possibility of embryonic lethality when such immune genes are overexpressed, such examples are well documented (Li et al., 2019). To further verify this hypothesis, in collaboration with Juan Camilo Ochoa, the same *Agrobacterium* preparation used for the Arabidopsis transformation was used to infiltrate tobacco leaves for transient expression. This generated a very strong localized cell death response upon infiltration while the empty vector did not elicit such a response (Figure 16). This led us to hypothesize that overexpression of *RPB1* in Col-0 may lead to a strong auto-immune response resulting in embryo lethality.

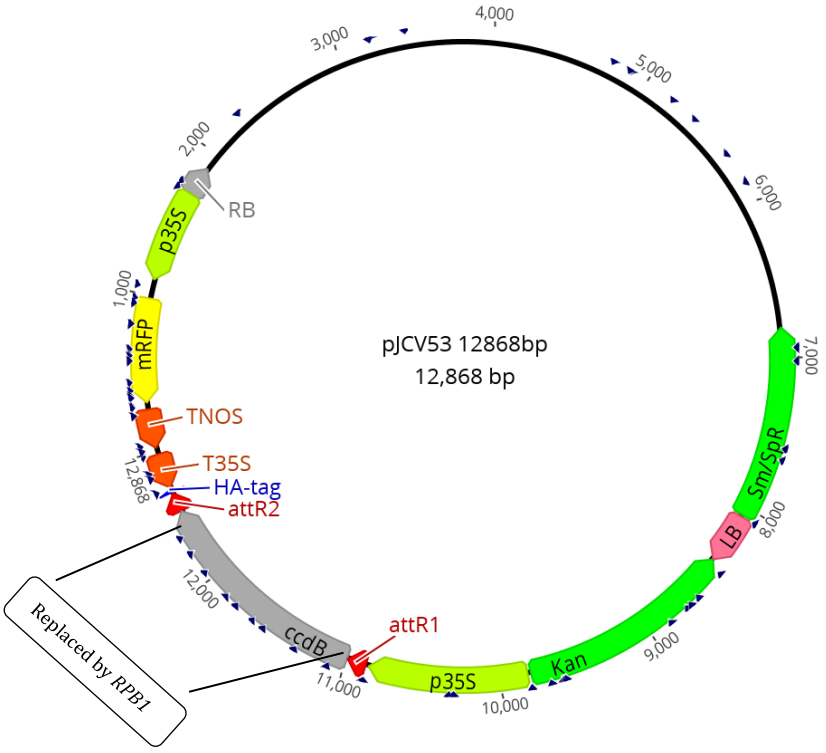


Figure 15: Vector map of pJCV53.

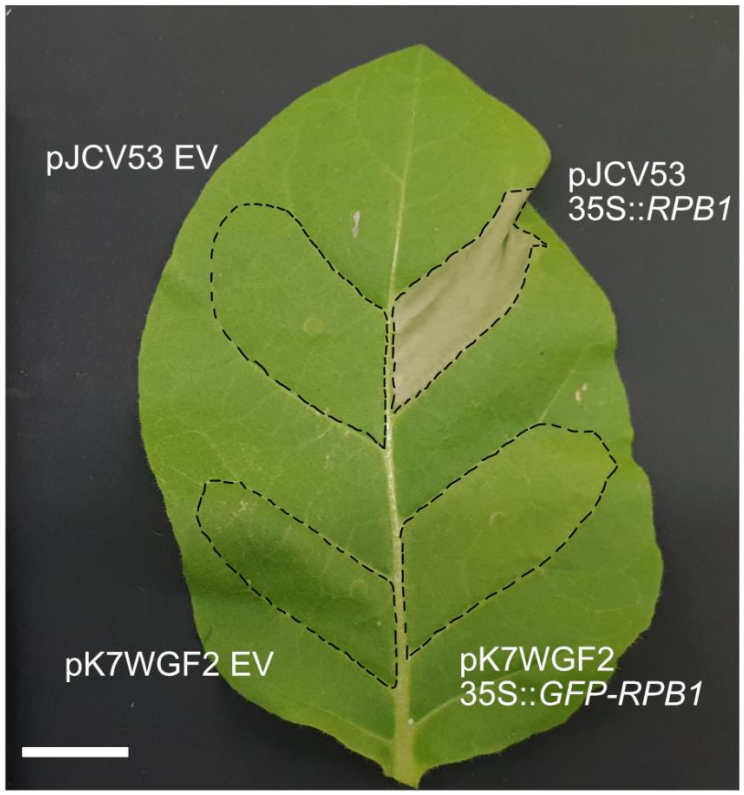


Figure 16: *Agrobacterium* mediated transient expression of *35S::RPB1* and *35S::GFP-RPB1* constructs in *Nicotiana tabacum* leaf three days after infiltration.

Scale bar represents 2 cm, EV represents empty vector control. (Photo credit: Juan Camilo Ochoa)

4.1.7 *GFP-RPB1* overexpression correlates with smaller gall and rosette size.

A second approach to characterizing the potential function of *RPB1* in Col-0 involved fusing a *GFP* tag at the 5' end of *RPB1* and expressing it under the 35S promoter. The aim was to observe the cellular localization of *RPB1* under a fluorescence microscope. Figure 17 shows the vector map of pK7FWGF2 which was used, replacing the ccdb region with the *RPB1* coding sequence (including STOP codon).

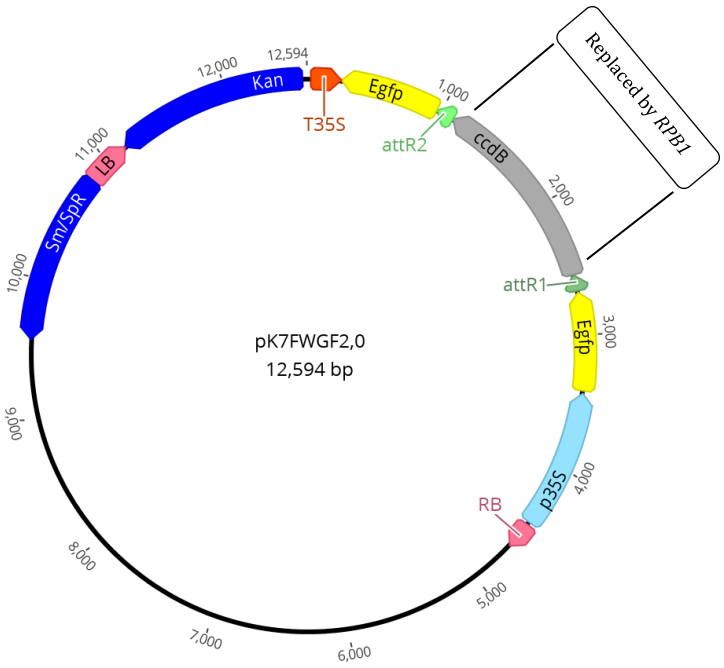


Figure 17: Vector map of pK7FWGF2,0

Transformation of Col-0 plants resulted in only few viable transgenic lines. Three independent lines (named A, B and C) were selected to generate T2 and T3 generations. The T2 generation plants exhibited a wide range of phenotypical abnormalities. Most plants were short, stunted and took a long time to flower. Some lines did not produce any seed. Figure 18 shows the spectrum of growth retardation in 3 transgenic lines. No GFP fluorescence was observed in any of the transformants. RPB1 possesses a predicted membrane bound domain, the absence of GFP signal may be due to the improper folding of the protein after fusion with RPB1. Viable but stunted transgenic lines compared to the absence of transformants for *35S::RPB1* without a tag indicates the possibility of a weakened immune signalling capability of RPB1 due to attachment of GFP which might interfere with RPB1 interacting partners. Unlike *35S::RPB1*, *35S::GFP-RPB1* did not elicit PCD in tobacco leaves (Figure 16). In order to assess the impact of *GFP-RPB1* expression on clubroot disease a large number of T3 plants were evaluated. For each of the three original transformants, several T3 plants representing a range of growth restriction phenotypes were chosen (though the most extreme examples did not produce seed) in one large experiment, evaluating both rosette

growth and clubroot disease. All plants were genotyped prior to infection as they were segregating. Transgenic plants that were deemed too small for analysis were removed at inoculation (17 days after germination). At 19 dpi galls were collected to assess pathogen growth. Simultaneously, the leaves were harvested for RNA extraction to determine the expression of *RPB1* and the defense marker gene *PR1*. For most T3 descendants, three plants were pooled for these extractions, for smaller T3 lines 4-6 plants were combined for analysis.

Figure 18: Phenotypic variation in rosette size and root / gall morphology in 3 *35S::GFP-RPB1* transgenic lines.

Pictures were taken from plants 19 days post inoculation.

The expression of *RPB1* was found to vary greatly in the leaves of T3 plants. The level of *RPB1* expression in leaves inversely correlates with the rosette size (Spearman rho = -0.76) and also with the pathogen DNA quantified in galls and the diameter of clubroot galls (Spearman rho = -0.61 and -0.83 respectively) (Figure 19). One possible explanation for the clubroot gall phenotype would be that expression of *GFP* tagged *RPB1* results in stunted plants and thus smaller galls perhaps due to the smaller root system available for infection and reduced photoassimilate available to fuel *P. brassicae* multiplication. However, the expression of *PR1* (Pathogenesis Related 1), a widely used immune marker associated with salicylic acid mediated responses (Papadopoulou et al., 2018) correlates positively with the *RPB1* expression level (Spearman rho = 0.66)

(Figure 19). This indicates that GFP-tagged *RPB1* over-expression can also generate a systemic activation of defense responses, and the response is dose dependent.

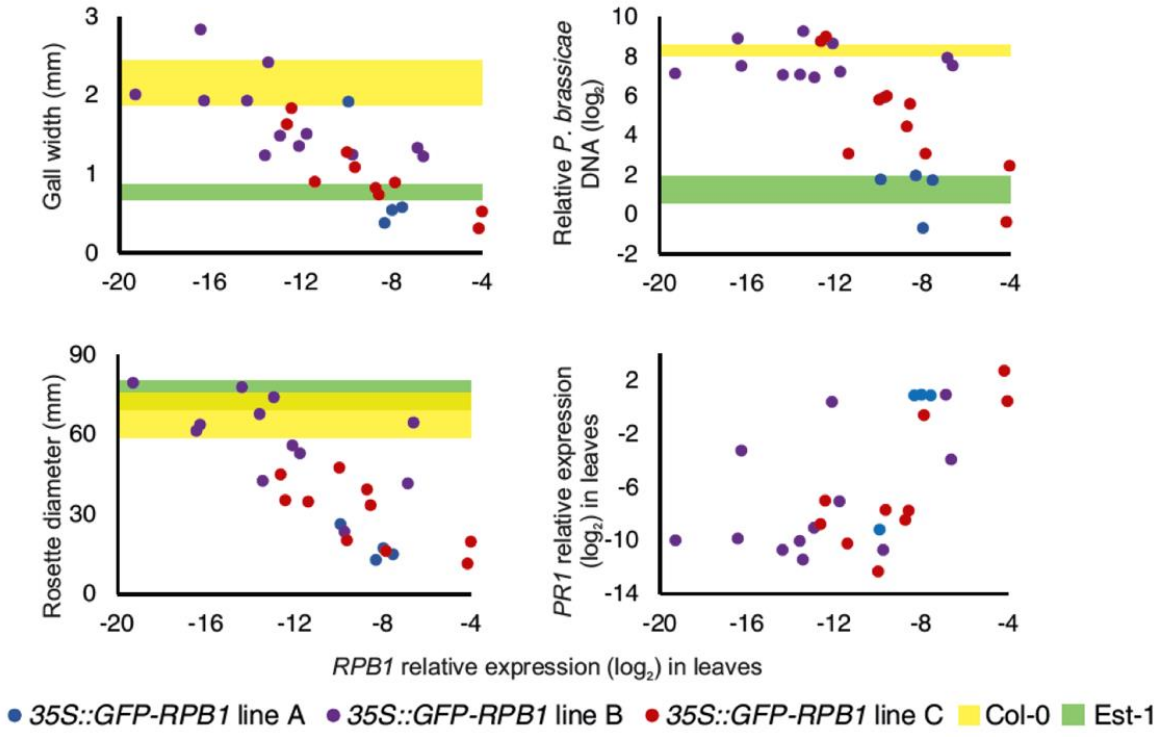


Figure 19: Correlation between *RPB1* expression and gall width/relative pathogen load/rosette diameter/*PR1* expression of *35S::GFP::RPB1* lines 19 dpi.

Coloured bands for Col-0 and Est-1 represent the 95% confidence interval. Each point represents the combination of 3-6 plants with a common T2 parent.

4.1.8 *pRPB1::RPB1* is not sufficient to confer clubroot resistance in Col-0

To circumvent the problem of autoimmune phenotypes observed with 35S driven over-expression, a third approach was adopted to ectopically express of *RPB1* along with 1000 bp upstream promoter cloned from Est-1. Figure 20 shows the vector map of pKGWFS7 which was used for making the construct. The ccdB was replaced by the 1024 bp upstream sequence + *RPB1* gene sequence (including a STOP codon).

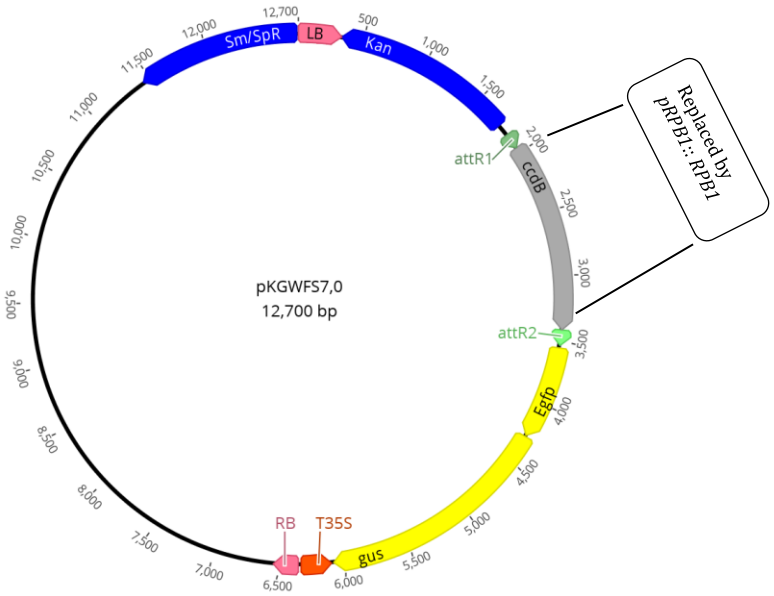


Figure 20: Vector map of pKGWFS7

The transgenic lines did not exhibit any phenotypic abnormalities or stunting, though some lines showed delayed seed maturation. Five independent transgenic lines were selected for evaluation- Lines 1, 4, 6, 7 and 9. *P. brassicae* infection assays were carried out for 2-4 independent inoculations, each comprising 3-9 plants for the transgenic lines along with Col-0 and Est-1 as controls. Seedlings used for the experiment were genotyped to confirm the presence of *RPB1* fragment prior to inoculation. Lines 1, 4 and 7 had statistically significant decreases in relative pathogen load, the other 2 lines also had lower mean pathogen load compared to Col-0 but were not significantly different (Figure 21). None of the transgenic lines could reach the level of resistance observed in Est-1 (Figure 22).

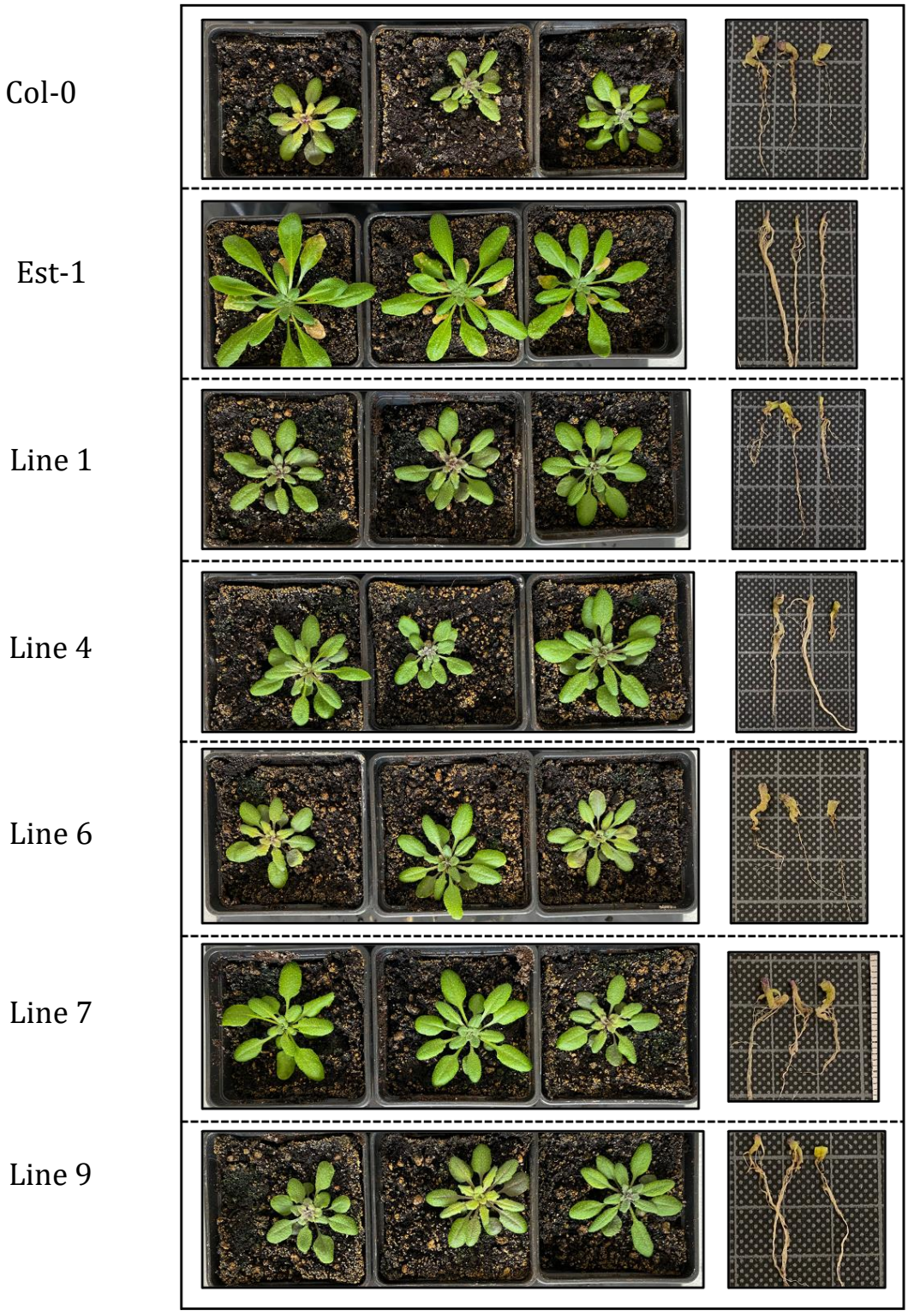


Figure 21: Pictures of rosette and root tissue of Col-0, Est-1 and 5 independent transgenic lines of Col-0 *pRBP1::RPB1*, 19 days post infection.

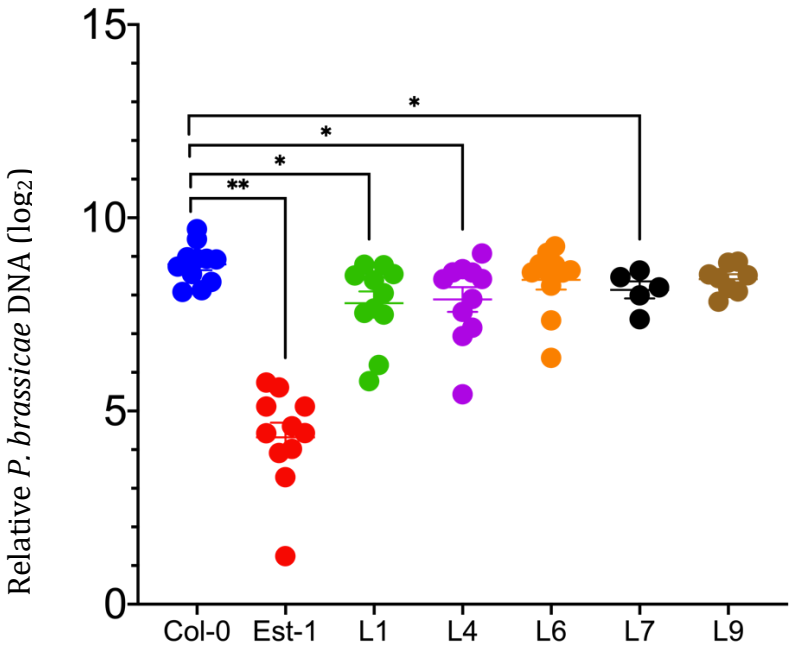


Figure 22: Relative pathogen load in Col-0, Est-1 and *pRPB1::RPB1* transgenic lines.

Each data point has been shown as dot plots with mean and standard error bars. Each dot represents a pool of 3 galls. Asterisks (*) indicate a significant difference ($p < 0.05$, ** for $p<0.005$) calculated by Student's T-Test.

Gene expression responses were assessed in mock and inoculated root tissues 7 dpi for each of the lines. Expression of *RPB1* was detected in all the inoculated samples, however, it could not be detected in every sample of uninfected transgenic lines (Figure 23). This might be explained by the tight regulation of immune genes in the absence of pathogen threat. In lines 6 and 9 where a statistical comparison with expression levels in mock treated samples could be made, there was a significant upregulation of expression in response to infection, though not to the same magnitude as in Est-1, showing that the Est-1 promoter is functional in Col-0. Interestingly, line 1 had a high level of *RPB1* expression even in the absence of *P. brassicae* infection. The responses of three immune markers- *AED1, CYP71A13* and J*AZ10* were also assessed. *AED1 (APOPLASTIC EDS1 DEPENDENT 1)* is induced locally by SA and during SAR signalling (Breitenbach et al., 2014) and it was selected from the Est-1 transcriptome dataset generated 7 days post infection (described in the following section). *CYP71A13* is a key

gene in the rate limiting step of camalexin biosynthesis (Zhou et al., 1999) and *JAZ10* is a marker for jasmonic acid mediated responses (Hoo et al., 2008). *AED1* was only induced in Est-1, line 1 and no other transgenic lines (Figure 23). *CYP71A13* was induced in Est-1, line 1, line 6 and line 7. This might indicate an initial trigger of defense responses upon infection which is not sustained as it is in Est-1. Line-1's elevated level of *AED1* and *CYP71A13* could be explained by its higher basal level of *RPB1* in mock treated plants. For *JAZ10*, the expression pattern of transgenic lines mirrored that of Col-0 rather than Est-1. These gene expression patterns indicate that suppression of SA mediated responses and elevation of jasmonic acid responses in transgenic lines upon *P. brassicae* infection remains very similar to wild type Col-0. The Est-1 *RPB1* promoter likely carries a motif or motifs that are targeted by transcription factors (TF) activated upon *P. brassicae* recognition. Figure 24 shows a TF binding site analysis of 1 kb upstream region of *RPB1* gene for three cis-elements associated with SA mediated signalling – the W-box element of WRKY TFs, the as-1 element bound by TGA TFs and the element associated with SARD1/CBP60g signalling (Chen et al., 2019; Garretón et al., 2002; Sun et al., 2018). But the absence of Est-1 like responses indicates that there could be missing components downstream of *RPB1a* in Col-0 that prevents the full scale ETI signalling.

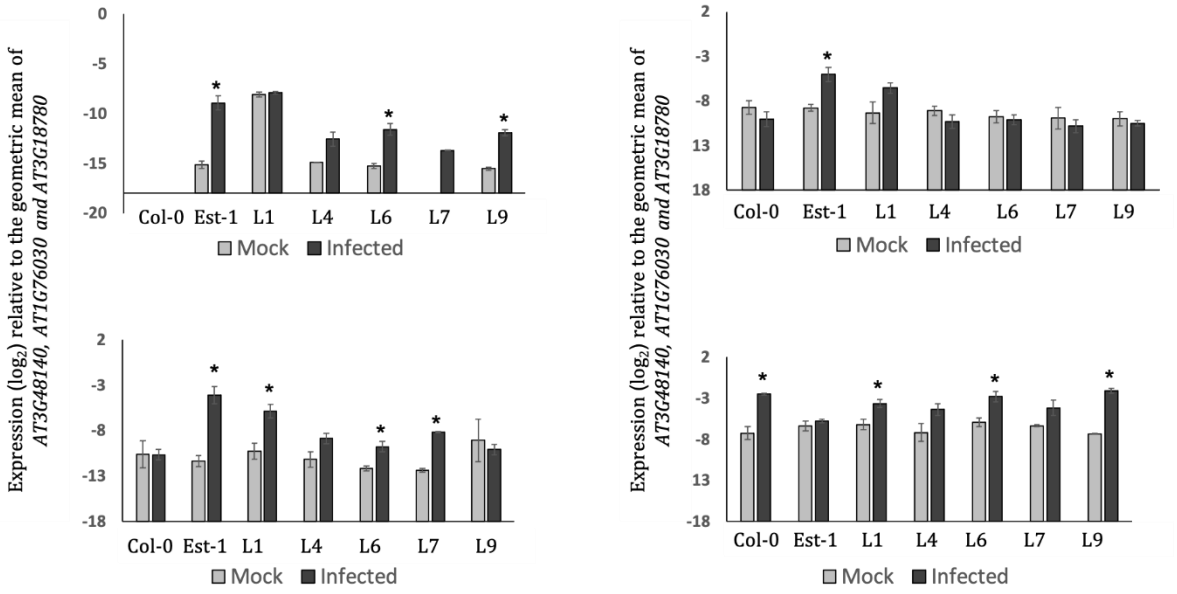


Figure 23: Relative (log 2) expression of *RPB1, AED1, CYP71A13* and *JAZ10* genes 7 dpi in Col-0 , Est-1 and *pRPB1::RPB1* transgenic lines.

The experiment was conducted for 2-4 biological replicates of each transgenic lines. Each biological replicate consists of 3 data points, each data point representing a pool of 3 plants. Asterisks (*) indicate a significant difference ($p < 0.05$) calculated by Student's T-Test.

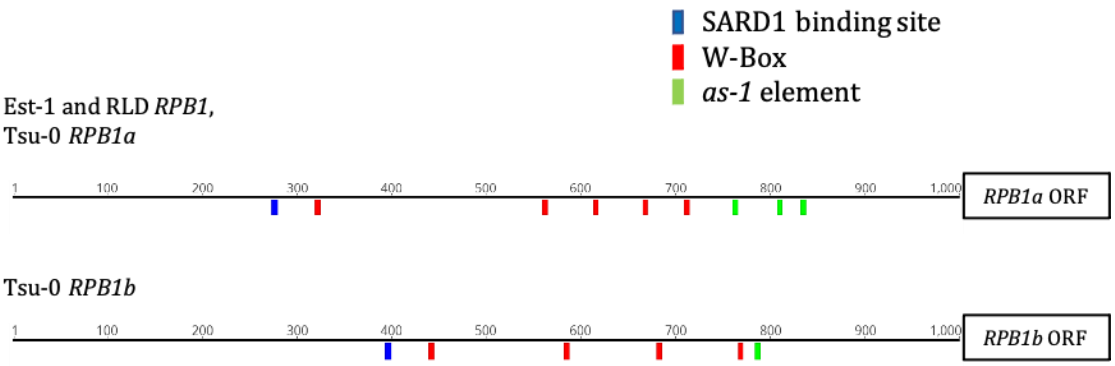


Figure 24: Predicted positions of 3 biotic stress responsive TF binding motifs in 1Kb upstream region of *RPB1* ORF in resistant lines Est-1, RLD and Tsu-0.

4.2 Transcriptome profiling of responses to *P. brassicae* infection in resistant and susceptible Arabidopsis accessions

4.2.1 Selection of Arabidopsis accessions and timepoint for RNASeq analysis

Screening of 142 Arabidopsis accessions for their interaction with *P. brassicae* pathotype P1+ revealed a wide spectrum of resistant and susceptible lines. Est-1, originally collected from Estonia, was found to be the most resistant. To characterize the transcriptional responses associated with clubroot resistance in Arabidopsis, Est-1 was selected for mRNA-Seq profiling. Col-0 is a widely used accession in clubroot research and is highly susceptible to the P1+ pathotype, thus being an ideal control for comparison with Est-1. 7 dpi was selected as an appropriate timepoint for the experiments as it represents a stage early in secondary infection (Liu et al., 2020).

4.2.2 Experimental design and RNAseq technology used

The experimental design consisted of two genotypes (Col-0 and Est-1) and two treatments (Mock and Infected). For each genotype-treatment combination, there were three independent biological replicates. Each biological replicate was a pool of the entire root system from 12 plants. 17 days old seedlings were inoculated with 2 ml of 10^6 ml^{-1} spores concentration (or water for mock treatment) and 7 days old infected plants were harvested. The 12 samples were sequenced on the BGISeq500 platform (Genomed). The sequencing generated ~25 million paired end reads of 100 bp per sample (Table 5).

Table 5: Lists the RNAseq samples with various metrices of sequencing reads.

Abbreviations: Number of sequencing reads generated (M Seqs – million sequences), read length (Length), percentage GC content of the reads (% GC) and amount of duplicated reads (%Dups). C stands for Col-0, E for Est-1, M for mock treated, P for *P. brassicae* infected, A/B/C indicates biological replicates and 1/2 denotes pair-end reads.

Sample Name	M Seqs	Length	% GC	% Dups
10CPC_1	25.8	100 bp	45%	63.2%
10CPC_2	25.8	100 bp	46%	61.7%
11EMC_1	26.0	100 bp	45%	63.8%
11EMC_2	26.0	100 bp	45%	63.5%
12EPC_1	23.2	100 bp	45%	62.5%
12EPC_2	23.2	100 bp	46%	62.1%
1CMA_1	26.5	100 bp	45%	62.4%
1CMA_2	26.5	100 bp	45%	63.8%
2CPA_1	26.6	100 bp	45%	60.9%
2CPA_2	26.6	100 bp	46%	62.4%
3EMA_1	25.7	100 bp	45%	62.0%
3EMA_2	25.7	100 bp	45%	64.0%
4EPA_1	25.7	100 bp	45%	61.3%
4EPA_2	25.7	100 bp	46%	63.8%
5CMB_1	25.7	100 bp	45%	61.1%
5CMB_2	25.7	100 bp	45%	62.8%
6CPB_1	26.3	100 bp	45%	60.8%
6CPB_2	26.3	100 bp	46%	62.6%
7EMB_1	26.9	100 bp	45%	61.4%
7EMB_2	26.9	100 bp	45%	63.1%
8EPB_1	26.3	100 bp	45%	63.3%
8EPB_2	26.3	100 bp	46%	62.7%
9CMC_1	22.3	100 bp	45%	61.3%
9CMC_2	22.3	100 bp	45%	61.0%

4.2.3 Metrics of data quality

The mean Phred score for each base position of the reads was above 35 (Figure 25), indicating a good quality sequencing run output. The trimmed read files were mapped to the TAIR 10 Arabidopsis genome with more than 97% of the reads mapping to the genome (Table 6).

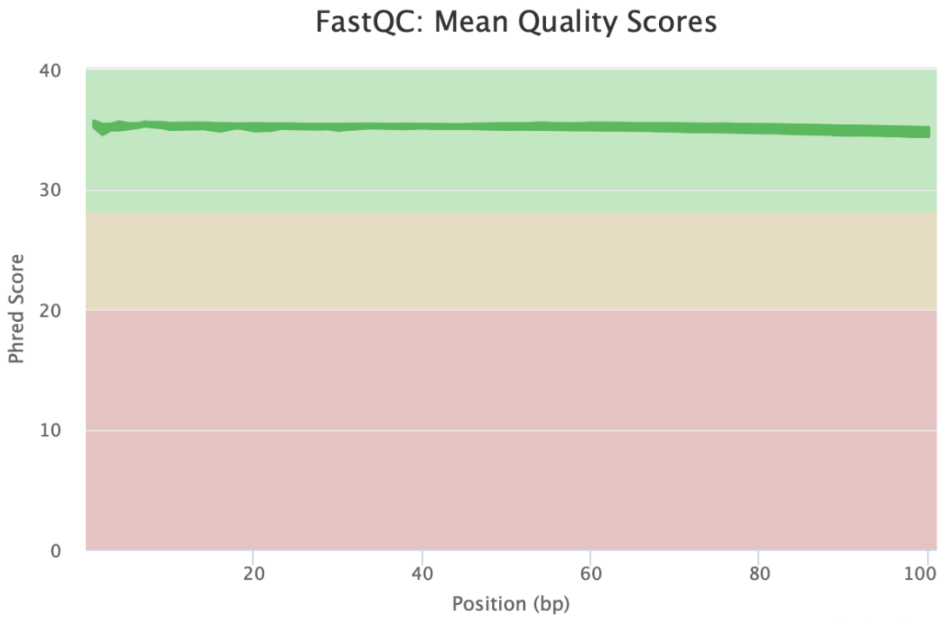


Figure 25: Shows the average Phred score for every position of the reads of RNASeq samples.

Due to high quality read output of every samples, every line appears to be merged in the graph.

Table 6: Lists the mapping stats for each RNASeq sample.

Abbreviations: millions of total paired-end reads mapped (M total reads), millions of reads aligned to the reference genome (M aligned), percentage of total reads aligned to the reference (% aligned), average sequencing depth across the genome (Mean Cov). C stands for Col-0, E for Est-1, M for mock treated, P for *P. brassicae* infected, A/B/C indicates biological replicates.

Sample Name	M Total reads	M Aligned	% Aligned	Mean cov
EMB	49.4	48.0	97.2%	73.7X
EPB	48.2	46.3	96.0%	71.3X
CMC	48.2	47.6	98.8%	71.2X
EMA	47.8	46.5	97.3%	70.7X
EMC	47.8	46.5	97.3%	70.7X
EPA	47.5	45.6	95.9%	70.2X
CPC	47.1	45.6	96.7%	66.2X
CPB	46.7	45.2	96.9%	68.4X
CPA	46.5	45.1	97.0%	67.1X
CMB	46.5	46.0	98.9%	66.9X
EPC	42.7	41.2	96.3%	63.5X
CMA	41.0	40.5	98.8%	57.9X

4.2.4 DESeq2 analysis reveals clustering of samples and number of differentially expressed genes

Principle component analysis of the unique read counts generated by the DESeq2 package shows a clear separation of the samples by genotype and treatment (Figure 26). The first two principal components account for 84% of the variability in the data, PC1 accounting for 71% of variance separates the Col-0 and Est-1 genotypes; PC2 accounts for 13% of variance and separates the mock and *P. brassicae* infected samples. The three biological replicates are well clustered together indicating consistency in the experimental design. In line with the principal component analysis, the greater number of differentially expressed genes were found in the comparisons between genotypes rather than in response to infection; with broadly equivalent numbers of genes being expressed more highly in one genotype or another. The response to infection lead to the significant up-regulation of more genes than those down-regulated with similar numbers of genes responding in Col-0 and Est-1 (Table 7). Subsequent analyses were performed using the list of 1.5-fold upregulated genes (if not stated otherwise) as 7 dpi is quite an early timepoint for a significant number of genes from a pathway to be strongly induced.

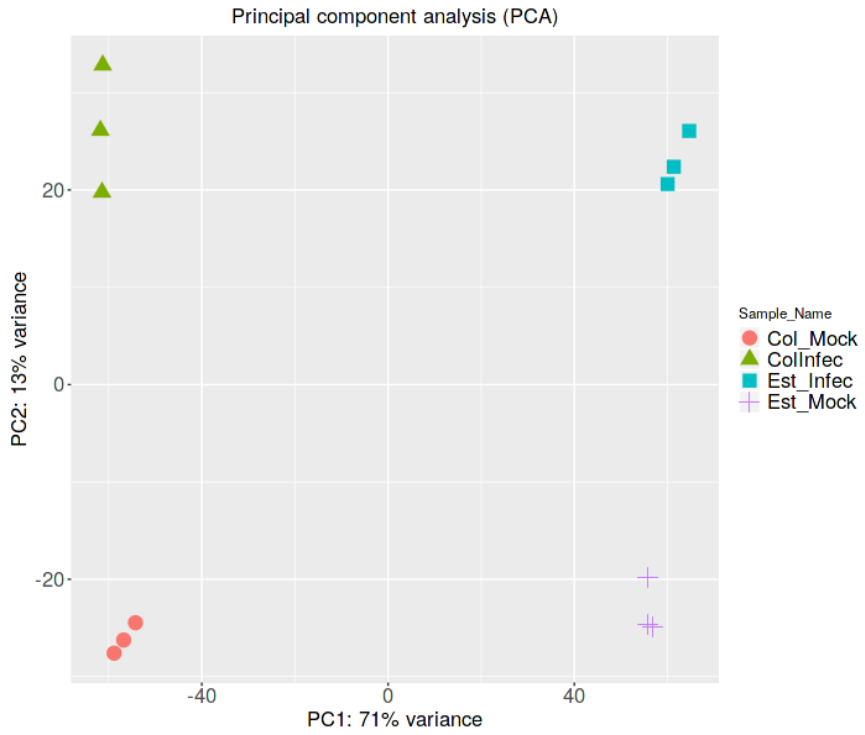


Figure 26: PCA plot of read counts associated with the genes of 12 RNAseq samples.

Table 7: Number of differentially expressed genes selected based on different cut-offs (2 fold/1.5 fold) and Benjamini Hochberg adjusted *p-value* < 0.05.

Significant changes at the 1.5-fold threshold

	UP	DOWN
Col-0 *P. brassicae* vs Col-0 mock	656	275
Est-1 *P. brassicae* vs Est1 mock	586	109
Est-1 mock vs Col-0 mock	982	1542
Est-1 *P. brassicae* vs Col-0 *P. brassicae*	1218	1731

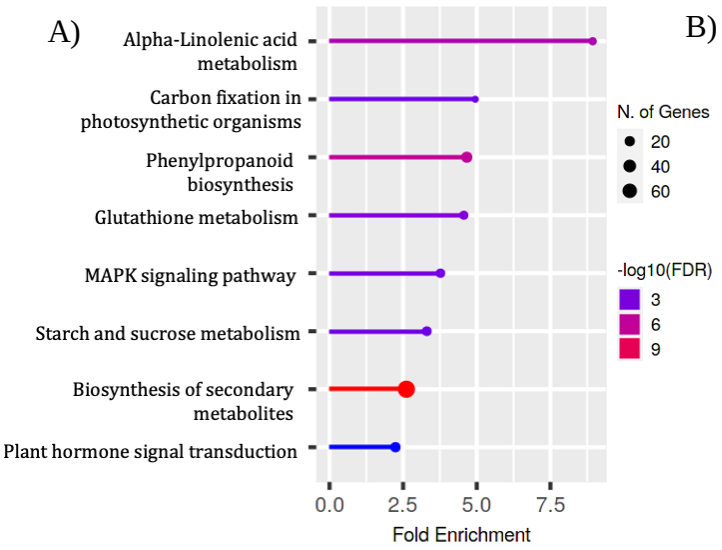
Significant changes at the 2-fold threshold

	UP	DOWN
Col-0 *P. brassicae* vs Col-0 mock	295	44
Est-1 *P. brassicae* vs Est-1 mock	273	12
Est-1 mock vs Col-0 mock	415	773
Est-1 *P. brassicae* vs Col-0 *P. brassicae*	559	885

4.2.5 KEGG pathway and GO enrichment analysis of genes upregulated in Col-0 and Est-1 upon infection

The list of genes significantly up-regulated in response to *P. brassicae* infection in Col-0 and Est-1 was analyzed by both gene set enrichment (Gene Ontology terms relating to Biological Function) and pathway enrichment (based on KEGG database enzyme classifications) analysis. Figure 27 shows the KEGG enrichment analysis of the upregulated genes in Col-0 infected plants. KEGG enriched pathways includes- 'Alpha-linolenic Acid Metabolism', 'Phenylpropanoid biosynthesis', 'Starch and Sucrose Metabolism', 'Plant Hormone Signal Transduction'. KEGG analysis of upregulated genes in Est-1 highlights the terms 'Phenylpropanoid biosynthesis', 'MAPK signalling pathway', 'Plant-Pathogen interaction', and 'Biosynthesis of Secondary Metabolites' (Figure 28). GO analysis additionally uncovered 'response to hypoxia' term which is an interesting common feature of both the accessions (Figure 27 B, Figure 28 B). It has been previously reported that hypoxia signalling aids in clubroot disease establishment (Gravot et al., 2016). It could be a feature due to early induction of hypoxia signalling

by pathogen effectors or as a byproduct of intra-cellular resource exploitation by the pathogen.

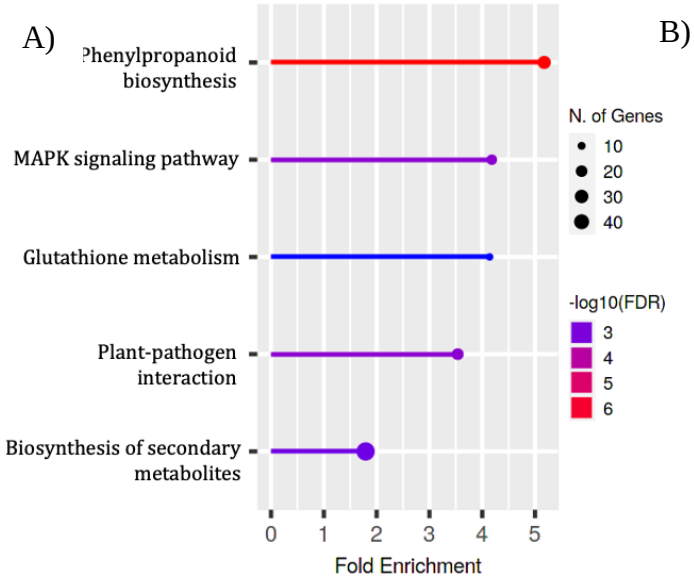


B)

No. of genes	FDR	GO term
33	2.2E-16	Response to fatty acid
32	2.9E-14	Response to wounding
32	3.1E-12	Response to hypoxia
108	5.2E-23	Response to biotic stimulus
79	2.3E-14	Immune response

Figure 27: Enriched KEGG and GO terms in Col-0 transcriptome upregulated in response to infection.

A) Enriched KEGG terms. Fold Enrichment is defined as the percentage of genes in the list belonging to a pathway, divided by the corresponding percentage in the background. B) Top 5 unique GO term hits sorted by fold enrichment.



B)

No. of genes	FDR	GO term
23	1.6E-17	Systemic acquired resistance
35	4.5E-16	Response to hypoxia
63	1.3E-26	Response to bacterium
122	1.2E-37	Response to external biotic stimulus
99	2.0E-29	Immune response

Figure 28: Enriched KEGG and GO terms in Est-1 transcriptome upregulated in response to infection.

A) Enriched KEGG terms. Fold Enrichment is defined as the percentage of genes in the list belonging to a pathway, divided by the corresponding percentage in the background. B) Top 5 unique GO term hits sorted by fold enrichment.

4.2.6 Jasmonic acid and ethylene biosynthesis/signalling pathways are upregulated in Col-0 upon infection

Enrichment of Alpha-Linolenic Acid metabolism pathways in Col-0 indicates activation of jasmonic acid biosynthesis and signalling. 3 LOX genes (*LOX1*, *LOX3* and *LOX4*) involved in JA biosynthesis are upregulated in our dataset. JAZ proteins function downstream of jasmonic acid induction. Their transcripts are a marker for JA accumulation and activation of JA signalling. The upregulated genes in Col-0 includes six JAZ genes- *JAZ5, JAZ6, JAZ8, JAZ9, JAZ10* and *JAZ13* (Figure 30). To examine the scope of the influence of JA on the response to *P. brassicae* 7 dpi, our dataset was compared with two groups of publicly available transcriptomics data: 1) Arabidopsis responding to exogenous jasmonate treatments 2) JA signalling mutants infected with pathogens. Figure 29 shows that 69% of the genes significantly up-regulated in response to *P. brassicae* were up-regulated in response to an exogenous JA application, highlighting induction of JA responsive pathways upon *P. brassicae* infection. Ethylene (ET) biosynthesis genes such as *ACS2, ACS8* (Ahmadizadeh et al., 2020) and 7 ethylene responsive transcription factors *ERF1,2,6,13,14,15,71* (Müller & Munné-Bosch, 2015) are also induced in Col-0 upon infection. JA/ET crosstalk has been shown to be antagonistic towards salicylic acid mediated responses (Yang et al., 2019), which might help *P. brassicae* establish its biotrophic lifecycle. *ERFs* are also essential components of the response to hypoxia (Giuntoli & Perata, 2018) which is also a prominent feature of the dataset.

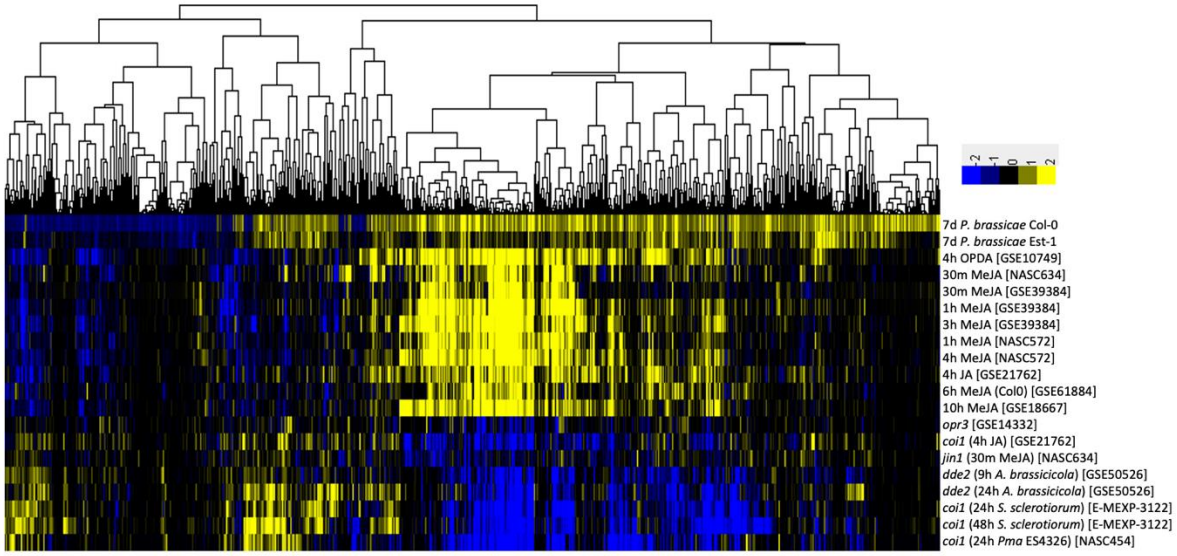


Figure 29: Hierarchical clustering of the genes upregulated in Col-0 with other publicly available transcriptomic datasets related to JA responses.

Yellow colour represents upregulation relative to mock treatment and blue signifies downregulation, scale is $\log_2$. Hierarchical clustering is based on complete linkage. Codes in brackets refers to experiment IDs in NCBI GEO, NASCARRAYS or EBI Arrayexpress databases.

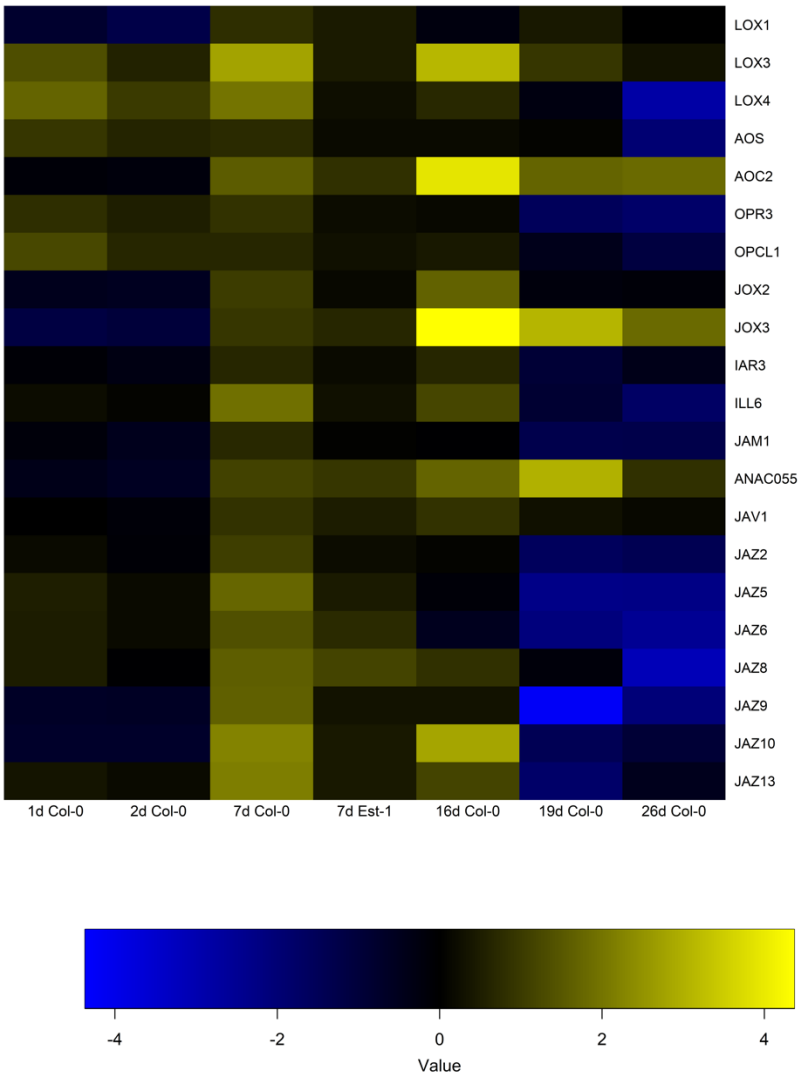


Figure 30: Heatmap of the JA biosynthesis / signalling genes which are up-regulated in inoculated Col-0 7 dpi, compared with the publicly available Col-0 transcriptome at 1 and 2 dpi (Zhao et al., 2017), 16 and 26 dpi (Rolfe et al., 2016), and unpublished data for the P1+ pathotype 19 dpi as well as infected Est-1 7 dpi.

Yellow colour represents upregulation relative to mock treatment and blue signifies downregulation, scale is $\log_2$.

4.2.7 Upregulation of flavonoid biosynthesis pathway in infected Col-0 roots

Phenylpropanoids are shikimate pathway derived compounds that play an important role in biotic defense responses. Several important metabolites including flavonoids, anthocyanins, tannins and lignins are derived from the phenylpropanoid biosynthesis pathway (Fraser & Chapple, 2011). Flavonoids have been reported to be involved in biotic stress responses, antioxidant activity, auxin transport, and pigment formation (Ferreyra et al., 2012). Anthocyanin biosynthesis genes including *CHALCONE SYNTHASE/TRANSPARENT TESTA 4, TRANSPARENT TESTA 5* AND *TRANSPARENT TESTA 8* are induced in Col-0 upon clubroot infection but remain uninduced in the Est-1 transcriptome. Indeed, there is a gradual reddening of the Col-0 galls as the disease progresses pointing to accumulation of flavonoids in infected tissue. Three different flavonoid compounds (naringenin, quercetin and kaempferol) have been previously reported to be accumulated upon clubroot infection (Päsold & Ludwig-Müller, 2013). It has been proposed that flavonoid accumulation might be involved in modulating auxin transport during gall formation. It is also possible that the antioxidant activity of flavonoids is part of a PTI response in Col-0 upon pathogen detection. Flavonoid production could also feed into anthocyanin accumulation or synthesis of phytoalexins as previously reported for other pathogen interactions (Ibraheem et al., 2010).

4.2.8 *P. brassicae* infection upregulates lateral root associated genes early in infection

There is a significant enrichment of lateral root (LR) biogenesis pathway genes (*GATA23, PUCHI, LBD16/17, PLT5, FLP, EXP1*) and other regulators of lateral organ size (*ARGOS, ARGOS-like1*). Involvement of the lateral root pathway was one prominent feature of the data, due to the upregulation of a significant number of genes from the same pathway at early stages of the disease and previous reports of the hijacking of lateral root pathway by other gall forming plant-pathogens. *LBD16* has been reported to be involved in formation of the gall like feeding structure induced by nematodes in the plant vasculature (Cabrera et al., 2014). *LBD16* is also crucial for rapid proliferation of cortical and pericycle cells during rhizobia mediated nodule formation in legumes (Bishopp & Bennett, 2019). Such evidences provide a link between different gall

forming microbes and nematodes and raises the possibility that the reprogramming of the lateral root initiation pathway facilitates the achievement of similar goals. Figure 31 highlights (red arrow) the role of the *P. brassicae* upregulated genes during LR initiation steps.

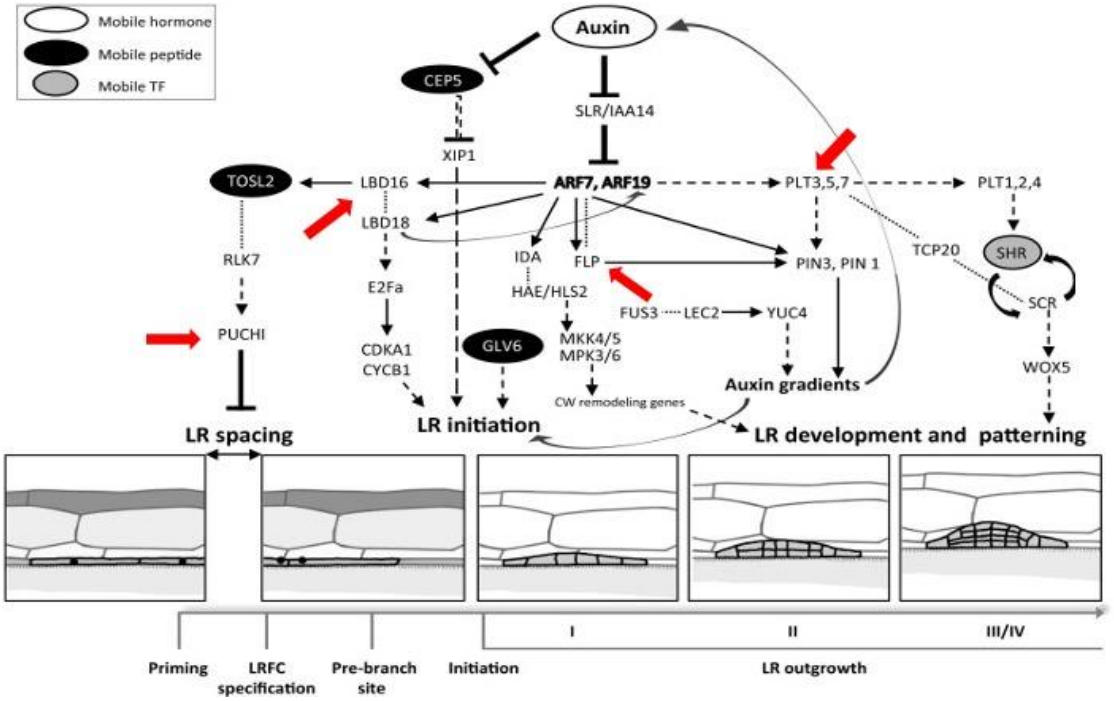


Figure 31: Highlights the role of the *P. brassicae* upregulated genes (red arrow) during LR initiation steps. (Adapted from (Banda et al., 2019))

4.2.9 Salicylic acid signalling pathways are upregulated in Est-1 upon infection

"Systemic Acquired Resistance (SAR)" (23 genes) is the top hit of the GO term analysis for the response to *P. brassicae* in Est-1. Salicylic acid (SA) mediated responses have

been shown to be effective against biotrophic pathogens including *P. brassicae* (Xi et al., 2021). The upregulated gene set in Est-1 include SA biosynthetic genes (*EDS5, SID2*), there are several SA responsive transcription factors including *WRKYs* and other immune related kinases. *WRKY70* and *WRKY40* are both upregulated which are essential for *SARD1* mediated immunity (Chen et al., 2021). Initiation of SAR points to SA mediated priming of the plant system for long-term resistance against the disease. To better understand the relationships of the groups of genes involved, the significantly over-represented GO terms for Est-1 induced genes were visualized using the Bingo plugin of Cystoscope software; Figure 32 shows the involvement of chitin responsive, SA responsive, SAR, toxin metabolism and programmed cell death signalling pathways. To restrict the analysis to the genes potentially responsible for resistance to clubroot disease the intersection between the significantly upregulated genes for Est-1 infected vs mock and Est-1 infected vs. Col-0 infected were identified. This intersection was clustered with two groups of publicly available transcriptomic data: 1) Arabidopsis plants responding to exogenous salicylic acid or chemical analogues and 2) SA signalling mutants infected with pathogens (Figure 33). The response in Est-1 to *P. brassicae* 7 dpi overlaps substantially with the responses to SA treatment datasets pointing to the activation of SA mediated signalling upon *P. brassicae* infection.

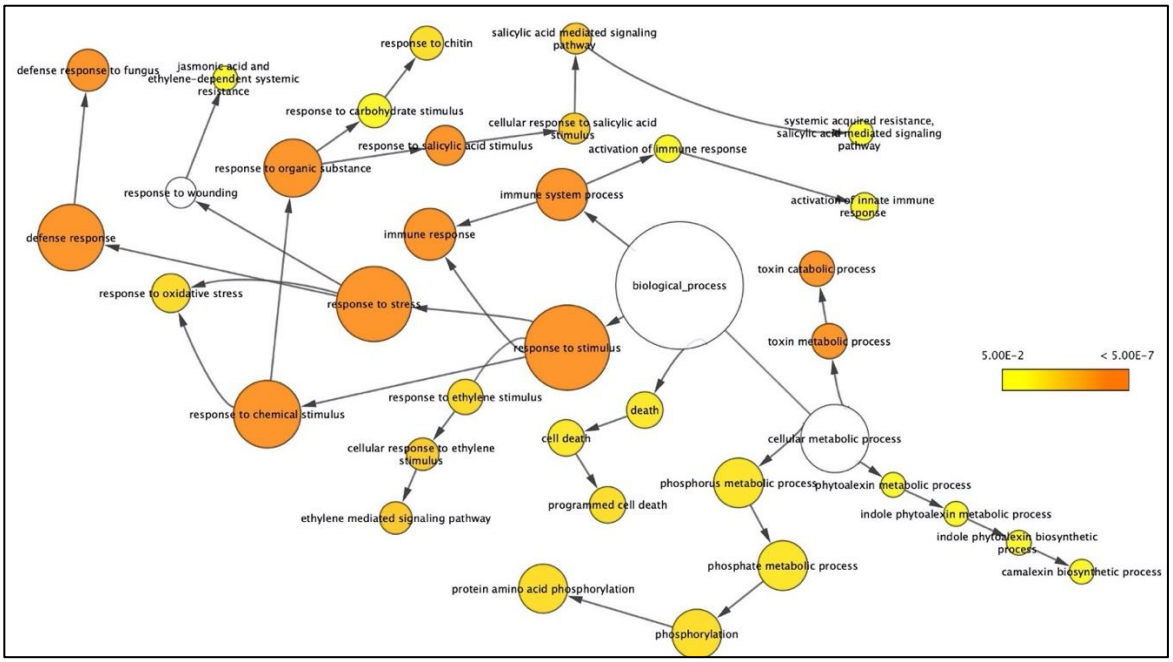


Figure 32: Cytoscape (Bingo) network of the GO terms of the genes upregulated in Est-1 upon *P. brassicae* infection.

The colour scale represents BH adjusted *p*-values with orange being the most significant.

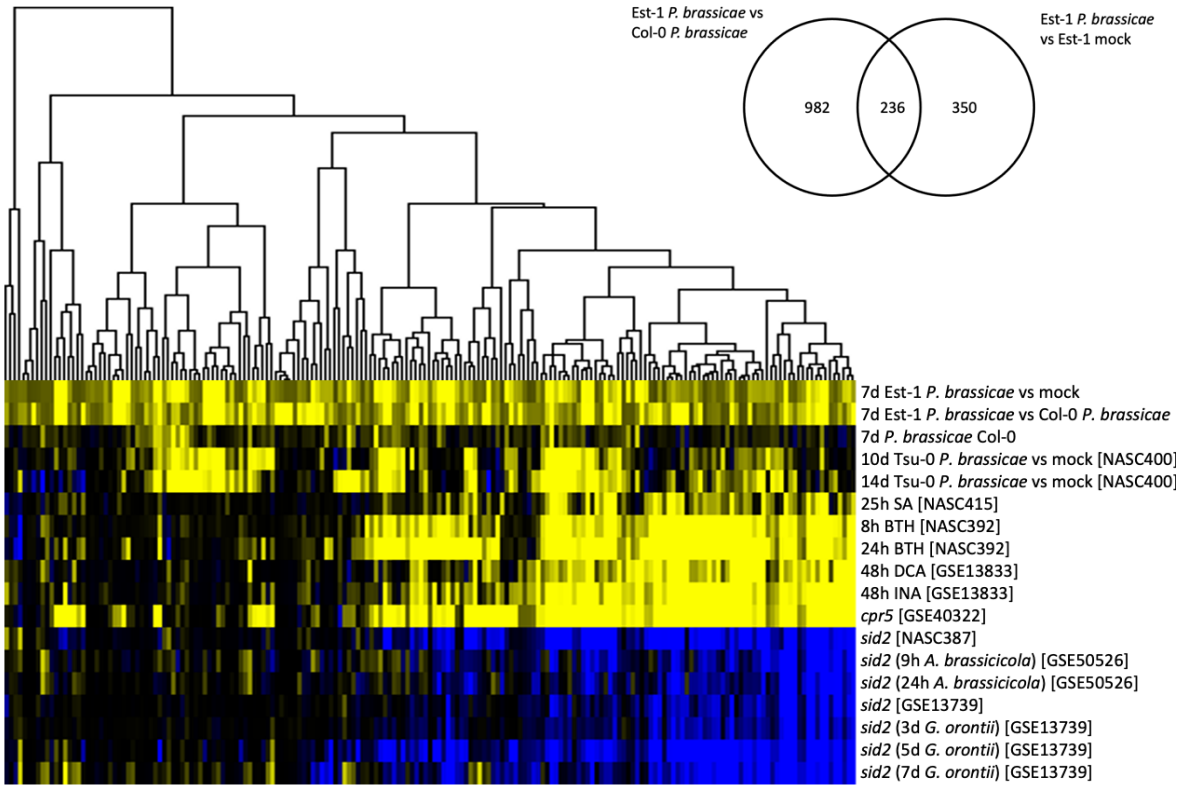


Figure 33: Hierarchical clustering of the resistance response to clubroot 7 dpi (common genes significantly upregulated for both Est-1 infected vs mock and Est-1 infected vs. Col-0 infected) with other publicly available transcriptomic datasets related to SA signalling.

Yellow colour represents upregulation relative to mock treatment and blue signifies downregulation, scale is $\log_2$. Hierarchical clustering is based on complete linkage. Codes in brackets refers to experiment IDs in NCBI GEO, NASCARRAYS or EBI Arrayexpress databases.

4.2.10 Upregulation of immune responsive genes in Est-1 infected plants

Hundreds of PTI, ETI and phytohormone responsive genes are upregulated in Est-1 upon clubroot infection. 3 *PATHOGENESIS RELATED* (*PR1, PR4, PR5*) genes, 7 Cysteine Rich RLKs, 9 Receptor-like Proteins (*RLPs*), 15 *WRKY*s ,7 *NAC* transcription factors and 14 Cytochrome P450s are some of the important biotic stress responsive genes that are induced. 7 ABC transporters were also induced including *ABCG40*, which was recently shown to be an important player in camalexin secretion (He et al., 2019). *CYP71A13* and

PAD3, which are key components of camalexin biosynthesis, are also upregulated. *CYP81F2*, which has been shown to be involved in production of indolic glucosinolate based antifungal compounds in Arabidopsis for deposition to the fungal entry site (Bednarek et al., 2009), is also induced in Est-1 infected plants. Based on this gene expression pattern and the capacity of *RPB1* to induce cell death, it appears that Est-1 mounts a strong ETI response against the invading *P. brassicae* early in the secondary infection stage.

4.3 Characterization of a putative chitin binding secreted protein from *P. brassicae*

Clubroot resting spores contain chitin as a major structural building block (25%) and during its complex lifecycle in the host, there are multiple times where chitin is presented to the host as a potential elicitor to initiate host defense signaling (Moxham & Buczacki, 1983). Chitinase activity has previously been reported as a feature of the responses to *P. brassicae* infection in Chinese cabbage that is tolerant of clubroot disease (Ludwig-Müller et al., 1994). A *Brassica rapa* genome wide scan for putative chitinases revealed 14 candidates upregulated in the resistant background upon infection. Such results highlight the possible involvement of chitin degrading enzymes in resistant hosts to dampen disease progression. As discussed earlier, *P. brassicae* genome contains multiple chitin binding putative virulence factors. Thus, it raises the possibility of an ongoing arms-race between plants and *P. brassicae* to detect/degrade or mask chitin respectively. The questions addressed by this study involved understanding the host's chitin associated responses by measuring the chitinase gene expression level and quantifying the chitinase enzymatic activity in root tissue at early timepoints of infection. Chitinase knock out lines were also screened to identify candidates impacting on clubroot disease progression.

4.3.1 Endo-chitinase activity increases in Est-1 upon infection

In order to quantify changes in host chitinase activity upon infection, the endo- and exo-chitinase enzymatic activity in the root tissue of Col-0 and Est-1 was assayed (Figure 34). Exo-chitinase activity did not significantly differ between treatments. Only endo-chitinase activity significantly increased in Est-1 following *P. brassicae* infection. This was followed up in Est-1 with more timepoints further into the disease progression (Figure 35). In all the three tested timepoints, endo-chitinase activity was significantly elevated in Est-1. This observation is in agreement with previous reports showing that endo-chitinases are induced in plants to suppress pathogen attack whereas exo-chitinases have limited effect (Roberts & Selitrennikoff, 1988). Though it remains

difficult to say whether the endo-chitinase induction is primarily due to the activation of the chitin sensing pathway or as a part of the broader PTI/ETI responses.

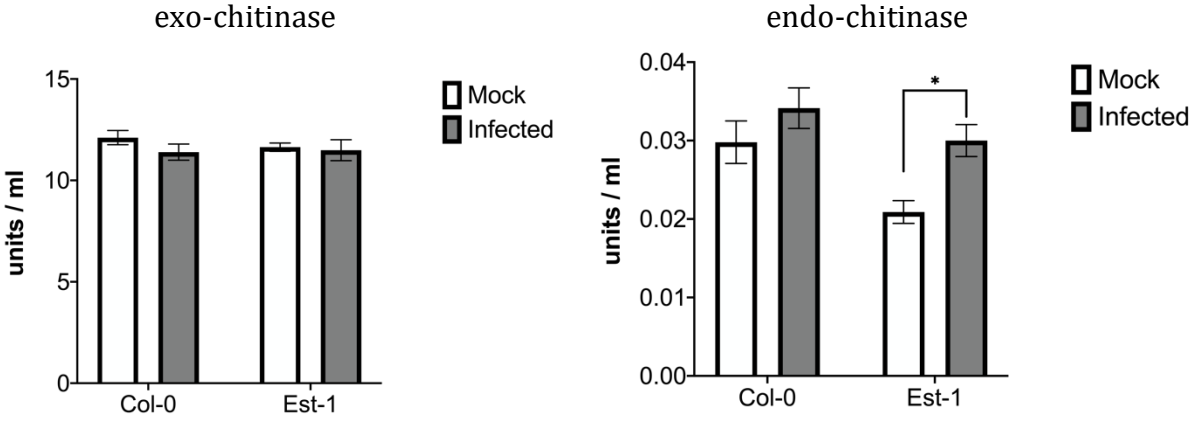


Figure 34: Chitinase assay in 7-day old roots of mock and infected Col-0/Est-1 plants

Each bar represents the average of 3 biological replicates consisting of 5 plants. The error bar is standard deviation. Asterisks (*) indicates significant difference ($p < 0.05$) calculated by Student's T-test.

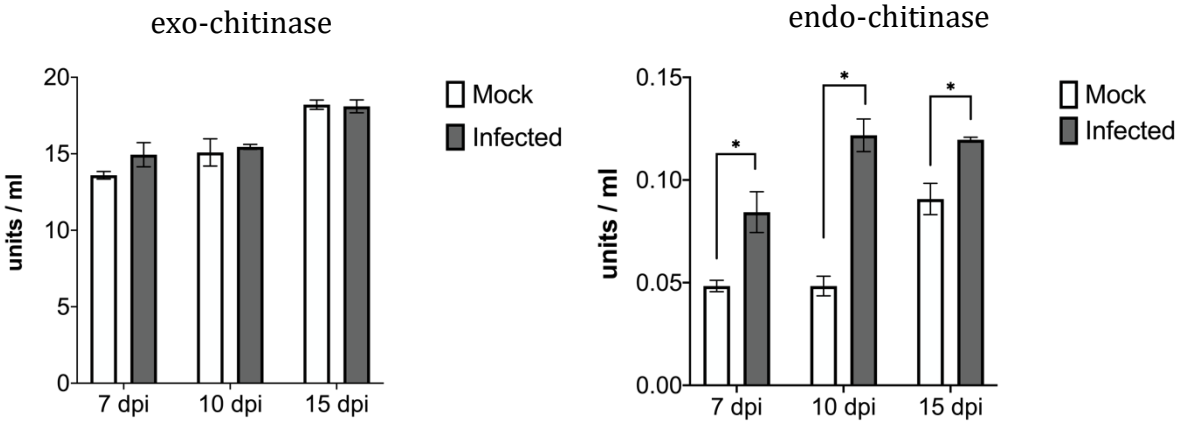


Figure 35: Chitinase assay in 7, 10 and 15 day old roots of mock and infected Est-1 plants.

Each bar represents the average of 3 biological replicates consisting of 5 plants. The error bar is standard deviation. Asterisks (*) indicates significant difference ($p < 0.05$) calculated by Student's T-test.

4.3.2 Arabidopsis chitinase gene expression upon *P. brassicae* infection

To gain insight into chitinase gene activation by clubroot disease, the expression patterns of these genes in various publicly available clubroot transcriptomic datasets were compiled. Figure 36 shows the expression pattern of 22 chitinase genes in various compatible (Col-0) and incompatible (Tsu-0) Arabidopsis-*P. brassicae* interactions. Based on the differences in expression pattern between compatible and incompatible interactions, five genes were selected for further gene expression study: *AT1G02360, AT2G43620, AT2G43570, AT3G54420, AT3G47540* (Figure 37). Of those five genes, *AT2G43570* was also upregulated in our Est-1 7 dpi transcriptome dataset. Relative gene expression change was measured at 2 dpi during primary infection and at 7 dpi during secondary infection. *AT3G47540* was upregulated 7 dpi in both Col-0 and Est-1, while *AT2G43570* was up only upregulated in Est-1. Based on the publicly available mutant lines and gene expression data, pathogen growth assays were conducted with 7 chitinase knock out mutants in Col-0 background (*AT1G02360, AT2G43570, AT2G43620, AT3G12500, AT3G47540, AT4G01700, AT4G19810, AT5G24090)* but none supported significantly different pathogen growth. Since single knock out mutants have no effect on pathogen growth, in future, it would be interesting to check a double knock-out mutant of *AT3G47540* and *AT2G43570* in both resistant and susceptible backgrounds.

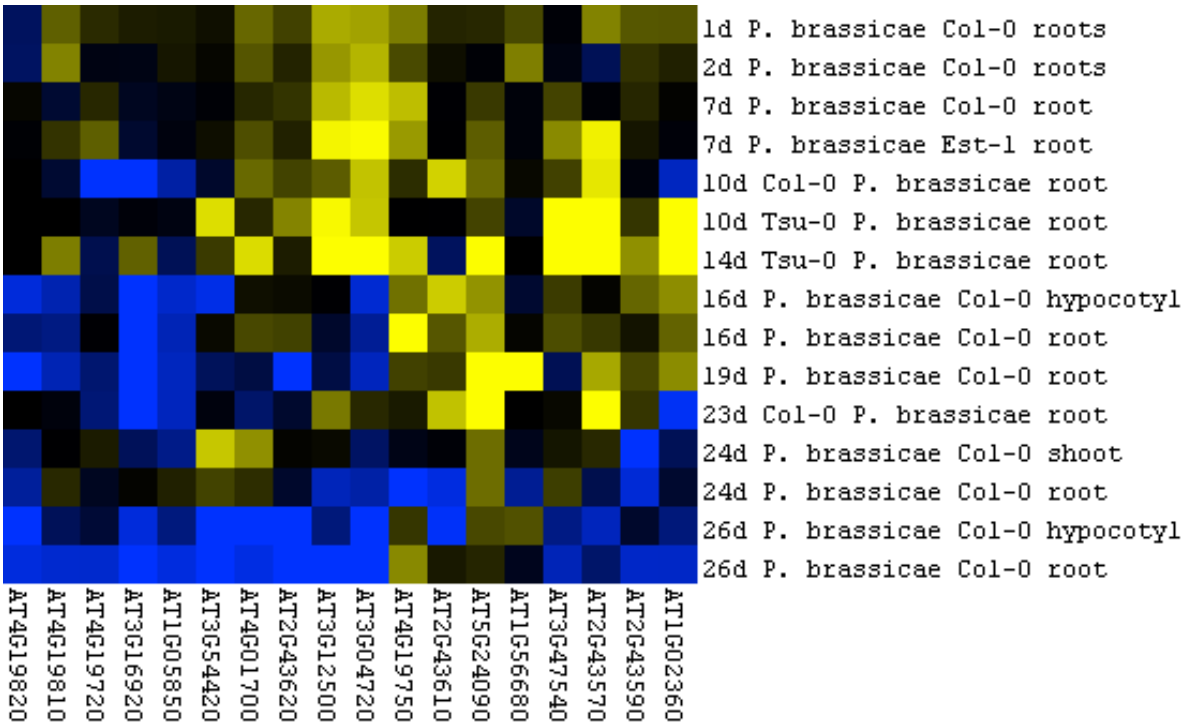
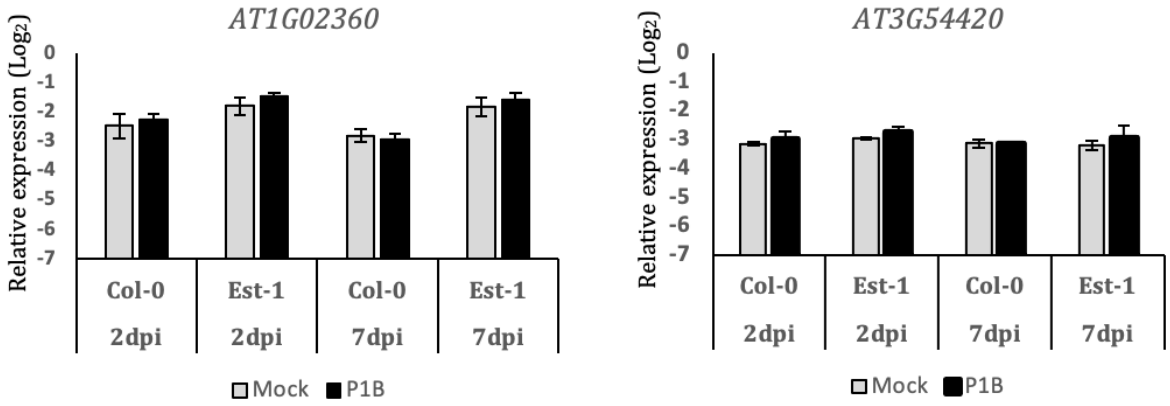


Figure 36: Heatmap of the chitinase genes selected from the publicly available transcriptomic datasets related to compatible and incompatible clubroot interactions at various timepoints (Zhao et al., 2017, Siemens et al., 2006, Rolfe et al., 2016).



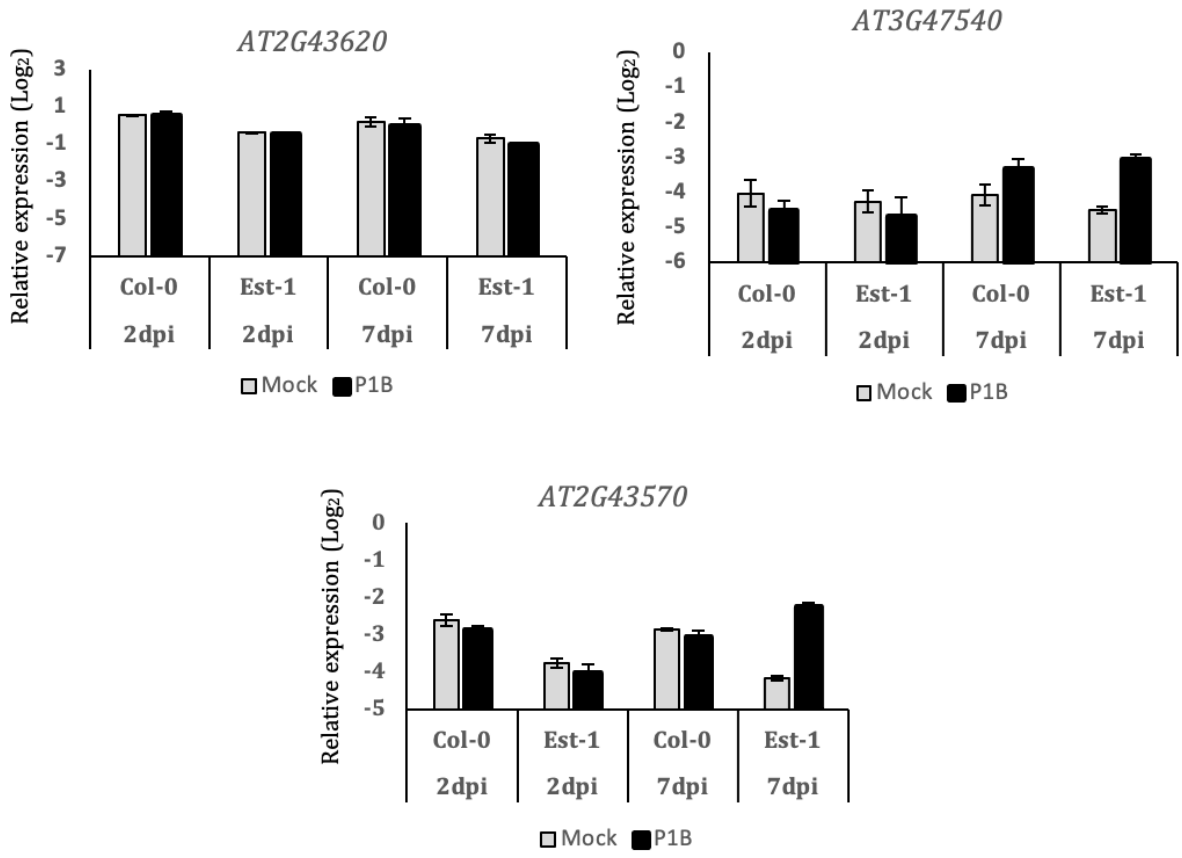


Figure 37: Expression pattern of five chitinases in mock and *P. brassicae* treated Col-0/ Est-1 plants.

The expression was normalized against *AT1G76030.* Each bar represents average of 2 biological replicates, each replicate consisting of 3 plants. Error bars are standard deviation and statistical tests have been omitted due to having fewer than 3 replicates.

4.3.3 Chitin responsive genes are suppressed at later time points of infection in Arabidopsis

Clubroot resting spores contain chitin as a major structural building block (25%) and during its complex lifecycle in the host, there are multiple times where chitin is presented to the host as a potential elicitor to initiate host defense signaling (Moxham & Buczacki, 1983). Clustered transcriptome data of 3550 genes significantly differentially expressed in response to chitin treatment of roots reveals that the degree of similarity in responses between early infection stages and PTI stimulated by chitin perception (30 m chitin – 24 h *P. brassicae* correlation = 0.25; 6 h chitin – 7 d *P. brassicae* Est-1 correlation = 0.49) drops dramatically by later stages of infection (chitin reponses – 26 d *P. brassicae* correlation = 0.03) (Figure 38). By 26 dpi, resting spores are already formed *in planta*. Therefore, downregulation of chitin responses is unusual, unless there is chemical 'masking' of chitin moieties or pathogen effector induced PTI shutdown.

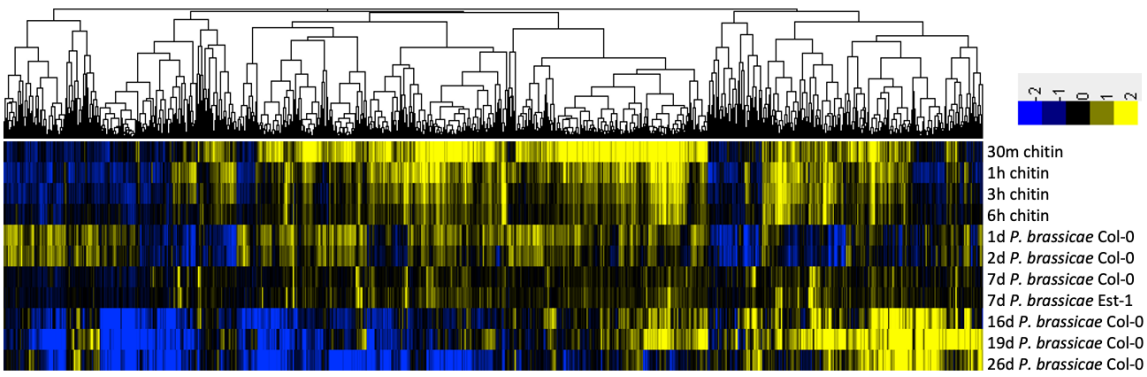


Figure 38: Hierarchical clustering of 3550 genes significantly differentially expressed in roots responding to chitin (NCBI SRA - PRJNA412447) with publicly available data for responses to *P. brassicae* (Zhao et al., 2017, Rolfe et al., 2016).

4.3.4 Mutants impaired in chitin perception do not show elevated pathogen load

Five LysM domain containing receptor-like kinases are present in the Arabidopsis genome, of which four have been identified as chitin binding receptors. These are *CERK1, LYK5, LYK4* and *LYM2*. *CERK1* forms a complex with *LYK5* and *LYK4* to transduce the signal, while *LYM2* acts independently (Xue et al., 2019). In order to investigate the potential for chitin perception mediated defense responses to restrict clubroot disease, the growth of the P1+ pathotype was assessed in various T-DNA knock lines of chitin receptors. None of the three mutants (*cerk1*, *lyk5* and *lyk4*) that were tested exhibited any significant difference in accumulation of pathogen compared to wild type Col-0 at 19 dpi (figure 39). The observed phenomenon could be partially attributed to *P. brassicae*'s ability to mask chitin or the suppression of the PTI machinery by *P. brassicae* secreted effectors.

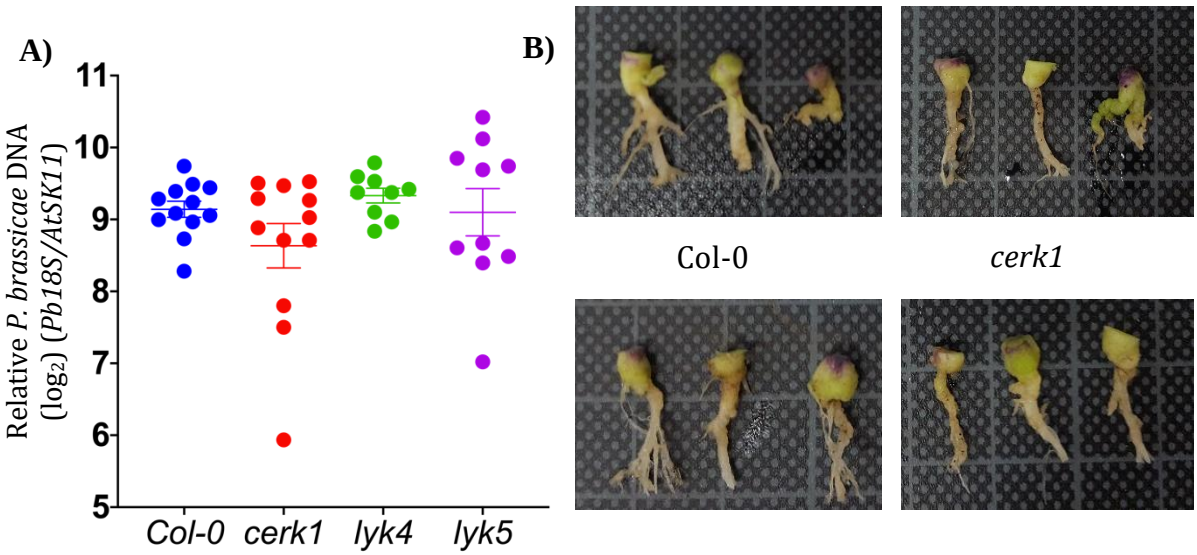


Figure 39: *P. brassicae* growth assay in chitin receptor mutants.

A) Relative pathogen DNA levels in wildtype Col-0, *cerk1, lyk4* and *lyk5* 19 dpi. The dots represent measurements in pools of 3 galls, lines indicate mean and standard error. The data was analyzed with a linear mixed model, but no significant difference was observed. B) 19 dpi galls of clubroot infected Col-0, *cerk1, lyk4* and *lyk5.*

4.3.5 Chitin pre-treatment does not enhance resistance to *P. brassicae* in Col-0 Arabidopsis

Treating Arabidopsis plants with exogenous chitin solution can trigger chitin responsive immune signalling (Huang et al., 2020). In order to determine if exogenous chitin application to the roots prior to the primary and secondary infection leads to elevated disease resistance, 2 ml of 10 $mgml^{-1}$ solution of crab shell chitin was supplied to the roots of wild type Col-0 and chitin receptor mutant *cerk-1* 1 day prior to inoculation and again 5 days after *P. brassicae* inoculation. No significant difference in pathogen DNA levels in the galls were observed between the treatments (Figure 40). This observation leads to the following possible explanations: 1) *P. brassicae* is very effective at suppressing PTI responses in Arabidopsis by deploying effectors to actively manipulate host signalling, 2) Perception of chitin in the root epidermis is limited and only chitin fragments that penetrate the cortex are effective as PTI elicitors, as (Zhou et al., 2020) showed unless the root epidermis is removed by ablation, exogenous chitin application is not effective at triggering a response.

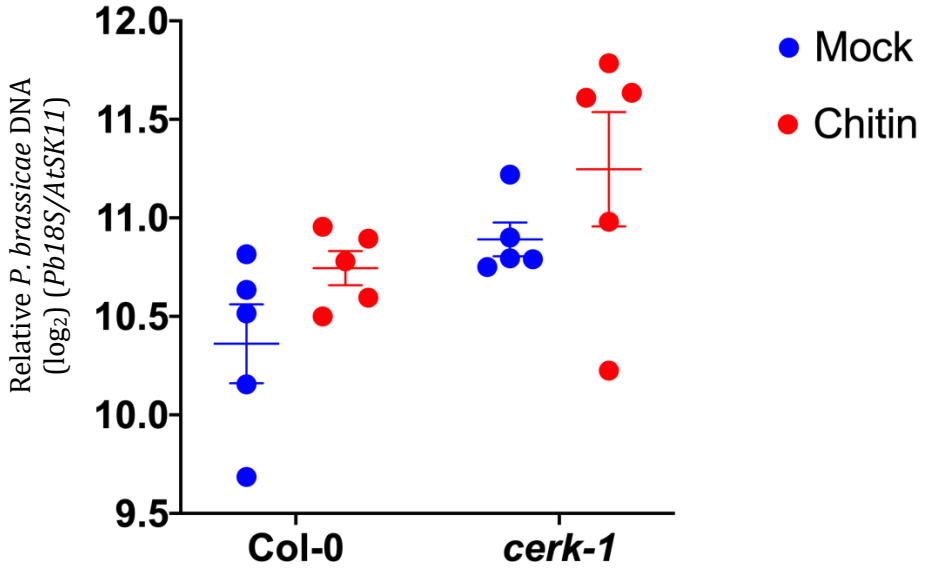


Figure 40: Relative pathogen load in Col-0 and *cerk1* upon mock and chitin treatments (19 dpi).

Each data point has been shown as dot plots with mean and standard error bars. Each dot represents average of 3 galls. Student's T-test was used for testing of significant difference.

4.3.6 Selection of a putative chitin binding deacetylase from *P. brassicae* secretome

Suppression of chitin mediated defense signalling in susceptible Col-0 plants at later stages of disease progression prompted an examination of the *P. brassicae* predicted secreted protein repertoire for potential chitin binding components. The *P. brassicae* secretome contains 16 chitin-CAZymes, of which 9 contain both chitin binding and chitin deacetylase domains (Muirhead and Pérez-López, 2022). Based on the expression pattern in 16 and 26 dpi transcriptome datasets, two chitin deacetylase genes: *PBRA_005081* and *PBRA_002551* were selected alongside *PBRA_004239* which is predicted to contain a tyrosinase domain along with a chitin binding domain (Figure 41). Our bioinformatic analysis was based on the reference 'e3' genome (ENA code PRJEB8376). However, upon attempted cloning of the three selected genes from pathotype P1+, two (*PBRA_004239* and *PBRA_002551*) could not be cloned either due to total absence from the genome or presence of premature STOP codon in the sequence making the resultant protein non-functional. The putative chitin deacetylase *PBRA_005081* was cloned without the predicted secretion signal peptide into the pJCV53 vector to be over-expressed in Col-0 under 35S promoter.

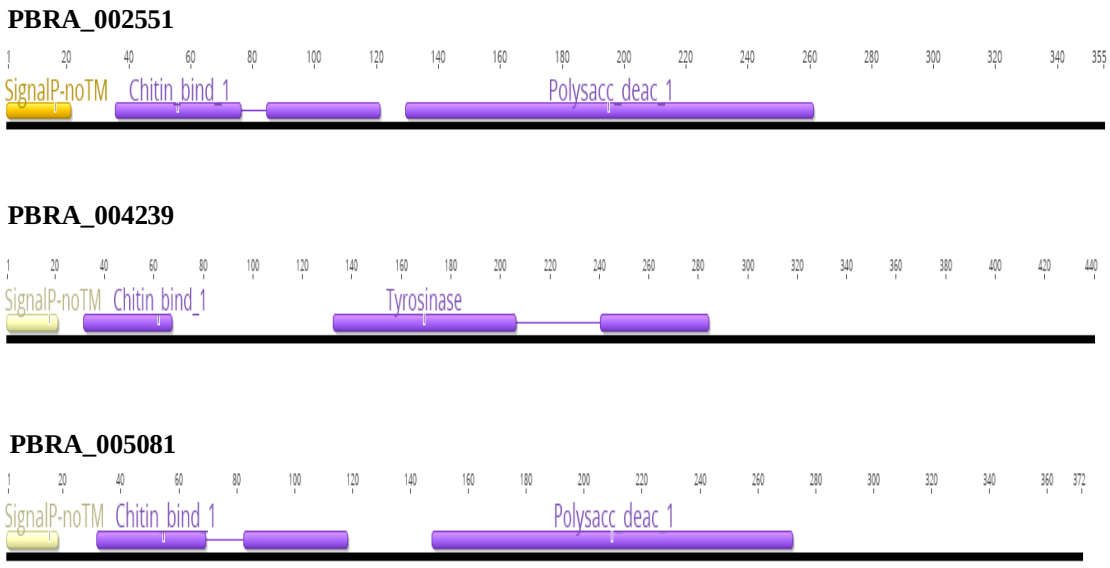


Figure 41: Diagram of 3 putative chitin binding *P. brassicae* proteins showing the various constituent domains.

4.3.7 Col-0 lines overexpressing *PBRA_005081* gene support increased pathogen load

Homozygous T3 seeds of 2 independent transgenic lines were obtained and characterized. Lines 1 and 3 overexpressing *PBRA_005081* accumulate significantly more pathogen DNA 19 dpi compared to the wild type plants (Figure 42 A). Figure 43 and Figure 44 compare the above ground part of wild type Col-0 and line 3 plants at 19 dpi and 32 dpi. Rosettes of line 3 plants shows enhanced susceptibility. It is interesting to note that, despite the *PBRA_005081* over-expressing line 3 having significantly reduced biomass in terms of its aerial tissue, it still supports significantly higher titers of pathogen DNA in the galls (figure 402 B).

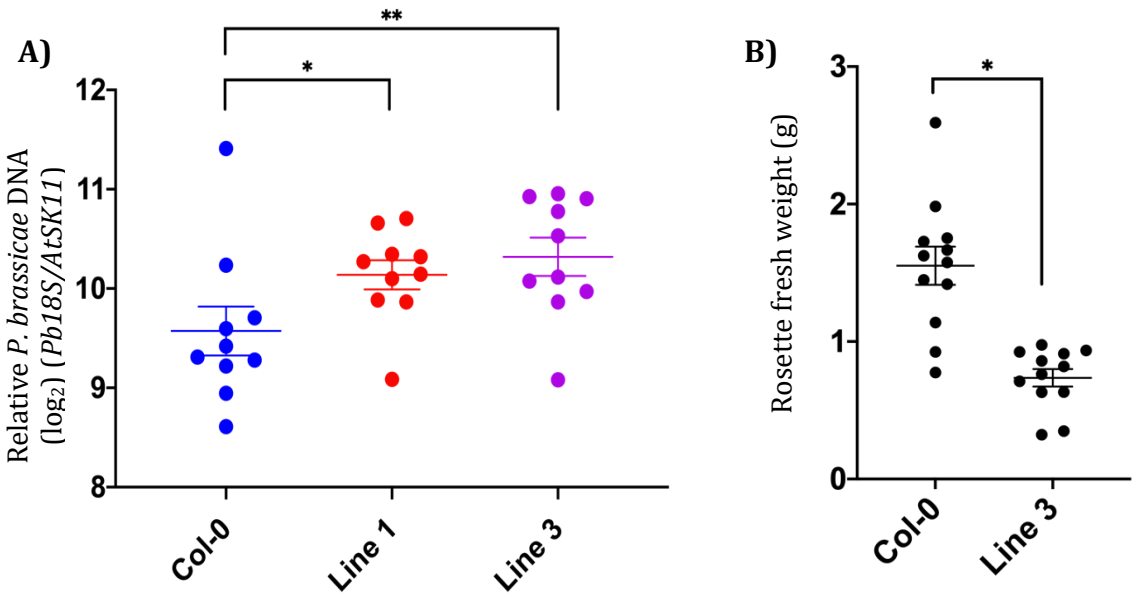


Figure 42: P. brassicae growth assay in wild type Col-0 and *PBRA_005081* overexpressing lines and difference in fresh weight between the wild type and line 3.

A) Relative pathogen load in Col-0, line 1 and line 3(19 dpi). Each data point has been shown as dot plots with mean and standard error bars. Each dot represents average of 3 galls. Asterisk (*) indicates significant difference ($p < 0.05$, ** for $p<0.005$) calculated by a linear mixed model taking genotype as fixed effect and experiment tray as random effect. B) Difference in fresh weight between aerial tissue of uninfected 37 days old Col-0 and line 3 plants. Each dot represents a single plant. Asterisks (*) indicates significant difference ($p < 0.05$) calculated by Student's T-test.

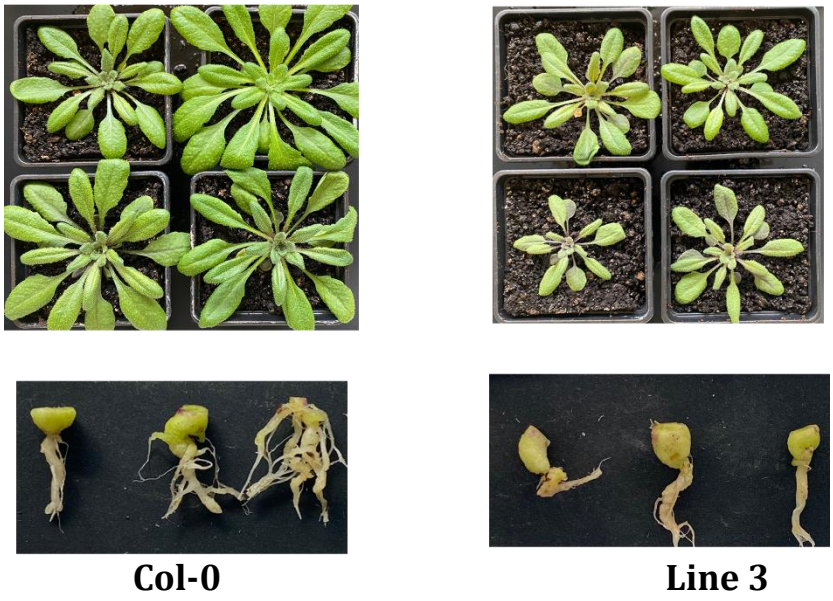


Figure 43: Pictures of rosette/galls of Col-0 and *PBRA_005081* overexpressing line 3, 19 dpi

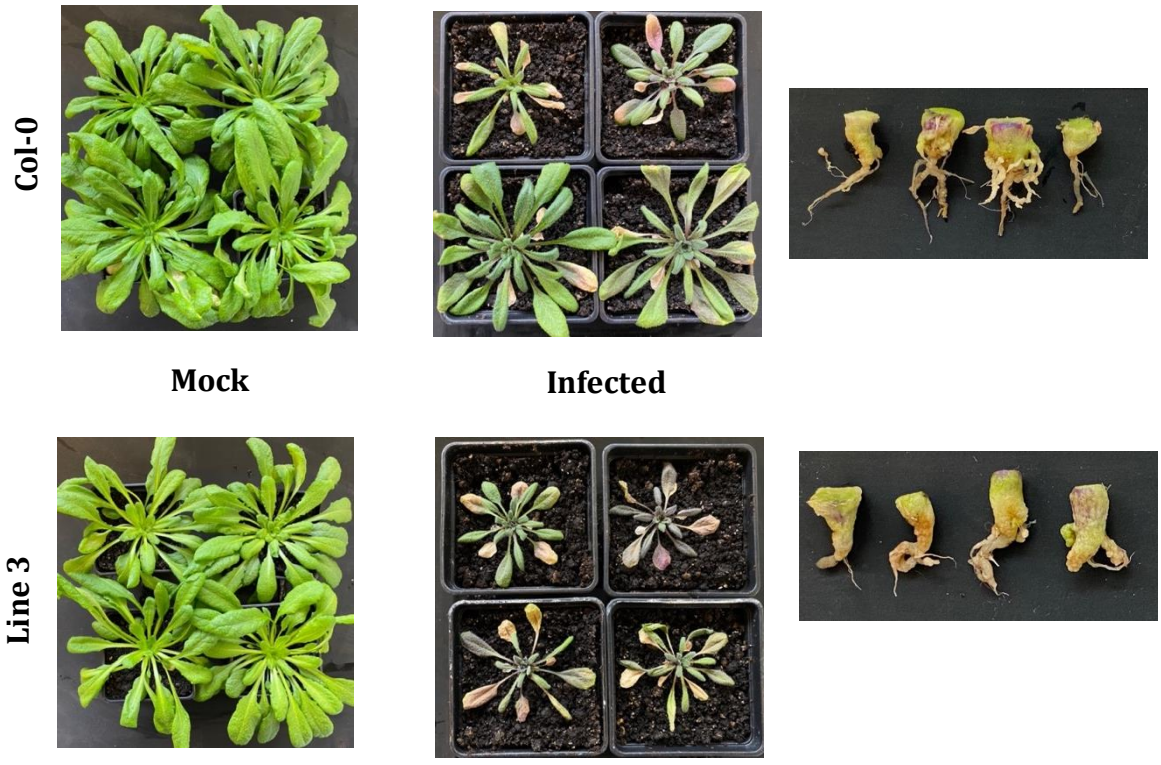


Figure 44: Pictures of rosette/galls of Col-0 and *PBRA_005081* overexpressing line 3, 32 dpi

Clubroot infection leads to suppression of xylogenesis which results in wilting of above-ground part (Malinowski et al., 2012). Therefore, measuring the relative water content in uninfected and infected plants could serve as a proxy for disease severity; the rosettes of wild type and *PBRA_005081* Line 3 rosettes were harvested and water content calculated from the difference between fresh and dried weight. Though both lines show significantly lower water content upon infection, the transgenic Line 3 infected plants had significantly lower relative water content compared to the wild type (Figure 45).

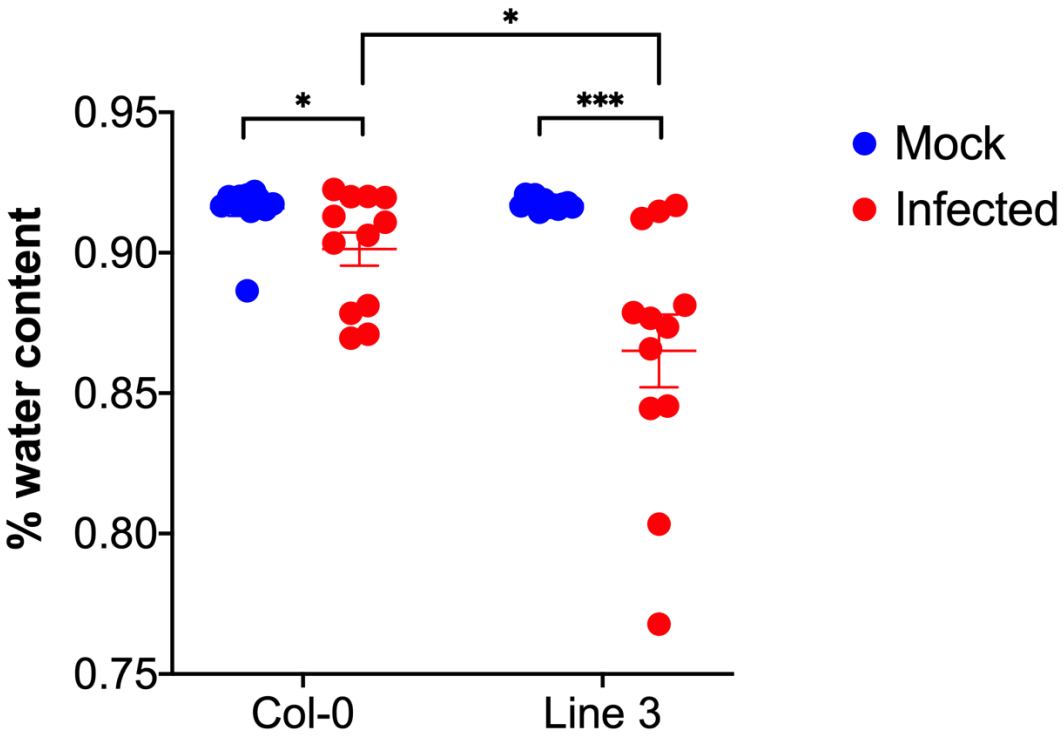


Figure 45: Relative water content in mock and infected wild type Col-0 and line 3 overexpressing *PBRA_005081*.

Each data point has been shown as dot plots with mean and standard error bars. 12 plants were used for every treatment. Asterisks (*) indicates significant differences ($p < 0.05$, *** for $p<0.0005$) calculated by Student's T-test.

4.3.8 Col-0 lines overexpressing a Trichoderma chitosanase do not show enhanced resistance

The Trichoderma group of fungi are well known bio-control agents (Mukhopadhyay & Kumar, 2020). Trichoderma has already been successfully used in control of clubroot in field conditions (Li et al., 2020). Trichoderma carries both chitinase and chitosanase genes which are thought to be detrimental towards other chitin carrying pathogens. If *P. brassicae* secretes chitin deacetylases to transform surface chitin to chitin as a less immunogenic moiety or to be less susceptible to host chitinolytic activity, then ectopically expressing Trichoderma chitosanase in Arabidopsis (which does not possess chitosanase enzymes) could be a way to slow down disease progression. The *AY571342* gene from *Hypocrea lixii* (teleomorph of Trichoderma) was cloned into the overexpression vector pJCV53. Eight independent transgenic lines were raised, out of which 4 were tested against *P. brassicae*. However, overexpression lines remained equally susceptible compared to the wild type plants in terms of pathogen DNA accumulation 19 dpi (Figure 46, 47).

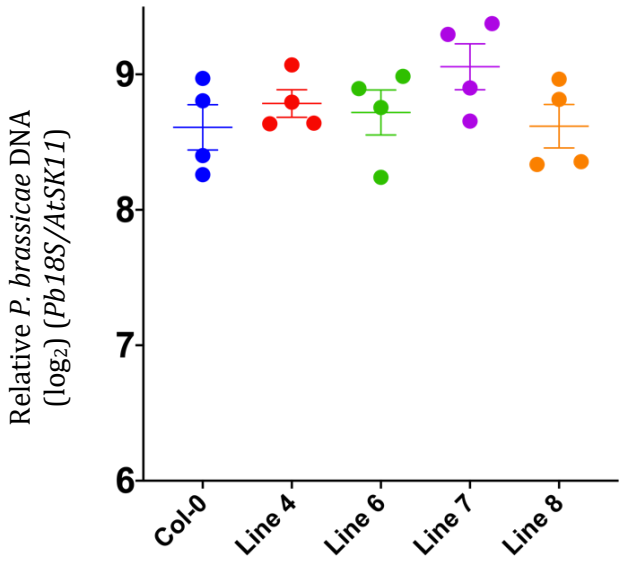


Figure 46: *P. brassicae* growth assay in Col-0 and Trichoderma chitosanase overexpressing lines

Each data point has been shown as dot plots with mean and standard error bar. Each data point represents a pool of 3 galls, Student's T-Test was used for testing statistical significance.

Figure 47: Pictures of rosette/galls of Col-0 and chitosanase overexpressing line 4, 19 dpi.

5. Discussion

Arabidopsis natural accessions display a wide spectrum of disease susceptibility to *P. brassicae*

Arabidopsis natural accessions have been exploited by GWAS to uncover various genetic factors underpinning different observed phenotypes (Lee et al., 2021). Arabidopsis being a Brassicaceae member could also be used to look for natural variation in disease susceptibility to *P. brassicae*. The 142 accessions used in our experiment, represent a wide variety of geographical areas covering Europe, Asia and North America. We used a P1+ strain of *P. brassicae* capable of breaking the resistance provide by the most common commercially used clubroot resistant oilseed rape cultivar 'Mendel'. There is a need to identify more sources of clubroot resistance to combat the rise of such resistance breaking strains. Screening of 142 accessions identified at least 10 accessions that were resistant to the P1+ train, which included the previously identified resistant accession Tsu-0 (Fuchs & Sacristán, 1996). We have identified new accessions such as Est-1, Fab-4, Uod-1 which display even greater level of resistance than Tsu-0, highlighting the importance of screening a larger population. Interestingly we found that pathogen DNA levels do not always fully reflect resulting size of the gall in susceptible accessions. The Pro-0 genotype accumulating high load of the pathogen developed average galls. Observed phenomenon may reflect different genotype specific capacity for host developmental plasticity.

RPB1 resistant locus has been identified by both traditional linkage mapping and GWAS

Previously the *RPB1* locus was identified by a traditional linkage mapping between the Tsu-0 and Cvi-0 accessions (Fuchs & Sacristán, 1996). This study identified the phenotype to be monogenic in inheritance. Subsequently a 12 kb resistance region responsible for the resistance containing multiple putative genes was cloned. While this data has been made publicly available, no publication describing the process of narrowing the region to 12 kb has been released. Interestingly, our GWAS approach pointed to the same region in other resistance accessions and closer inspection showed

that this genomic fragment is missing in numerous susceptible forms including the Col-0. Subsequently we found that 2 ORFs from this genomic stretch identified as *RPB1* and *RPB1-like-4* were found to be expressed in our resistant accessions Est-1 and Uod-1 and were even up-regulated in response to infection. These two genes were targeted for deletion by CRISPR-Cas9 genome editing by another researcher in our group; knock-out lines for *rpb1* in Est-1 and Uod-1 were found to be completely susceptible to P1+ and accumulated *P. brassicae* DNA levels as high as in Col-0. However, *rpb1-like-4* mutants remained resistant and a double *rpb-1 rpb1-like-4* line was no more susceptible than the single *rpb1* mutants (Ochoa, 2022). These results establish that *RPB1* is essential for clubroot resistance. The fact that the same genomic region responsible for resistance to *P. brassicae* infection in Arabidopsis has been independently identified in Germany and Poland points out common evolutionary origin and selective pressure between used pathotypes. Naturally those strains (e3 and P1+) evolved in very close proximity and probably carries the same effector molecules that are recognized by the resistant hosts. It would be interesting to establish if the *RPB1* mediated resistance holds towards *P. brassicae* pathotypes from distant regions. By identifying compatible and incompatible interactions it may be possible to identify candidate avirulence factors in the genomes.

Additionally, it is worth mentioning that accessions that do not carry the *RPB1* locus still exhibit variations in the level of resistance. As previously noted in GWAS studies of Brassica lines against *P. brassicae*, such observed variation in susceptibility in our Arabidopsis set could be polygenic in nature (Dakouri et al., 2018). As *P. brassicae* is a biotrophic pathogen, such variation in pathogen growth could be explained by host's ability to induce PTI more efficiently, induction of various transporters of nutrients for spore growth, differences in vascular growth pattern, or the presence/absence of susceptibility factors. One way to obtain such allelic variation would be to perform a GWAS analysis without resistant accession and to increase the number of susceptible accessions that do not have *RPB1* mediated resistance.

Complementing susceptible Col-0 with *RPB1* fragment is not sufficient to confer full scale resistance: missing downstream components or presence of susceptibility factors?

As overexpressing *RPB1* led to cell death in *Nicotiana* leaves and probable embryonic lethality in Arabidopsis, we decided to express it in Col-0 under the control of its native promoter from Est-1. *RPB1* transcript could be detected in Col-0 transgenic lines after *P. brassicae* infection. It's also interesting to note that in *rpb1* mutant lines, *RPB1* promoter (measured in the expression of 5'UTR sequences upstream of the deletion site) is still activated by *P. brassicae* infection (as observed by other researchers in our group). *RPB1* contains no known immune recognition domain, thus it is unlikely that *RPB1* is involved in direct recognition of pathogen secreted effectors. Therefore, it is likely that Col-0 carries the upstream immune receptor necessary for recognition of the effector molecule which further triggers *RPB1* induction. The *RPB1* promoter contains 5 WRKY boxes and some WRKYs are upregulated in the transcriptome of the Col-0 infected plants. Therefore, we cannot rule out the possibility that *RPB1* is co-expressed in response to a broader PTI response as well. Whichever the case, it appears that the induction of *RPB1* in Col-0 is unable to confer full scale resistance as offered by Est-1. We have also frequently noted random lesions and signs of senescence in Est-1 plants but not in other accessions. We could hypothesize that Est-1 already has an elevated basal defense response which is triggered easily when it faces abiotic/biotic stresses. Thus, it is a possibility that the superior resistance showed by Est-1 compared to the other *RPB1* containing resistance accessions could be due to a combined effect of pre-priming of the defense sector and *RPB1* mediated ETI. As *RPB1* is absent from Col-0, possibly due to transposon related genome rearrangements, it remains to be seen if it still carries necessary downstream components to effectively mount an immune response upon detecting the critical avirulence factor(s). Other possible explanation could be the presence of susceptibility factors as seen in other patho-systems (He et al., 2018) which are essentially host negative immune regulators that can be hijacked by pathogen to dampen host immunity. At present we do not possess a clear answer why in the course of evolution *RPB1* mediated resistance was lost from Col-0 even though there has always been a survival pressure from *P. brassicae*. One possible explanation

could be that carrying *RPB1* in the genome resulted in fitness penalty which was greater than the threat of clubroot disease in the long run. We also do not know if the P1+ class of pathotypes has emerged recently in the evolutionary timescale and therefore certain accessions still don't have any resistance mechanism against it.

Screening a broader range of pathotypes against *RPB1* resistance might contribute to a better understanding of the broad-spectrum usefulness of the locus. Though immune receptors are some of the most evolutionarily diverse genes in plant genomes, *RPB1* is highly conserved among multiple resistant and susceptible accessions. Susceptible accessions including An-1, Kyoto and C24 possesses *RPB1* with 99.9 - 100% amino acid identity compared to the Est-1 *RPB1*. One possible explanation would be the observed sequence variability in the promoter region that might interfere with the expression pattern. Therefore, it would be interesting to check the expression pattern of *RPB1* in susceptible accessions upon infection. Although Col-0 carrying the $p_{Est\text{-}1}$*RPB1::RPB1* transgene offers limited pathogen restriction, we do not know what the situation would be if we were to transform susceptible accessions other than Col-0.

RPB1 is an enigmatic small protein with no known domains. Further characterization of this immune gene might uncover previously unknown sectors of the programmed cell death signalling cascade. Finally, the ultimate application of this gene would be to make economically important brassica crops resistant. There are predicted *RPB1* orthologues in Brassicaceae, but no CR (Clubroot Resistance) genes yet identified match *RPB1*. As *RPB1* can confer resistance to pathotypes capable of breaking resistance in 'Mendel' cultivars, transfer of this resistance to routinely used brassica cultivars would be of great interest if the other genetic components necessary for resistance can be identified.

Col-0 and Est-1 show contrasting phytohormone mediated signalling responses upon infection

As clubroot disease is associated with significant developmental changes of the host, much prior research on Arabidopsis has focused on the later stages of the disease. We

selected the 7 dpi timepoint to identifying pathways that are differentially regulated in resistant and susceptible accessions. Our data clearly points to the JA-SA antagonism that appears to be crucial for the outcome of the disease. Biotrophic pathogens colonize apoplastic or intracellular spaces and deliver effector molecules that can shut down salicylic acid mediated PCD (Huang et al., 2020). In Est-1 brownish spots can often be observed on the roots when they are harvested 19 dpi, this may be indicative of hypersensitivity/localized cell death to prevent *P. brassicae* spread. *RPB1* mediated ETI responses in Est-1 leads to SA mediated response which is reflected in the upregulation of various Wall-Associated Kinases, Receptor Kinases, and Pathogenesis-Related genes. Interestingly, some jasmonic acid biosynthesis genes like *LOX2* (Mochizuki et al., 2016) are also induced in Est-1. Clearly, the abundance of JA responsive genes in Est-1 is significantly lower than Col-0 but it highlights a low level JA associated response that is accompanied with primary SA mediated signalling. It has been previously shown that NPR3 and NPR4 mediated JA accumulation is necessary for ETI response (Liu et al., 2016a). Lemarie *et al.* have previously reported involvement of both SA and JA for the resistance against *P. brassicae*, our transcriptomic data seems to corroborate such a finding (Lemarié, et al., 2015).

Taking the overlap between the Est-1 and Col-0 upregulated gene lists, we find three Cysteine-Rich Receptor-like Kinases (*CRK9, CRK10, CRK18*), five Ethylene Response Factors (*ERF2, ERF14, ERF15, ERF94, ERF113*), seven Glutathione-S-Transferases (*GSTF2, GSTF3, GSTF6, GSTU11, GSTU12, GSTU3, GSTU4*), six Peroxidases (*PER10, PER33, PER37, PER52, PER54, PER59*). We also see the induction of the SA master regulator *NPR1*, SAR response mediator *MES1* (Manosalva et al., 2010), antimicrobial *PR5*, indicating that Col-0 is capable of mounting a primary PTI response against the invading pathogen but fails to sustain it longer, potentially due to the absence of an *RPB1* mediated ETI trigger.

Col-0 transcriptome shows signs sign of early developmental changes upon infection

Though we did not find significant number of cell cycle regulators, auxin/cytokinin responsive genes or development associated transcription factors being differentially expressed in susceptible background at 7 dpi, lateral root (LR) initiation and the ARGOS family of genes were significantly enriched. In Arabidopsis, auxin binds to its receptor *TIR1/AFB* which in turn degrades the *AUX/IAA*s, the Auxin Response Factors (*ARF*) repressor (Santos Teixeira & ten Tusscher, 2019). This signaling frees the *ARF*s to be able to induce downstream signaling to induce the formation of LR founder cells. *GATA23* which is a prime target of those ARFs is upregulated in our dataset (Banda et al., 2019). Next, *ARF7* and *FLP* (upregulated in our study) maintain local concentrations of *PIN3* protein in the founder cell region to initiate LR formation (Santos Teixeira & ten Tusscher, 2019). *LBD16, 18, 33* transcription factors are downstream targets of the previously mentioned module and are necessary for nuclear migration and asymmetric cell division which is a prominent feature of LR initiation. Subsequent induction of *LBD16* and *PUCHI* has been shown to be indispensable for lateral root initiation (Goh et al., 2019). *LBD16*, its homologue *LBD17* and *PUCHI* are all induced upon *P. brassicae* infection. LBD16's involvement in nematode mediated gall formation, rhizobia mediated nodule formation and spontaneous callus formation (Fan et al., 2012) when overexpressed *in planta* is a very interesting observation. Did phytopathogens from various kingdoms evolve to exploit the LBD16 hub to stimulate rapid cell proliferation? Is this an example of convergent evolution in action? The losses in crop production of clubroot infected oilseed rape are due to the pathogen's manipulation of host development – the gall creates a resource sink that diverts photo-assimilate away from seed production and the reprogramming of vascular development and reduction of xylem formation leads to severe drought stress (Walerowski et al., 2018). Therefore, chemical or genetic interventions that might alter the early stages of signalling cascade would be tremendously beneficial in developing more tolerant plants capable of producing seeds. As the pathotypes capable of breaking resistance to currently available commercial varieties on the market spread to wider geography, there would

be increased demand for tolerant plants which would not suffer an almost 100% yield penalty.

Other interesting development associated genes included *ERECTA-like 1* and *ERECTA-like 2* which were strongly upregulated at 7 dpi. The *ERECTA* genes have been implicated in auxin mediated cell expansion in the Arabidopsis hypocotyl (Qu et al., 2017). Such phenomenon would be useful for the pathogen to increase intra-cellular space for growth and reproduction.

P. brassicae has adapted to evade chitin recognition

P. brassicae genome is predicted to contain 13 Chitin Synthase (CHS) genes (Schwelm et al., 2015). Chitin is an essential component of the *P. brassicae* spores and it being an intra-cellular pathogen, chitin is surely presented to the host multiple times during the course of infection. Though we found induction of few chitinases and chitin receptors in Col-0 7 days post infection, comparison with transcriptomic datasets from later stages of disease indicate significant suppression of the chitin response sector. A recent publication has identified 16 proteins in *P. brassicae* genome having domains associated with binding to chitin moieties (Muirhead & Pérez-López, 2022). They classified the proteins into 4 main categories- Chitin Binding (PbChiB) which carries only the chitin binding CBM18 domain, Chitin Binding Deacetylase (PbChiBD) which carry chitin binding CBM18 and chitin deacetylase CE4 domins, Chitinases (PbChi) having only a chitinase domain and Chitin Deacetylase (PbChiD) having only the CE4 domain. It was reported that *P. brassicae* (and other Plasmodiophorids) are unique in having an abundance of CBM18 domain containing proteins which is rare in other closely related Rhizarias such as *Bigelowiella natans*, *Reticulomyxa filosa* and other biotrophic fungi / pathogenic oomycetes (Schwelm et al., 2015). Thus *P. brassicae* genomes show signs of adaptation to a pathogenic lifestyle where chitin masking proteins have been acquired. Our attempt to boost host chitin responses prior to *P. brassicae* infection did not make any significant difference in the relative pathogen load. We hypothesize that various chitin binding proteins from *P. brassicae* bind to external chitin moieties to prevent recognition by other CBM18 containing plant chitin

receptors. Arabidopsis mutants lacking such functional receptors (*LYK4, LYK5* etc.) remained equally susceptible to *P. brassicae*. This shows the possible evasion of chitin triggered immunity when presence or absence of chitin perception machinery did not have any significant impact on disease progression.

The PbChiBD group of proteins are the largest group of chitin binding proteins (6 entries) in the *P. brassicae* genome. To clone two such chitin binding deacetylases and one chitin binding tyrosinase, we designed primers from 'e3' genome publicly available at that time. One PbChiBD gene and the other tyrosinase gene were absent or pseudogenised in our pathotype of interest, P1+. Pathogen effector proteins often show polymorphism and local duplications as those are constantly under selection pressure (Kourelis & van der Hoorn, 2018). As more *P. brassicae* genomes from distant geographies become publicly available, comparative transcriptomics and phylogeny analysis would shed light on the key chitin binding proteins that are conserved across lineages and most essential for pathogenicity. Identification of such key genes would allow us to unravel the exact chemical modifications to the *P. brassicae* chitin oligomers.

PbChiBD2's possible role in pathogenicity

A recent publication has presented a nomenclature system for the putative chitin binding proteins present in *P. brassicae* genome (Muirhead & Pérez-López, 2022). We initially adopted the gene naming system provided by Schewlm et. al in the initial *P. brassicae* genome paper (Schwelm et al., 2015) while characterizing the gene. In the recent publication, *PBRA_005081* has been named as *PbChiBD2*, streamlining the classification system and removing the confusion around different naming systems later adopted by other genome papers (Rolfe et al., 2016; Stjelja et al., 2019). As overexpression of *PbChiBD2* led to increased pathogen growth compared to the wildtype plants, we hypothesized 3 possible roles for the protein-

1. It contains CBM18 chitin binding domains and which out-competes the plant host's own CBM18 containing receptors to bind to *P. brassicae* chitin moieties.

2. As *P. brassicae* colonizes the intra-cellular space it may present chains of chitin oligomers. PbChiBD2's deacetylase domain deacetylates the surface chitin and

any detached free-floating oligomers into the less immunogenically potent form chitosan that has less potential to stimulate the host chitin perception machinery and may also become more resistant to degradation by the basal host chitinolytic activity present in the cytosol and apoplastic spaces.

3. Chitosan layers have been reported to be part of the yeast spores (Bemena et al., 2017). Though not much information is available on the detailed chemical composition of *P. brassicae* spores, it is possible that PbChiBD2 also has role in producing chitosan as part of the structural integrity of the spore wall.

Unfortunately, there is no established method of transforming *P. brassicae* with foreign genetic material, thereby limiting direct testing of possible hypothesis. Future experiments would involve assessing the deacetylation capacity of PChiBD2 *in vitro*. It would be interesting to determine out if PbChiBD2 has multiple roles in protecting and producing *P. brassicae* spores.

Summary of the research

This study involved probing multiple levels of the interaction between Arabidopsis and *P. brassicae*. We screened a wide variety Arabidopsis accessions and mutants to determine the genetic basis underpinning host clubroot resistance mechanisms. We also investigated the pathogen's genetic repertoire to identify putative virulence factors that might be involved in suppression of host immune responses, Figure 48 summarizes the research.

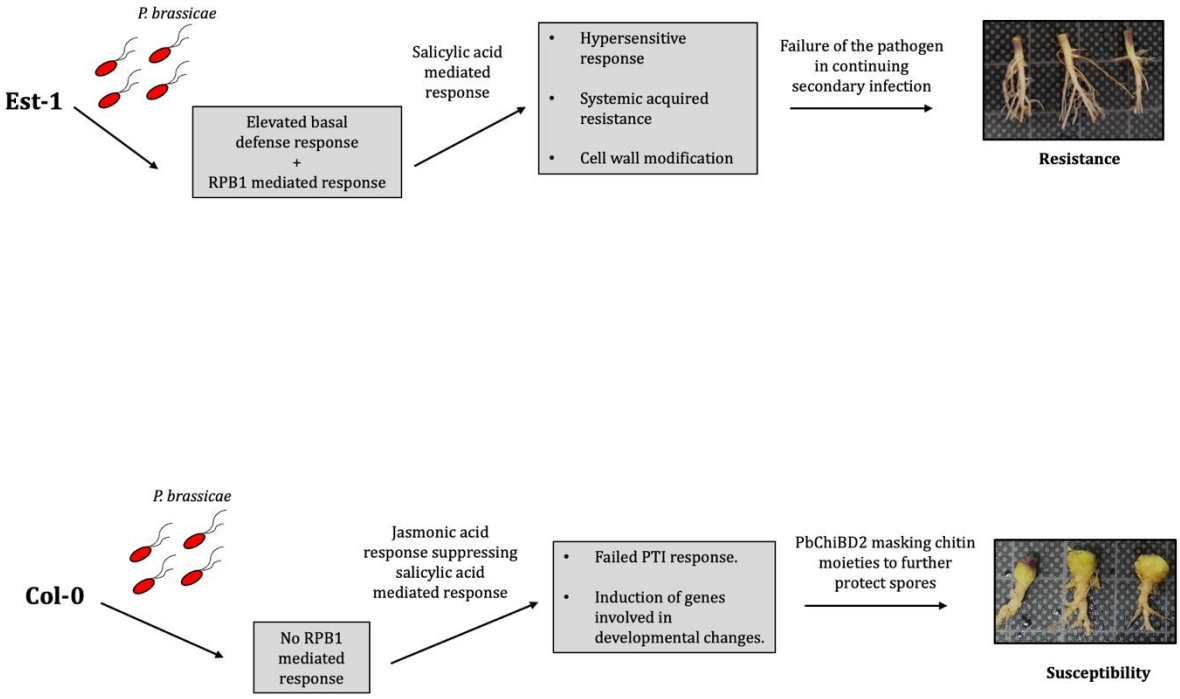


Figure 48: A schematic of the summary of the research describing the various modes of resistance and susceptibility against *P. brassicae*.

6. Conclusion

1. Investigation of natural germplasm variation of *Arabidopsis thaliana* has been useful in identifying sources of resistance against a virulent strain of *P. brassicae.*

2. *RPB1* is an immune gene (with no known domains) involved in clubroot disease resistance and overexpression of the gene possibly leads to embryo lethality.

3. Complementing Col-0 with *pRPB1::RPB1* is not sufficient for full scale resistance comparable to Est-1 indicating the requirement of additional signalling components.

4. Transcriptomics study at an early timepoint of secondary infection provided a snapshot of the hormonal crosstalk and various defense/development related genes differentially responding in resistant/susceptible backgrounds.

5. *P. brassicae* infection leads to suppression of chitin mediated defense responses in the susceptible background. A putative chitin binding and deacetylating protein (PbChiBD2) has been identified that might be involved in protection and production of *P. brassicae* spores.

"There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."

-Charles Darwin, *On the Origin of Species*

7. List of figures

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9. Bibliography

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