Defense Gene Responses Toward Necrotrophic Fungi in *Arabidopsis thaliana*

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Cover: Drawing of an *Arabidopsis thaliana* seedling.
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Abstract

Leptosphaeria maculans an ascomycete in the diverse class of Dothideomycetes, is an devastating pathogen on Brassica crops. In this thesis, tools and knowledge in *Arabidopsis thaliana* was explored to enhance our knowledge on plant defense to this fungus. Mechanisms that to large extent are shared with other necrotrophic fungi. Plants have evolved disease resistance (*R*) genes encoding for nucleotide-binding site (NB) and leucine-rich repeat (LRR) proteins with N-terminals represented by either Toll/Interleukin-1 receptor (TIR) or coiled-coil (CC) domains. A genome-wide study of presence and diversification of CC-NB-LRR and TIR-NB-LRR encoding genes, and shorter domain combinations in 19 Arabidopsis thaliana accessions and *Arabidopsis lyrata*, *Capsella rubella*, *Brassica rapa* and *Eutrema salsugineum* were performed. Out of 528 *R* genes analyzed, 12 CC-NB-LRR and 17 TIR-NB-LRR genes were conserved among the 19 *A. thaliana* genotypes, while only two CC-NB-LRRs, including ZAR1, and three TIR-NB-LRRs were conserved when comparing the five species. The resistance to *Leptosphaeria maculans* 1 (*RLM1*) locus confers resistance to *L. maculans* and has experienced conservation and diversification events particularly in *B. rapa*. RLM3 is unique in comparison to other characterized R proteins due to its three *brevis radix* (BRX) domains adjacent to the TIR and the NB domains. Three homologs were found in *Camelina sativa* that diverged from *Arabidopsis* ~17 million years ago. The BRX domains are able to interact with RLM3 possibly regulating downstream defense signaling responses.

Two *A. thaliana* mutants, being susceptible to *L. maculans* (*lms1* and *lms5*) were further characterized. *LMS1* encodes a lipid phosphate phosphatase-like protein but lacks phosphatidic acid phosphatase activity. LMS1 is localized in the plasma membrane where it interacts through the third motif of the phospatidic phosphatase domain with the salicylic acid binding protein 3 (SABP3). SABP3 mutagenesis abolishes the interaction with LMS1. The *lms1* mutant displays enhanced susceptibility to diverse pathogens, and high endogenous levels of SA and JA were found upon pathogen challenge. The LMS1-SABP3 interaction is central to maintain accurate JA and SA hormone homeostasis and defense signaling to necrotrophic pathogens. Mapping and genomic resequencing of the *lms5* mutant revealed a mutation in a prolyl oligopeptidase (*POP1*) gene. POP1 interacts with the glycolate oxidase 1 protein, a regulator of hydrogen peroxide production. Upon fungal challenge levels of callose and indole-acetic acid (IAA) were elevated in *lms5* compared to wild-type. The data highlight the importance of a balanced redox and auxin homeostasis to counteract invasion of *L. maculans.* In conclusion, this thesis work has revealed new defense genes, intricate interactions and signaling forming an important platform for future research and application in molecular breeding.

Keywords: Arabidopsis, *Brassicaceae*, defense responses, fungi, hormones, *R* genes

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Courage is not the absence of fear, but rather the judgment that something is more important than fear. The brave may not live forever, but the cautious do not live at all.

Meg Cabot

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Peele HM, Guan N, Fogelqvist J, Dixelius C. (2014) Loss and retention of resistance genes in five species of the *Brassicaceae* family. *BMC Plant Biol.* 14:298.

II Peele HM, Bejai S, Montiel V, Dixelius C. The resistance gene landscape in An-1 and the importance of the BRX domains (manuscript).

III Peele HM, Bejai S, Oide S, Persson M, Guan N, Montiel V, Dixelius C. LEPTOSPHAERIA MACULANS SUSCEPTIBLE 1 modulates salicylic acid and jasmonic acid defense signaling to necrotrophic pathogens in Arabidopsis (manuscript).

IV Bejai S**,** Guan N, Peele HM, Kaliff Stenberg M, Dixelius C. POP1, a prolyl oligopeptidase essential for the redox homeostasis in response to the fungus *Leptosphaeria maculans* (manuscript).

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The contribution of Hanneke Peele to the papers included in this thesis was as follows:

I Taken part in the planning and performed all the experiments and most of the analysis. Wrote a large part of the paper.

II Taken part in planning, performed a large part of the experiments and analysis. Wrote part of the paper.

III Taken part in planning, performed a subset of the experiments and analysis. Wrote part of the paper.

IV Taken part in planning, performed a subset of the experiments and analysis. Wrote part of the paper.

1 Introduction

During its lifetime, a plant is constantly challenged with abiotic (e.g. drought, wind, frost) and biotic stresses (insects and micro-organisms). The latter comprise mainly fungi, but also bacteria, oomycetes, nematodes and viruses. In agriculture, the losses due to pathogen infections can be extensive, which is particularly severe for people relying on a single crop as a food source mainly within developing countries (Oerke, 2005; Savary *et al.*, 2012). To minimize disease in crop plants in a profitable and sustainable way, resistant varieties need to be used and in most plant breeding programs the selection of these varieties is a major element. New tools are continuously developed to dissect complex traits (reviewed in Araus and Cairns, 2013), but since pathogens change over space and time, resistant crop varieties can often only be used for a short period of time. Producing crops that are resistant over a longer time period is one of the most difficult aspects in producing pathogen resistant crops (Strange and Scott, 2005). Introducing resistance genes from a resistant crop into a susceptible crop can be very time consuming, taking often more than over 10 years and is therefore costly. Within this timespan the pathogen might already have overcome the resistance to be deployed. Overcoming resistance in plants has also been attributed on the reduced genetic variety in crops used for plant breeding in comparison to their wild relatives. Genetic bottlenecks are not unusual for crops that underwent domestication (Ladizinsky, 1998). Thus to ensure global food security, understanding plant-pathogen interaction is of great importance to battle the fast evolving pathogenic populations (reviewed in Boyd *et al.*, 2013).

This thesis focuses on different aspects in plant immunity, highlighting the evolution of resistance genes within the family *Brassicaceae*. Additionally a gene important in the switch between two phytohormone responses in plant defense against necrotrophic filamentous pathogens; salicylic acid (SA) and jasmonic acid (JA) and a prolyl oligopeptidase involved in defense against the fungus *Leptosphaeria maculans* is described.

1.1 The *Brassicaceae* family

1.1.1 Taxonomy

The *Brassicaceae* family includes many economically important crop species used as oilseed (feed and biofuel), vegetable, condiment and fodder comprising approximately 321 genera and about 3660 species (Al-Shehbaz, 2012). Within the family nearly all members contain six stamens, often two shorter than the others, petals in the shape of a cross (or cruciform) and two-part fruits referred to as siliques (Beilstein *et al.*, 2006). Within the *Brassicaceae*, the genus *Brassica* including mustard, broccoli, cauliflower, turnip, rapeseed and several varieties of cabbage represent the best known crops being grown and consumed worldwide. Radish, horseradish, cress, watercress, rucola and wasabi that belong to a different genus are examples of other widely used crops.

The acknowledgement of the benefits of different species within the *Brassicaceae* has not stopped there. With the climate change and depleting fossil fuel stocks the importance of alternatives has been an interesting field of research for decades. In the case of aviation fuel used for planes, expenditure is steadily rising covering a large sector of the fossil fuel consumption (Kallio *et al.*, 2014). A reduction of greenhouse gas emissions with biodiesel usage (Li and Mupondwa, 2014) would diminish the environmental impact of aviation fuel significantly. Several promising candidates are currently exploited as a biofuel in planes. For example, *Brassica carinata* was used to fuel a plane in 2012 without the addition of kerosene (APA). As well as *Camelina sativa* which was used in a mixture with kerosene in several flights (Kagale *et al.*, 2014a). Field pennycress (*Thlaspi arvense*) which can overwinter as a rosette and flowers in spring, produces up to 1300kg/hectare of seed usable for oil production (Sedbrook *et al.*, 2014). Additionally it can be grown on soil used for summer annual crops increasing the usage of arable land. Very recently, the genome sequence of *T. arvense* became available with the aim of domesticating this new winter oilseed crop (Dorn *et al.*, 2015).

The *Brassicaceae* family includes several model species, with *Arabidopsis thaliana* as the most extensively used within the different disciplines of plant biology. Other increasingly important model species are *Arabidopsis halleri* and *Noccaea caerulescence* which have been used to study tolerance to heavy metals and hyper-accumulation, while the *Eutrema*/*Thellungiella* species have been studied in salt stress response (reviewed in Amtmann, 2009; Pollard *et al.*, 2014). The origin of the adaptation to extreme conditions is an important emphasis in all plant research (Koenig and Weigel, 2015).

1.1.2 Genomes and evolution

The clear phylogeny within the *Brassicaceae* family and the small genome sizes motivated the sequencing of different members in this family in the last decade (Table 1). Sequencing efforts has also included usage of different accessions or genotypes of the same species. *Brassicaceae* was probably shaped by an ancient whole-genome-duplication (*At*-α) ~47 mya (Kagale *et al.*, 2014b), distinguishing it from the *Aethionemeae* family (Figure 1). Most of the

Table 1. Genomic sequences released within the *Brassicaceae* family.

Species	Genome size	Gene models	Reference
Aethionema arabicum	240 Mb	23,167	Haudry *et al.*, 2013
Arabidopsis halleri	220 Mb	26,911	Akama *et al.*, 2014
Arabidopsis lyrata	230 Mb	27,379	Hu *et al.*, 2011
Arabidopsis thaliana	135Mb	28,710	AGI, 2000
Arabis alpina	370 Mb	22,032	Lobréaux *et al.*, 2014
Boechera stricta	197 Mb	43,370	Boechera stricta v1.2, DOE-JGI
Brassica napus	712 Mb	101,040	Chalhoub *et al.*, 2014
Brassica oleraceae	696 Mb	59,225	Ayele et al., 2005; Liu *et al.*, 2014; Parkin et al., 2014
Brassica rapa	529 Mb	41,174	Wang *et al.*, 2011
Camelina sativa	785 Mb	89,418	Kagale *et al.*, 2014a
Capsella rubella	216 Mb	26,521	Slotte *et al.*, 2013
Capsella grandiflora	112 Mb	26,561	Slotte *et al.*, 2013
*Eutrema salsugineum**	314 Mb	26,521	Yang *et al.*, 2013b
Leavenworthia alabamica	316 Mb	30,343	Haudry *et al.*, 2013
Neslia paniculata	113 Mb	-	Slotte *et al.*, 2013
Raphanus raphanistrum	515 Mb	38,174	Moghe *et al.*, 2014
Raphanus sativus L.	573 Mb	61,572	Kitashiba *et al.*, 2014
*Schrenkiella parvula***	140 Mb	28,901	Dassanayake *et al.*, 2011
Sisymbrium irio	262 Mb	28,917	Haudry *et al.*, 2013
Thlaspi arvense	539 Mb	27,390	Dorn *et al.*, 2015

* previously *Thellungiella halophila;* ** previously *Thellungiela/Eutrema parvula*/*parvulum*
- (unknown).

duplicated genes were however lost before the divergence of the 3660 species (Edger and Pires, 2009; Schnable *et al.*, 2012; Haudry *et al.*, 2013) resulting in an ancestor with a medium-sized genome of ~500Mb with a diploid karyotype of 8 chromosomes split into 24 crucifer genomic blocks (A-X) (Schranz *et al.*, 2006; Lysak *et al.*, 2009). Three lineages have been described in the core depending on their evolutionary history (Beilstein *et al.*, 2006; Franzke *et al.*,

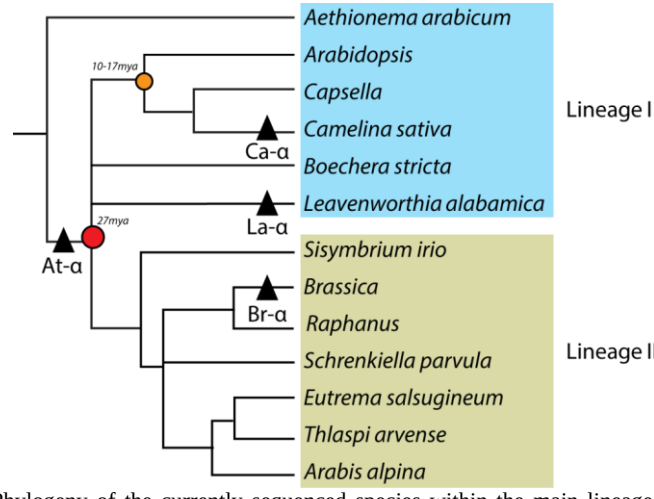

Figure 1. Phylogeny of the currently sequenced species within the main lineage I and II in *Brassicaceae*. Data from figure is a compilation of results from Beilstein *et al.*, 2006; Busch *et al.*, 2012; Franzke *et al.*, 2011; Haudry *et al.*, 2013; Kagale *et al.*, 2014b; Moghe *et al.*, 2014. The whole genome duplication distinguishing *A. arabicum* with the core *Brassicaceae* (At-α), the whole genome triplication in *Brassica* species (Br-α), *L. alabamica* (La-α) and *C. sativa* (Ca-α) are given. The coloured dots represent split estimates.

2011). Currently only the genomes from lineage I and II have been sequenced (Franzke *et al.*, 2011). Lineage I is represented by the model species *A. thaliana* and the more well-known *Brassica* genus can be found in lineage II.

Many characteristics within *Brassicaceae* have originated independently, including the transition to selfing in several of the species and the removal of fertilization in the *Boechera* species (Rushworth *et al.*, 2011). Numerous chromosomal rearrangements occurred between the *Brassicaceae* genomes exemplified in a comparison between *Arabidopsis lyrata* and *A. thaliana* and between *B. napus* in comparison to *B. oleracea* and *B. rapa* (Chalhoub *et al.*, 2014). However, some genomes revealed high similarity, as was observed in a comparison between the genomes of *B. oleracea* and *B. rapa* (Parkin *et al.*, 2014). The sequencing of the three *Brassica* crops also gave some insights in genes selected during domestication, revealing *FLOWERING LOCUS C* (*FLC*) with one copy in *A. thaliana*, four in *B. rapa* and *B. oleracea* and nine in *B. napus* corresponded to QTLs in flowering time (Zou *et al.*, 2012). After divergence, several species underwent triplication, represented by the *Brassica* genus, *C. sativa* and *Leavenworthia alabamica* (Ba-α, Ca-α and La-α). In *Brassica* species this was followed by massive gene loss and reshuffling of the genomes.

1.1.3 *Arabidopsis thaliana*

Since the first paper published on *A. thaliana* (Laibach, 1943), this flowering plant has been increasingly exploited in numerous aspects within plant biology and is considered to be the major biological model for plants (Sommerville and

Koornneef, 2002). The different *A. thaliana* accessions, representing the *A. thaliana* plants found throughout Europe, Asia and North America have adjusted to many different environments and resulted in extensive diversity within several physiological traits e.g. flowering time and tolerance to different abiotic stresses (e.g. Zhen and Ungerer, 2008; Brachi *et al.*, 2013). The vast collection of accessions especially within Europe (http://1001genomes.org) resulted in the functional annotation of many different genes using forward genetics. For example the *RESISTANCE AGAINST L. MACULANS 3* (*RLM3*) gene was found absent in the accession An-1 resulting in a diseased phenotype when challenged with *L. maculans* (Staal *et al.*, 2006).

Even with the extensive *A. thaliana* accession collection, the Columbia or Col and to some extent the Landsberg *erecta* (L*er*) accessions have predominantly been used (Alonso-Blanco and Koornneef, 2000). Especially the Col-0 accession which the reference genome is based on (AGI, 2000). The availability of thousands of mutants in this background as well as other data including microarray and information on many different physiological (e.g. development of different organs) and biochemical characteristics (e.g. hormone levels) are outstanding resources in plant biology.

Since numerous genes have been discovered using *A. thaliana*, it has become increasingly apparent that the use of only one wild type creates a bias. In the recent sequencing of 18 different accessions of *A. thaliana*, novel genes with similarity to *A. lyrata* and other *Brassicaceae* species were identified (Gan *et al.*, 2011). Among 180 accessions collected in Sweden, 200-300 additional genes not identified in Col-0 were identified (Long *et al.*, 2013), underlying again the great resource the different natural accessions represent. Therefore the discovery of genes expressed in a variety of accessions but not in the wild type suggested the importance of sequencing and re-annotating individual genomes.

1.1.4 The *Brassica* genus

The genus *Brassica* and its varieties have a tremendous morphological variability within the roots, stems, leaves, buds, flowers and seeds (Warwick, 2011). The well-known cultivated *B. oleracea* for example can be divided in kales (var. *acephala*), cabbage (var. *capitata*, *sabauda* and *bullata*), kohlrabi (var. *gongylodes*), inflorescence kales like cauliflower, broccoli (var. *botrytis*, *italic*), branching bush kales (var. *fruticose*) and Chinese kale. Most of the *Brassica* species originated in the Mediterranean-Middle Eastern area, while the differentiation of *B. rapa* and *B. juncea* was believed to have taken place in China (Prakash and Hinata, 1980). Of the cultivated crops, *B. napus* (oilseed rape) is one of the most important edible oil crop produced worldwide, mainly in Europe, Asia and Canada (FAOSTAT). In total, an area of more than 36 million hectares was used for *B. napus* production in 2013 which reached over 70 million tons of harvested rapeseed.

The widely cultivated *Brassica* species are described according to their genome composition referred to as the A, B and C genomes forming the

'triangle of U' (U, 1935). Within this triangle three diploid species are represented by *B. rapa* (2n = 20, A genome), *B. nigra* (2n = 16, B genome) and *B. oleracea* (2n =18, C genome). Through hybridization, the amphidiploid species *B. juncea* (2n =36, A and B genomes), *B. napus* (2n =38, A and C genomes) and *B. carinata* (2n =34, B and C genomes) were formed. These six widely cultivated *Brassica* species are excellent models to study polyploidy in evolution (Nagaharu, 1935; Mizushima, 1950). Recently, the genomes of *B. rapa*, *B. oleracea* and *B. napus* have become available, giving insights into the conservation and evolution of the A and C genomes (Ayele *et al.,* 2005; Wang *et al.,* 2011; Chalhoub *et al.,* 2014; Liu *et al.,* 2014; Parkin *et al.*, 2014).

1.2 Sequencing and crop improvement

1.2.1 Advances in next generation sequencing

Sequencing and assembling plant genomes has been challenging, largely due to the large genome sizes accompanied by repetitive sequences, transposable elements and a higher frequency of polyploidy and heterozygosity (Claros *et al.,* 2012). In addition, plants tend to have multi-gene families with high number of paralogous genes (Lockton and Gaut, 2005). *A. thaliana*, completed by the Sanger sequencing method was the first plant genome to be sequenced and was a multi-year and costly endeavor (AGI, 2000). Thanks to advances in next-generation sequencing (NGS) technology as well as improvements in bioinformatic algorithms and *ab initio* gene structure prediction programs it is now possible to produce a draft genome sequence in just a few months with just a fraction of the costs (Weigel and Mott, 2009). The main advance in the NGS technology was the ability to remove additional time consuming steps, mainly the subcloning and clone amplification in bacterial hosts (Claros *et al.,* 2012).

NGS technology is commonly divided in second and third NGS, relying on similar methods with the usage of either a PCR step or not (reviewed in Knief, 2014). A single template is directly used to generate millions of bases and the protocol is much more straightforward than Sanger sequencing. Combining different NGS technologies as well as assembly technology was shown to be a very effective tool to produce high quality draft sequences (Aury *et al.,* 2008; Kumar and Blaxter, 2010) and was employed in the sequencing of *B. oleracea*, *B. napus*, *Capsella rubella* and *Schrenkiella parvulum* (Dassanayake *et al.,* 2011; Chalhoub *et al.,* 2014; Slotte *et al.,* 2013; Liu *et al.,* 2014; Parkin *et al.,* 2014). Additionally, technology for assembling genomes has advanced tremendously, needing substantial expertise and effort and it has become clear that no single technique can be used for all sequencing projects (Schatz *et al.,* 2012). While NGS technology has major advantages, shortcomings still remain (reviewed in Claros *et al.,* 2012). One of the shortcomings is the high inaccurate base calling and short sequence lengths generated. The latter is

becoming less of a problem with longer reads being obtained reaching up to 5000bp.

To date only *A. thaliana* is considered 'finished' if you ignore the lack of sequence data in the highly repetitive regions, which still remain cumbersome for sequencing and assembly (Hamilton and Buell, 2012). Major focus is put in the production of draft sequences and less into producing 'complete' genomes. The reduced efforts could hinder functional genomics in the future and subsequently the improvements to crop quality (Feuillet *et al.*, 2011; Claros *et al.*, 2012). Therefore aiming for a high quality sequence would be highly beneficial for crop improvement.

1.2.2 Crop improvements

Understanding the gene regulatory networks that are shared between plant species is instrumental in improving cultivated crop species as well as aiding in understanding of crop evolution (reviewed in Dhanapal and Govindaraj, 2015). A major challenge in producing a crop that is resistant against all possible stresses is the constantly changing environment influencing the regulation of multiple genes including genes with very small effect and epigenetics. A further understanding of the interactions between abiotic and biotic stress as well as with the biochemical pathways in the plant is needed to continue producing crops that can be grown in large scale without major losses (Fleury *et al.*, 2010). The available sequence data from *A. thaliana* unraveled the basic mechanisms in plant development and has provided knowledge into tolerance to abiotic and biotic stress situations. The information from *A. thaliana* was for example used to improve the tolerance to freezing, salt and drought tolerance in tomato, rapeseed, strawberry, rice and wheat (Zhang *et al.*, 2004). In disease resistance however, the situation is much more complex with multiple genes playing minor roles, but together form the strongest response (Poland *et al.*, 2008). Identifying each individual factor involved in resistance response remains challenging (Raghuvanshi *et al.*, 2010). Currently the introduction of both QTLs associated with disease resistance and major resistance (*R*) genes into crop varieties is the best way to achieve effective and durable resistance (Boyd *et al.*, 2013). The increasing availability of genomic sequence data has resulted in the increase of available markers and the identification of regions within the genomes important in defense responses.

Other major problems reside in the decline of genetic diversity within crops through domestication of plant species leading to a collection of highly genetically similar cultivars (reviewed in Gepts, 2014). For example in rice, only 10 to 20% of the genetic diversity was retained from wild rice in cultivated rice (Zhu *et al.*, 2007). The resulting crops are often less fit to adapt to a changing environment, making them more vulnerable to new diseases (Brown and Funk, 2008; Turner *et al.*, 2009). Improvements have to be made in the current selection of crop species to ensure protection against biotic and abiotic stresses. To this end the use of landraces, rare breeds and wild relatives with novel resistance traits are currently exploited. Traditional breeding

requires two species to be inter-fertile creating a major limitation in improving a crop. However, bridge-crosses could be used to overcome this. Unfortunately, crossing often introduces potential undesirable traits, which becomes greater with distantly related species or wild species. Additionally, the introduced resistance could make the plant more vulnerable to other pathogens or abiotic stress like drought (Huot *et al.,* 2014). Thus, crossing is not always as simple as it sounds, often resulting in several years of back-crossing and selection before the desired product is achieved (reviewed in Boyd *et al.,* 2013). In the case of potato (*Solanum tuberosum*) introduction of late blight resistance was achieved using the wild species *Solanum bulbocastanum*, which took more than 30 years (Haverkort *et al.,* 2009).

1.2.3 Pathogens that attack Brassica crops and their genomes

Sequencing efforts on pathogens infecting one or more of the *Brassicaceae* species has aided in a better understanding of plant-pathogen interaction. *L. maculans* causing stem cankers on several *Brassica* species results in major economic losses each year (Fitt *et al.,* 2006). The release of the *L. maculans* genome sequence aided in the identification of two avirulence genes *AvrLmJ1* and *AvrLm11*, the latter representing the first cloned *Avr* gene with a corresponding resistance gene, *RLM11* identified in *B. rapa* (Balesdent *et al.,* 2013; Van de Wouw *et al.,* 2014). And *RLM11* was introduced to susceptible oilseed rape genotypes with the aim to use in fields were >95% of the *L. maculans* population was shown to contain the *AvrLm11* gene. So far the improved oilseed has not been commercially released (Zander *et al.,* 2013), therefore the effectiveness of the resistance is unknown.

The research in *Hyaloperonospora arabidopsidis* has been used as a genetic model to study plant-microbe interactions, with the hope of using it in other pathosystems within the *Brassicaceae* (Holub, 2008; Coates and Beynon, 2010). The sequenced genome of *H. arabidopsidis*, causing downy mildew in *A. thaliana*, contains 134 potential secreted proteins (Baxter *et al.,* 2010). These potential proteins were subsequently used in different functional experiments, revealing their localization *in planta*, interaction with plant proteins and induction of plant defenses as well as being able to enhance susceptibility in the plant (Baxter *et al.,* 2010; Fabro *et al.,* 2011; Mukhtar *et al.,* 2011; Caillaud *et al.,* 2012a; 2012b; 2013; Wirthmueller *et al.,* 2015).

Additionally, the availability of the genomes of other pathogens revealed insight in their infection strategies, like the *AMR1* transcription factor in *Alternaria brassicicola* (Cho *et al.,* 2012), the *VELVET* gene and chitin synthase genes in *Botrytis cinerea* (Morcx *et al.,* 2013; Schumacher *et al.,* 2012) to name a few. Additionally the obligate biotrophic clubroot pathogen *Plasmodiophora brassicae* impacting numerous species within the *Brassicaceae* family was recently released (Schwelm *et al.,* 2015), predicting effector candidates which likely will aid in a better understanding of this devastating protist and close relatives in the future.

1.2.4 Effectoromics and transcriptomics

Effector proteins are secreted by pathogens to aid in infection through suppressing immunity and altering plant processes and are generally not well conserved between species (Jones and Dangl, 2006). Functional genomic strategies employing effectors from pathogens probed against plant germplasms to detect *R* genes and other interacting proteins are an important part of modern breeding today (reviewed in Vleeshouwers and Oliver, 2014). A great example of usage of such a strategy is in the breeding of resistant potato against *Phytophthora infestans*, a devastating pathogen for this crop (Vleeshouwers *et al.,* 2008). Wild *Solanum* species were tested against effectors from the oomycete and genotypes displaying HR response were selected for crossing resulting in the resistant crop currently used in cultivation. Durable resistance is more attainable using 'core effectors' found in a wide range of the population rather than dispensable effectors that are easily lost (Dangl *et al.,* 2013). It is now feasible to sequence different field isolates to determine effector content. For example, the highly conserved *AvrBs2* effector identified in *Xanthomonas* species and its R protein Bs2 in pepper was successfully employed to induce resistance in tomato against *Xanthomonas perforans* (Tai *et al.,* 1999). However, resistance of this particular gene was overcome in pepper, due to point mutations in the *AvrBs2* gene (Stall *et al.,* 2009; Dangl *et al.,* 2013) highlighting the advantage of stacking multiple *R* genes rather than focusing on just one. However there is the possibility of silencing with the use of gene stacking as was observed for the powdery mildew resistant in wheat lines (Stirnweis *et al.,* 2014). A better knowledge of effector function within plant defense will aid in the overall understanding of plant disease and will aid in producing crops with increased durable disease resistance.

Studying plant-pathogen interaction at the transcriptional level gives insight into the transcriptional changes caused by a pathogen. RNA sequencing has greatly enhanced transcriptomic analysis and also functions as a screening method to find new players in response to a pathogen (reviewed in Wolf, 2013). To this end different pathogen strains with different effector repertoires and plant genotypes with differences in disease phenotype (resistant, susceptible or moderate) are used. Additionally, sequencing of non-coding RNAs and small RNAs provides information on possible induced silencing by a pathogen. In tomato RNA isolated from resistant and a susceptible breeding lines against the tomato yellow leaf curl virus had the additional advantage that novel genes were recovered not present in the reference genome sequence (Chen *et al.,* 2013). RNA sequencing therefore provides an additional tool to find interesting genes for crop improvement when only a partly sequenced genome is available.

1.3 Defense responses

1.3.1 Resistance genes and their evolution

Plants have evolved several defense responses including the action of resistant genes or *R* genes. These genes are involved in protection against a spectrum of pathogens, such as bacteria, viruses, fungi, nematodes, insects and even parasitic plants (Dangl and Jones, 2001; Gururani *et al.,* 2012; Michelmore *et al.,* 2013). *R* genes also have been shown to be involved in response to cold and drought stress (Chini *et al.,* 2004; Yang *et al.,* 2010). The largest class of *R* genes encode proteins with a 'nucleotide-binding site and leucine-rich repeat' domains (NB-LRR, NL) (Dangl and Jones, 2001; Hulbert *et al.,* 2001). The NB domain is involved in binding to ATP or GTP and includes highly conserved motifs, such as the P-loop, kinase-2 and Gly-Leu-Pro-Leu motifs (Tan and Wu, 2012). The LRRs, found in a diversity of proteins is involved in the interaction with proteins, ligands and carbohydrates and are under diversifying selection (Jones and Dangl, 2006). The LRRs are divided in eight specific classes, depending on sequence length and similarity within the variable segment and LRRs are suggested to have evolved several times within a variety of different species (Kajava, 1998; Miyashita *et al.,* 2013).

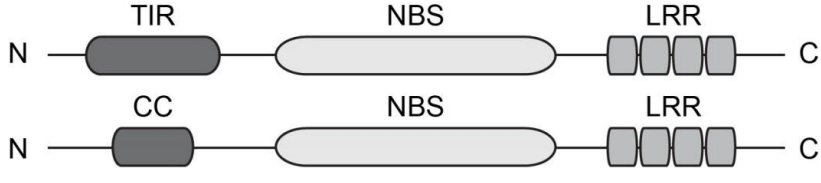

Figure 2. Schematic representation of the domains found in the two subclasses of R proteins. The upper drawing depicts the subclass consisting of a Toll and mammalian interleukin-1 receptor (TIR) flanking the 'nucleotide-binding site and leucine-rich repeat' (NB-LRR), while the lower drawing shows the subclass replacing the TIR with a coiled-coil (CC) domain.

The NB domain and LRRs have a very ancient origin since several genes in the eubacteria species (e.g. *Streptomyces coelicolor*, *Nostoc punctiforme*) are identified encoding either a NB domain or LRRs (Yue *et al.,* 2012; Jacob *et al.,* 2013). The fusion between NB and LRR is not observed until the moss *Physcomitrella patens.* The NLs are further subdivided into two subclasses, based on the N-terminal structural features. The first subclass contains an N-terminus referred to as the *Drosophila* Toll and mammalian interleukin-1 receptors or TIR (TNL), while the second subclass contains a coiled-coil (CC) domain (CNL, Figure 2). The subclasses can induce different signaling pathways within the cells to aid in disease resistance (Aarts *et al.,* 1998). It is predicted that the combination of the TIR domain with the NL has evolved earlier than its CNL counterpart (Yue *et al.,* 2012). The two gene families have expanded extensively within the different plant families not correlating with genome size. However in monocots, *TNL* genes seemed to have been lost completely while in dicots there is a preference for *TNL* genes (Tarr *et al.,* 2009).

Most plant *R* genes are found to cluster together in plant genomes and are divided in two types depending on how the cluster is shaped. The first type contains *R* genes that have experienced tandem duplications, while ectopic duplication, transposition or large-scale segmental duplication and rearrangements are typical for the second type (Hulbert *et al.*, 2001; Leister, 2004). *R* genes are more diverse than can be expected from random genetic drift suggesting major selective pressures working on this gene family (Marone *et al.*, 2013). Indentification of R gene clusters in plant genomes can be usefull to find new functional *R* genes and was employed in a study with the rice blast pathogen *Magnaporthe oryzae* (Yang *et al.*, 2013c). In this study three rapidly evolving *R*-gene families shaped by recent duplications were identified in maize, sorghum and Brachypodium. Several of the fast evolving genes were found to induce resistance towards *M. oryzae* when introduced into rice.

Genes encoding only the TIR domain or TIR and NB domain are identified in every node of the plant kingdom and there is growing evidence that these genes also play important roles in plant defense response (Nandety *et al.*, 2013). Several *A. thaliana* TN genes confer resistance towards *Pseudomonas syringae* and *Fusarium oxysporum* and show high conservation between distantly related species. In yeast-two-hybrid studies several were found to interact with effector proteins from a wide variety of pathogens, suggesting that this class of genes is directly involved in recognizing a pathogen. Additionally, the TN proteins were suggested to work as adapter proteins interacting with TNL proteins functioning as downstream signaling components in defense response (Meyers *et al.*, 2002).

1.3.2 Salicylic acid and other phytohormones

Phytohormones play essential roles within plant development, reproduction, growth and are associated with the response towards many different types of stresses. The regulation of phytohormones has to be tightly regulated resulting in a complex signaling network that uses both antagonistic as well as synergistic crosstalk within the different hormone pathways (Robert-Seilaniantz *et al.*, 2011; Kazan and Lyons, 2014). Within plants, three hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the most predominate players regarding defense signaling. Even though exceptions exist, SA has been mostly described in defense against biotrophic pathogens (feeding on living tissues); while the JA and ET have been associated with pathogens with a necrotrophic lifestyle (feeding on dead tissues).

After a pathogen encounter, SA levels often increase rapidly leading to the expression of *PATHOGENESIS-RELATED* (*PR*) genes (Klessig and Malamy, 1994; Pieterse and Loon, 1999; Shah, 2003). NONEXPRESSER OF PR GENES 1 (NPR1) plays a vital part within SA defense response and is known to interact with several members of the TGACG motif binding (TGA) family of basic leucine zipper transcription factors to activate expression of the *PR* genes (Jakoby *et al.*, 2002). Several effectors from pathogens are able to bypass SA-triggered defense responses in the plant and induced disease

(Djamei *et al.*, 2011; Asai *et al.*, 2014). Controversies still exist on how exactly NPR1 functions within SA response and more in-depth studies on this topic is on-going (Boatwright and Pajerowska-Mukhtar, 2013). While *A. thaliana* has been the model species for investigating hormone defense responses, alternative model species, like rice have revealed complex differences (De Vleesschauwer *et al.*, 2013; Yang *et al.*, 2013a). In rice, the SA biosynthesis is not well understood and does not seem to be influenced by pathogen attack (Silverman *et al.*, 1995). Orthologs within the SA pathway from *A. thaliana* have different functions in rice (De Vleesschauwer *et al.*, 2014). In case of the rice NPR1 homolog, it functions as an energy switch limiting resources for growth to handle a pathogen attack.

Between SA and JA the antagonistic effects have been described extensively and found conserved among 17 plant species from various taxonomic groups (Robert-Seilaniantz *et al.*, 2011; Thaler *et al.*, 2012). Increasing evidence is accumulating revealing the existence of synergistic effects relating to possible threshold levels of the two hormones (Spoel and Dong, 2008). ET generally has been described to work in concert mainly with JA, but ET is also described to positively interact with the SA pathway (van der Ent and Pieterse, 2012; Derksen *et al.*, 2013). Other hormones, like abscisic acid, gibberellins, auxins, cytokinins and brassinosteroids are increasingly shown to positively or negatively affect the defense pathways (Bari and Jones, 2009; Cao *et al.*, 2011; Naseem *et al.*, 2015).

2 Aims of the study

The emphasis of the work was to increase our understanding within the different levels of plant defense at a functional and evolutionary level. With this in mind the specific aims were:

- Gain deeper knowledge in *R* gene conservation and expansion within the *Brassicaceae* family
- Functionally characterize the *RLM3*, *LMS1* and *LMS5* genes and their different roles in plant defense.

3 Results and Discussion

3.1 *R* gene repertoires

3.1.1 A core set of 29 *R* genes in nineteen *A. thaliana* accessions (I)

The *R* gene repertoires identified in plant genomes represent one of the largest gene families (Jacob *et al*., 2013). The *R* gene expansions have been shaped through the host and pathogen co-evolution resulting in unique *R* gene sets in each species (Michelmore *et al.,* 2013). The expansion seems to be unrelated to phylogeny or genome size and evolves rapidly through duplication. In the *A. thaliana* reference genome Col-0, 81 genes encoding TIR-NB-LRRs (TNLs) and 51 CC-NB-LRRs (CNLs) are present (Meyers *et al.,* 2002; Meyers *et al.,* 2003) with 24 genes associated with specific plant defense responses known to date. The expanding knowledge of natural variation within *A. thaliana*, like the accessions sequenced by Gan *et al.* (2011) gave the opportunity to investigate the conservation and expansion of the *R* gene repertoires in this model species. In a subset of the *R* genes described by Meyers *et al.* (2002; 2003) a wide distribution of *R* gene-repertoires was identified, discovering a core set of 29 conserved TNL and CNL encoding genes. Since none of the known genes in the conserved core set directly interact with a pathogen protein, the core set might be part of downstream signaling rather than directly recognizing a pathogen. The 51 *R* genes that were not conserved were either absent or encoding proteins lacking one or two domains. Evolving *R* genes are important in creating new *R* gene pools for resistance to keep up with fast evolving pathogen virulence proteins also referred to as effectors (Yang *et al.,* 2013c).

3.1.2 Five *R* genes are conserved in five *Brassicaceae* species (**I**)

The availability of sequence information from a growing number of species related to *A. thaliana*, including *A. lyrata*, *C. rubella, B. rapa* and *E. salsugineum* gave us the opportunity to study *R* gene conservation and expansion within one family and two lineages. In these five species we found a

great variation of the predicted *R* genes, ranging from 67 in *E. salsugineum* to 135 in *B. rapa*. Between species a greater conservation of the TNL subclass in comparison to the CNL subclass was observed and a core set of three CNLs and three TNLs were identified with orthologs in all five species. *HOPZ-ACTIVATED RESISTANCE 1* (*ZAR1*) and possibly *RESISTANT TO P. SYRINGAE 2* (*RPS2*), both important in defense against *P. syringae* were the only genes with known function in this core set. ZAR1 interacts with HOPZ-ETI-DEFICIENT1 (ZED1), the latter a non-functional kinase interacting with the *P. syringae* effector HopZ1a (Lewis *et al.,* 2013). *ZAR1* homologs were also identified in *Vitis vinifera* and *Solanum* species that belong to other families mainly within the Rosid clade. The conservation of the *R* genes with no known function in the core set could hint to a possible important function either in plant defense or other pathways. The expected higher numbers of conservation within lineages was not observed, emphasizing the different selection forces shaping the diversity of *R* gene family repertoires in each individual species rather than at the lineage level.

3.1.3 The *RLM1* locus is partly conserved

Several genes have a role in *L. maculans* resistance in *A. thaliana.* The *RLM1* locus consists of seven *R* genes (*RLM1A* to *RLM1G*) (Staal *et al.,* 2006). The major *RLM1A* gene in the resistance response was conserved in 20 different *A. thaliana* accessions (**I**), including An-1 (**II**). Additionally, a potential homolog was identified in *C. rubella* as well as in the *B. napus* cv. Surpass 400 (**I**). In *B. napus* cv. Surpass 400 used to control blackleg disease, the resistance towards *L. maculans* was overcome in 2004 (Larkan, *et al.,* 2013) Whether the *RLM1* homolog had any influence on resistance in *B. napus* cv. Surpass 400is unknown. Within the different accessions and in *A. lyrata*, *C. rubella*, *B. rapa* and *E. salsugineum* diversification in the *RLM1B* to *RLM1D* was observed. The *RLM1E* to *RLM1G* genes were well conserved between the 19 accessions and orthologs of *RLM1F* and *RLM1G* were identified in *A. lyrata* and *B. rapa*. *C. rubella* only contained *RLM1G*. In the case of *RLM1F*, it might be part of defense responses specifically in flowers where it is primarily expressed in *A. thaliana* (Tan *et al.,* 2007).

3.1.4 RLM3 interacts with the BRX protein

Plant genomes contain multiple genes encoding for R proteins lacking one or more domains or have included additional domains. During the evolution several domain fusions have occurred. For example fusions between a α/β hydrolase and a NL domain in the liverwort *Marchantia polymorpha* a species that diverged from land plants 480 mya and a fusion between a protein kinase with a NL in *P. patens* diverging from land plants 450 mya (Xue *et al.,* 2012). In *A. thaliana* the *RESISTANT TO RALSTONIA SOLANACEARUM 1* or *RRS1* gene encodes a TNL and a C-terminal WRKY domain. The WRKY domain is able to directly interact with effector proteins (Sarris *et al.,* 2015). In the case of RLM3, instead of the LRR domains flanking the TN it has three BREVIS

RADIX (BRX) domains mainly known for its presence in a root elongation protein (Mouchel *et al.*, 2004). RLM3 is an important player in defense towards a variety of pathogens, including *B. cinerea* and *Alternaria* species (Staal *et al.*, 2008). It is therefore surprising that *RLM3* is completely deleted in six out of 19 analyzed *A. thaliana* genomes (**I**). The deletion is likely the result of a single event, revealing only minor differences in the *rlm3* accessions at this locus. Homologs also exist in *A. lyrata* and *C. sativa* (**I+II**), the latter containing three copies as a result of whole genome triplication (Kagale *et al.*, 2014a). One of the homologs in *C. sativa* is likely a pseudogene, due to the presence of several stop codons and the presence of a transposase domain. The history of the evolution of *RLM3* remains unclear since no homologous gene or gene fragments were identified with high similarity with the NB and BRX domain of RLM3 (**I**). However with the growing sequence and annotation information available this could very well be solved in the near future.

While there is no involvement of RLM3 in root growth, a resistant phenotype is achieved when using only the C-terminal region of the RLM3 protein containing solely the three BRX domains (**II**). Additionally, in the plasma membrane RLM3 interacts with the BRX protein, the latter consisting of two BRX domains. The BRX protein functions as a transcription factor and probably controls numerous of genes (Scacchi *et al.*, 2009). Nuclear interaction between RLM3 and BRX still needs to be resolved. The possibility of the RLM3-BRX interaction in the nucleus provides an interesting mechanism for RLM3 to regulate the expression of genes in response to a pathogen signal.

3.2 *LMS1* and its role in defense (III)

3.2.1 LMS1 and necrotrophic filamentous pathogens

Identifying components important in defense responses in plants is an ongoing process and more and more information is gathered through the study of different pathogen systems. The usage of mutants displaying an altered phenotype when challenged by different pathogens has aided greatly in identifying genes needed for defense response. In the EMS *lms1* mutant disease symptoms were observed when challenged with *L. maculans* (Bohman *et al.*, 2004) and to the additional pathogens; *A. brassicicola*, *B. cinerea*, *Bipolaris sorokiniana* and *P. infestans*. Bacteria and biotrophic pathogens on the other hand could not infect *lms1*, indicating that the mutation affected only the response towards filamentous pathogens with a (partly) necrotrophic lifestyle. The *LMS1* gene responsible for the diseased phenotype was subsequently identified to encode a protein with similarity to lipid phosphate phosphatases, but no phosphate phosphatase activity was observed for LMS1.

3.2.2 LMS1 interacts with a SA-binding protein

Several pathogens have evolved clever ways to directly inhibit or alter host responses in favor of the pathogen including manipulations of phytohormones.

In the *lms1* mutant the levels of SA were elevated 5.6-fold after pathogen challenge compared to the wild type. Induction of several SA associated genes; *SALICYLIC ACID INDUCTION DEFECIENT 2* (*SID2*), *PHYTOALEXIN DEFICIENT 4* (*PAD4*) and *PR-1* were subsequently found. Crosses between *lms1* and *sid2* or *pad4* revealed a resistant response, further highlighting their importance in the *lms1* susceptible phenotype. Increase in SA levels has been associated with successful colonization of necrotrophs. The increase in SA levels in *lms1* would subsequently lead to the observed susceptible phenotype when challenged with pathogens with a necrotrophic lifestyle. Whether any of the pathogens directly influence the SA levels in the *lms1* mutant remain unknown. A component important in the defect in SA response in *lms1* was identified as a carbonic anhydrase or SA binding protein 3 (SABP3) described to play important roles in SA perception and signaling (Du and Klessig 1997). SABP3 interacts with LMS1 at the plasma membrane, where LMS1 is primarily located, while the truncated LMS1 found in *lms1* could not interact. From our data it was additionally shown that SABP3 is relocated upon pathogen attack from its primary chloroplast location to the plasma membrane.

3.2.3 Insights into antagonistic effects between SA and JA

Increase in JA levels has been associated with defense responses towards necrotrophic pathogens and the repression of JA through SA has been observed numerous times (Spoel *et al*., 2003; 2007; Flors *et al*., 2008). Unexpectedly, the levels of the active component jasmonoyl-L-isoleucine (JA-Ile) was found constitutively high in the *lms1* mutant compared to the wild type, suggesting that a resistant response instead of a diseased phenotype should have been observed when challenged with the necrotrophic pathogens. The introduction of the *coi1* mutant defective in JA-signaling in the *lms1* mutant did not affect the susceptible response indicating that JA has no role in the observed susceptibility in the *lms1* mutant. From our data it can be suggested that LMS1 has an inhibitory effect on JA levels, either its biosynthesis or its break down. This inhibitory affect is repressed after a successful interaction with SABP3, resulting in increase in JA responses and decrease in SA-responses.

3.3 A prolyl oligopeptidase is involved in defense against *L. maculans* (IV)

Pathogens like *L. maculans* with a necrotrophic lifestyle use phytotoxins, cell wall degrading enzymes and other enzymes to cause cell death in the plant (Howlett *et al*., 2001; Laluk and Mengiste 2010; Pedras and Yu 2009). Furthermore, low-molecular weight phytotoxic metabolites, which can be

either host-specific or have similar effects on a variety of plant species are also produced as well as necrosis-inducing phytotoxins (van Kan, 2006). To overcome the effects of these molecules a plant has developed several lines of defense responses. One of the candidate genes selected to study in its response against the *L. maculans* fungus and its toxins is the gene encoding a putative *PROLYL OLIGOPEPTIDASE* (*POP*) gene predicted to be involved specifically in the cleavage of peptide substrates smaller than 30 amino acids in length (Polgár, 1994). This particular gene could play an important function in hydrolyzing these smaller molecules as a defense response. The *POP* gene, which we named *POP1* was found to be mutated at the R36H position in the *L. maculans* susceptible *lms5* mutant. Two individual T-DNA insertion mutants; *pop1-1* and *pop1-2* with down-regulation and almost complete absence of *POP1* expression have a similar weak disease response in comparison to the *lms5* mutant when challenged with *L. maculans*. Higher fungal quantification revealed successful colonization. The results from these two mutants demonstrate the involvement of the *POP1* gene in defense against *L. maculans.* Complementation of the *lms5* mutant with the Col-0 *POP1* gene was attempted but no transformants were generated, even after repeating the dipping several times and screening thousands of seeds. Since the *lms5* mutant is in the L*er*-0 background, an accession with low transformation efficiency (Desfeux *et al.*, 2000), this was not completely unexpected. In a pull down assay with POP1-HA overexpressing plants several weak bands were identified, one of these bands was glycolate oxidase 1 (GOX1) interacting with the POP1 protein. GOX proteins are known to take part of the production of hydrogen peroxide during both gene-for-gene and non-host resistance responses (Rojas *et al*., 2012). So far, direct interaction with a fungal protein effector with POP1 was not observed and further studies are ongoing.

4 Conclusions

- The *R* gene repertoire between several *A. thaliana* accessions, *A. lyrata*, *C. rubella*, *B. rapa* and *E. salsugineum* has a large variation.
- The *RLM1* locus with seven genes has undergone conservation and diversification within 19 accessions and five species.
- The *RLM3* gene is conserved within *Arabidopsis* and *Camelina* but absent in seven *A. thaliana* accessions.
- The C-terminal region of RLM3 containing three BRX domains is involved in plant defense response independent from the TIR and NB domain.
- LMS1 is important in defense against filamentous plant pathogens with a necrotrophic stage in their lifecycles.
- LMS1 interacts with SABP3 at the plasma membrane upon pathogen contact and is involved in the regulation of SA and JA defense signaling.
- POP1 interacts with GOX1 possibly to protect against *L. maculans* infection.

5 Future perspectives

A list of analyses to improve the manuscripts in the thesis and to gain more knowledge on *RLM3*, *LMS1* and *POP1* in defense responses.

- Investigating difference between the S-nitrosylation status (active vs. inactive protein) of SABP3 and the interaction with LMS1
- Test for a role of JA levels using a JA biosynthesis mutant (*dde2* mutant) in LMS1 defense
- Determining the interaction between RLM3 and BRX-like proteins
- Crossing *rlm3* mutants and *brx* and *brx-like* mutants and investigating defense response.
- Immunoprecipitation experiments with RLM3 to identify novel interacting proteins.
- Test the $35S_{pro}$:$RLM3_{1333}$-HA overexpressing lines to other pathogens, e.g. *B. cinerea, L. maculans*.
- Test auxin and brassinosteroid involvement in the RLM3-BRX interaction.
- Silencing the *POP1* gene to link the mutation in *lms1* with the POP1 gene.
- Determining interaction between the POP proteins and fungal proteins from *L. maculans*, extending with the available sequence information from *L. maculans*
- BIFC analysis between POP1 and GOX1 to confirm interaction
- Complementation of *pop1-1* and *pop1-2* mutants with POP1::POP1 to determine resistance towards *L. maculans*
- Measure GOX activity in *pop1-1gox1-1* mutant.

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