Defense Gene Responses Toward Necrotrophic Fungi in *Arabidopsis thaliana*

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Cover: Drawing of an *Arabidopsis thaliana* seedling.
(photo: H. Peele)
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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 phosphatidic 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
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This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:


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).

Papers I is reproduced with the permission of the publishers.
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 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.,

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

* previously *Thellungiella halophila*; ** previously *Thellungiella/Eutrema parvula/parvulum* - (unknown).
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 (Ler) 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 where >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 AMRI 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).

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).

![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.](image-url)
Most plant R genes are found to cluster together in plant genomes and are divided into 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). Identification of R gene clusters in plant genomes can be useful 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 400 is 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 $\alpha/\beta$ 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 DEFCIENT 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 Ler-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 35Spro:RLM31333-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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