The interplay between a *Phytophthora* RXLR effector and an Arabidopsis lectin receptor kinase

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‘*Felix qui potuit rerum cognoscere causas*’
(Vergilius, Georgica II, 29 B.C.)

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General introduction

1.1 Introduction

The impact of plant diseases

Ever since the first agricultural activities – which emerged approximately 10.000 years ago from the Fertile Crescent in the Middle East (Heun *et al*., 1997; Zohary and Hopf, 2000) – plant diseases have been a major constraint to crop production. Even in present times of post-Green Revolution agriculture, plant diseases cause still large yield losses. In the period 2000-2003 the actual losses worldwide due to damage by plant pathogens in the six major crops (i.e. wheat, rice, maize, potato, soybean, cotton) amounted between 8 and 21% (Oerke, 2006). The estimated loss potential due to pathogens is even more staggering with the highest loss rate for potato that can reach up to 30%. Moreover, climate change and instability is increasing the severity and spread of plant diseases (Evans *et al*., 2008; FAO, 2008). Together with the need for more food due to the rapid expansion of the world population – which will total approximately 8.3 billion people in 2030 – it seems like an almost insolvable problem (FAO, 2003). Disease control is one of the vital tools to combat economic losses to agriculture and the long-term global food security. In the past, farmers were able to prevent crop losses by making use of pesticides and resistant crop plants. Nowadays, the use of many agrochemicals is restricted and the tendency is to use as little as possible to relieve the environment. Unfortunately, many plant pathogens have become tolerant to pesticides and host-plant resistance. In this respect, new sustainable and environmentally safe crop protection methods should be developed. By obtaining more insight in the molecular mechanisms underlying plant–microbe interactions, and in the biology of pathogens and their hosts, it should be possible to generate crop plants with more durable types of resistance and to design novel methods for disease control.

Infection and defense

Everywhere plants grow they can be affected by pathogenic organisms. Some are pathogenic on multiple plant species, while others are restricted to only particular species or derived varieties. Plant pathogens commonly interfere with the normal physiological functions of a plant; they can cause malfunctioning of water uptake, affect photosynthesis or prevent flowering and fruit set. The main “goal” for every plant pathogen is to successfully infect its host and to complete its reproduction cycle. This can only be achieved when the pathogen is able to convert plant biomass into energy. The strategy to reach and hit the energy source is, however, not the same for every pathogen. Plant pathogens can be divided into groups based on their level of parasitic adaptation. So-called facultative pathogens have a

largely saprophytic lifestyle and are capable to colonize plant tissue under certain circumstances only. Many of these pathogens do not have specific strategies to invade plants and often infect damaged tissue or enter plants after primary infections by other pathogens. Necrotrophic plant pathogens rely on host cell killing enzymes or toxins and subsequently live saprophytic (Box 1; **Chapter 1.2**). Others – including the powdery and downy mildews, the rust fungi and all plant viruses – are biothrophic plant pathogens, and are partially or sometimes entirely dependent on living plant tissue (Box 1; **Chapter 1.2**). In contrast to the necrotrophs – that thrive on plant cells after having killed them – (hemi)biotrophic pathogens depend on more sophisticated infection strategies. The majority of these pathogens penetrate host cells directly by breaching the cell wall. Subsequently, specialized infection structures or haustoria are formed to establish an intimate host-pathogen relationship and to increase the interface between the pathogen and its host. This increase in area of contact not only enables efficient nutrient flow between host and pathogen, but is also essential for sustaining its biotrophic association with its host.

Box 1: Lifestyles of plant pathogens

Plant pathogens can be divided into groups based on the different strategies they employ to colonize plants (Agrios, 2004). Obligate biotrophs grow and reproduce in living plant tissue and obtain energy by uptake of plant nutrients. They are entirely dependent on living cells and can therefore only survive by establishing a nondestructive relationship with their host. Among the obligate biotrophs are various fungi (e.g. the ascomycetes *Blumeria graminis* and *Sphaerotheca pannosa*, and the basidiomycete *Ustilago maydis*) and several oomycetes, such as the downy mildews (e.g. *Hyaloperonospora arabidopsidis* and *Bremia lactucae*) and white rusts (e.g. *Albugo candida*). Necrotrophic pathogens (e.g. *Botrytis cinerea* and *Leptosphaeria maculans*) feed on dead plant tissue. Before colonizing plant tissue, they kill cells by secreting toxins or cell wall degrading enzymes. A third strategy is an intermediate lifestyle called hemibiotrophy. The first stage of hemibiotrophy is based on an intimate relationship of the pathogen with its host. Later during the infection process, hemibiotrophic pathogens switch to a destructive necrotrophic lifestyle (e.g. *Colletotrichum lindemuthianum* and *Phytophthora infestans*).

MAMP-triggered immunity

Unlike animals, plants lack an adaptive immune system; however, they are not completely defenseless and acquired other sophisticated manners to withstand pathogen attack. The first line of induced plant defense is a resistance response which can be triggered by host-derived fragments – for example plant cell wall components such as cellulose, cutin and oligogalacturonides – that are released upon pathogen infection and tissue damage, or by microbe-associated molecular

patterns (MAMPs). MAMPs represent conserved microbial molecules that are common and essential for microbe viability, and are found in both pathogenic and non-pathogenic microorganisms. The best known examples of MAMPs include lipopolysaccharide, flagellin, and the elongation factor EF-Tu from bacteria, chitin and xylanase from fungi, as well as the transglutaminase-epitope PEP13 and the cell wall glucan WGE of oomycetes (reviewed by Schwessinger and Zipfel, 2008). MAMP detection is mediated by plasma membrane-associated pattern recognition receptors (PRRs), which upon elicitation induce primary defense responses, including enhanced deposition of callose and lignin and the accumulation of defensive enzymes. Unlike the broad range of MAMPs only few cognate PRRs have been identified thus far. Well-studied PRRs are FLS2 and ERF that recognize the MAMP epitopes of flagellin (flg22) and EF-Tu (elf18), respectively, and thus activate MAMP-triggered immunity (MTI) (Gomez-Gomez and Boller, 2002; Zipfel *et al*., 2006). Both FLS2 and EFR are receptor-like kinases (RLKs) and it is anticipated that many other plant RLKs perceive yet unknown MAMPs.

Effector proteins modulate plant cellular pathways

In order to colonize a plant successfully, pathogens secrete proteins that interfere with plant cellular processes and defense mechanisms including the suppression of MTI (Figure 1). Because of their presumed role in virulence these pathogen-secreted proteins are termed effectors. Our knowledge of how effectors suppress MTI is largely based on studies with type III secretion-dependent effectors of the bacterial plant pathogen *Pseudomonas syringae*. For example, AvrRpm1 and AvrRpt2 are able to suppress flg22-mediated responses via interaction with the plant protein RIN4. Inactivation of RIN4 due to phosphorylation by AvrRpm1 or proteolytic cleavage by AvrRpt2 increases virulence (Kim *et al*., 2005). The *P*. *syringae* effector AvrPto inhibits kinase activity of several plant kinases, including FLS2, EFR, and BAK1. The latter functions as a key signaling partner of multiple developmental and defense associated RLKs (Shan *et al*., 2008; Xiang *et al*., 2008). Another *P*. *syringae* effector, AvrPtoB, degrades host protein kinases, such as the Fen kinase of tomato which plays a crucial role in resistance against *Pseudomonas syringae* pv. *tomato* by means of its E3 ubiquitin ligase activity (Rosebrock *et al*., 2008). In a similar manner, AvrPtoB targets the kinase domain of the Arabidopsis LysM receptor kinase CERK1 for degradation (Gimenez-Ibanez *et al*., 2009). CERK1 is essential in the perception of the fungal MAMP chitin and subsequent fungal resistance (Miya *et al*., 2007; Wan *et al*., 2008), although its functionality in bacterial disease resistance is not clear yet. Avr4, an effector of the tomato leaf mould fungus *Cladosporium fulvum*, binds chitin to gain protection against plant chitinases (Van den Burg, 2006). Transgenic plants expressing *Avr4*

show enhanced disease susceptibility against chitinious fungi, and *Avr4*-deficient *C*. *fulvum* lines are compromised in virulence (Van Esse *et al*., 2007). Similarly, expression of the effectors ATR1 and ATR13 of the downy mildew *Hyaloperonospora arabidopsidis* (an oomycete pathogen previously known as *H*. *parasitica*) in Arabidopsis enhances susceptibility towards *P*. *syringae* (Sohn *et al*., 2008). Effectors are also found to suppress elicitins, a family of small cysteine-rich proteins secreted by oomycete pathogens that trigger defense reactions in plants. For example, the elicitin INF1 of *P*. *infestans* induces cell death in the non-host species *Nicotiana benthamiana*, and is suppressed by Avr3a, an effector which in itself is recognized by the resistance protein R3a from *Solanum demissum* (Bos *et al*., 2008; Armstrong *et al*., 2003).

Another way to gain control over the plant is to hack the host transcriptional regulation of its defense pathways, as was demonstrated for the type III effector XopD, a SUMO protease of the bacterial tomato pathogen *Xanthomonas campestris*, which suppresses disease symptom development during late stages of infection (Kim *et al*., 2008).

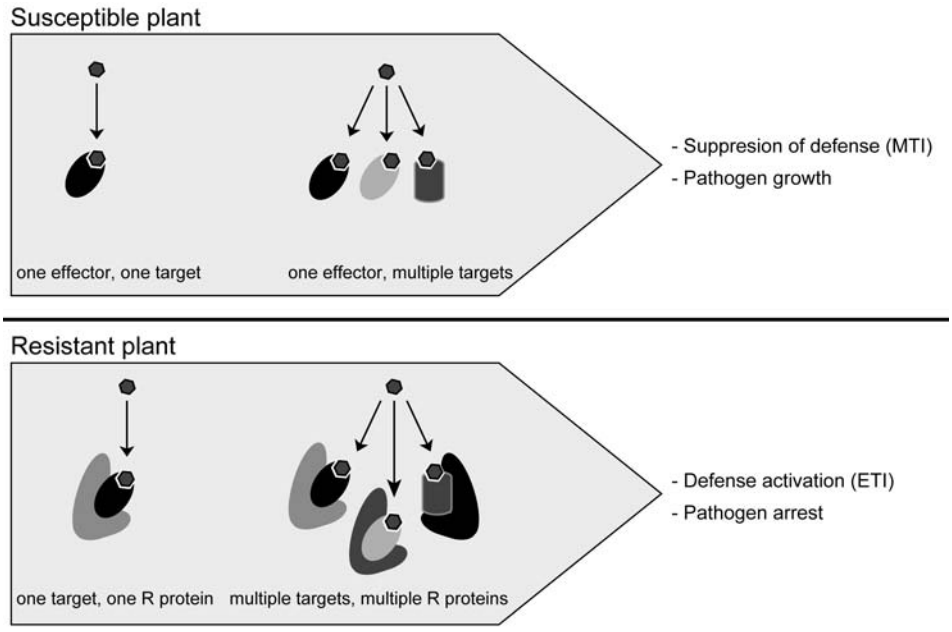

Figure 1. Pathogen effectors interact with one or more host proteins (virulence targets) in order to suppress defense responses (e.g. MAMP-triggered immunity (MTI)) thereby facilitating infection. Susceptible plants lack resistance (R) proteins that sense effector-mediated modification of virulence targets. In contrast, resistant plants are able to sense these alterations and mediate effector-triggered immunity (ETI) thereby arresting growth of the pathogen.

Effector-triggered immunity

Once MTI is overcome or avoided, the pathogen may face a second line of defense. During evolution plants acquired cognate resistance (R) proteins that can prevent effector-mediated suppression of MTI by recognizing effectors (i.e. directly or indirectly), hence named avirulence (Avr) factors. This recognition triggers a R protein-mediated cascade of events leading to effector-triggered immunity (ETI) and is often associated with a hypersensitive response (HR) that blocks further growth of the pathogen (Figure 1). A considerable number of R proteins belongs to the nucleotide-binding site leucine-rich repeat (NBS-LRR) superfamily. NBS-LRRs are ubiquitous in plants. In Arabidopsis 150, and in rice and poplar over 400 *NBS-LRR* genes have been reported, and it is likely that in larger plant genomes these numbers are even higher (Meyers *et al*., 2003). NBS-LRRs can be divided into two distinct subfamilies, based on the prescene of Toll/Interleukin-1 receptor (TIR) or coiled-coil (CC) motifs in the amino-terminal end of the NBS domain. Hitherto, several *NBS-LRR* genes conferring resistance to viruses, fungi and oomycetes have been cloned from a variety of plant species, including Arabidopsis and several crop plants (reviewed by Tameling and Takken, 2008). NBS-LRR-mediated effector recognition and the subsequent activation of ETI can also be circumvented, as was shown for the *Fusarium oxysporum* effector Avr1. This effector triggers – on the one hand – the tomato R protein I-1, but on the other hand suppresses the activity of I-2 and I-3, which recognize the cognate avirulence factors Avr2 and Avr3, respectively (Houterman *et al*., 2008).

Although the majority of the R proteins described to date are intracellular NBS-LRR proteins (DeYoung and Innes, 2006), ETI can also be mediated by membrane-bound receptor-like kinases (RLKs) and receptor-like proteins (RLPs). RLKs functioning in disease resistance are, for example, the rice lectin receptor kinase Pi-d2 conferring resistance to the blast fungus *Magnaporthe grisea* (Chen *et al*., 2006), and the Arabidopsis ERECTA protein. The latter not only has a role in ETI to *Ralstonia solanacearum* and *Plectosphaerella cucumerina*, but also functions as a signaling component in plant organ development (Godiard *et al*., 2003; Llorente *et al*., 2005). *RLPs* implicated in disease resistance include *HcrVf2* from apple providing resistance to scab (Belfanti *et al*., 2004) and the tomato *Cf* and *Ve* genes that confer resistance against *C*. *fulvum* and *Verticillium* species, respectively (Kruijt *et al*. 2005; Fradin and Thomma, 2006). Various other RLPs with a comparable function are found in other plants. It is remarkable though, that the majority of Arabidopsis RLP knock-out lines did not show a notable defect in disease resistance, suggesting a high degree of functional redundancy (Wang *et al*., 2008).

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1.2 Scope of this thesis

Plants display a range of alterations upon exposure to stress. Notorious stress-inducers are the fungus-like oomycetes which are causal to a variety of devastating crop diseases. Oomycete diseases are difficult to control, on the one hand because many of the compounds successful as fungicides do not affect oomycetes, and on the other hand because oomycetes have the ability to quickly defeat genetic resistance. Among them are around hundred *Phytophthora* species, over hundred *Pythium* species, and a variety of obligate biotrophs, including downy mildews and white rusts. The review in **Chapter 1.3** gives an update of the latest discoveries and insights in oomycete biology and pathology, and summarizes the available genomic resources. It also describes the impact of the various strategies that have been used to accelerate oomycete gene discovery and provides a perspective on oomycete genomics. The experimental research described in **Sections 2**, **3** and **4** of **this thesis** deals with two oomycete species, *Phytophthora infestans* and *Phytophthora brassicae*, and the interactions with their host plants. The first one, *P. infestans*, causes late blight on potato – the fourth most important crop in the world – and has considerable economic impact. Historically, it is known as the cause of the Irish potato famine in the mid-nineteenth century (Box 2). The second one, *P. brassicae* is of minor economic importance. It is pathogenic on Arabidopsis, and as such the *P. brassicae*–Arabidopsis interaction features as a model pathosystem (Box 3). In **Section 5** of **this thesis** the obtained experimental data are discussed in a broader context.

The *Phytophthora infestans*–potato interaction

P. infestans is well-known as one of the most destructive pathogens of potato (*Solanum tuberosum*). In addition, it forms a significant threat to tomato production. Recently, tomato growers along the east coast of the USA encountered a very severe late blight epidemic (http://civileats.com/2009/07/28/battling-late-blight-in-the-north-east/). *P. infestans* has also been reported to infect other cultivated and wild solanaceous species, like pear melon (*S. muricatum*), cape gooseberry (*Physalis peruviana*) and the climbing nightshade *Solanum dulcamara* (Adler *et al*., 2002; Vargas *et al*., 2009; Cooke *et al*., 2002). Infection by *P. infestans* starts via wind-blown sporangia that land on plant tissue and release biflagellate zoospores. Upon encystment, a germ tube is produced of which the tip develops into an appressorium that pierces the cuticle and penetrates an epidermal cell. Hereafter, the infection extends to neighboring mesophyll cells via hyphal growth through intercellular spaces. The infected foliage becomes water-soaked and turns yellow. After several days, the infected tissue necrotizes and the onset of sporulation

starts. Sporangiophores will emerge through stomata that produce numerous sporangia, which are capable to initiate new infections. *P. infestans* can also reproduce sexually. This requires two compatible mating types (A1 and A2) and is dependent on the interplay between release and perception of mating hormones (Harutyunyan *et al*., 2008). After fertilization a thick-walled sexual spore is formed, the oospore, which can survive for many years in the soil. Upon stimulation, dormant oospores will germinate and form a sporangium that can directly infect plant tissue. Sexual reproduction generates genetic variation and can thus greatly influence disease epidemics and durability of genetic resistance.

Combating late blight disease is a difficult enterprise, due to susceptibility of the majority of potato cultivars. Many of these cultivars are the end product of selective potato resistance breeding with race-specific late blight resistance (*R*) genes originating from the Mexican wild species *Solanum demissum* and *S. stoloniferum* (Müller and Black, 1952; Schick *et al*., 1958; Black *et al*., 1953; Malcolmson and Black 1966). A differential set consisting of eleven dominant *S. demissum R* genes (*R1*-*R11*) has been used for decades by potato breeders to introgress late blight resistance into new potato cultivars (Mastenbroek, 1953; Solomon-Blackburn, 2007; Trognitz and Trognitz, 2007). Unfortunately, attempts to achieve durable resistance showed to be unsuccessful due to the rapid adaptation of *P. infestans* to introduced *R* genes (Wastie, 1991). Already in the early 1950s it was reported that introgressed resistance was overcome for four *S. demissum R*-genes (i.e. *R1*, *R2*, *R3* and *R4*) (Howatt and Hodgson, 1954). Nowadays, late blight disease management largely relies on protective and curative fungicides, which have an adverse effect on the environment and are costly. As a consequence, development of new high level and broad spectrum resistant potato cultivars has received renewed attention in many modern potato breeding programmes. Hence, it is not surprising that novel *R*-genes from other *Solanum* species are being mapped and cloned (Śliwka *et al*., 2006; Van der Vossen *et al*., 2005; Park *et al*., 2005; Lokossou *et al*., 2007). One of the late blight resistance genes that received a lot of attention – and was originally isolated from *S. bulbocastanum* by two independent groups in 2003 – is *Rpi-blb1* (alternatively named *RB*), which appeared to be able to block growth of all tested *P. infestans* isolates that were virulent on the differential set carrying R1 to R11 (Van der Vossen *et al*., 2003; Song *et al*., 2003). Recently, Vleeshouwers and co-workers (2008) identified via an effector genomics approach the matching *P. infestans* avirulence gene *Avr-blb1*, which triggers *Rpi-blb1*-mediated resistance. Surprisingly, this avirulence gene (*Avr*) turned out to be *ipiO*, one of the first identified *in planta* induced *P. infestans* genes (Pieterse *et al*., 1993). The *ipiO* gene was shown to be highly expressed during growth of the pathogen in association with its host, i.e. in leaf tissue, whereas its expression during *in vitro* growth was below detection level (Van West *et al*., 1998). It took another 15 years

to discover that IPI-O shares a common motif with proteins that were identified around 2003 as oomycete avirulence factors. The *Avr* genes that were isolated by labour intensive map-based cloning procedures appeared to belong to a very large gene family. These genes encode RXLR-dEER effectors, which were named after their common conserved motif that functions as an effector translocation signal for delivery into host plant cells (Jiang *et al*., 2008; Whisson *et al*., 2007; **Chapter 1.3**). There is now ample evidence that the RXLR effectome comprises most, if not all, effectors that interact in a gene-for-gene mannner with *R* genes.

Box 2: The Irish Potato Famine (1845-1849)

Already 200 years ago, farmers in Ireland were growing a range of potato varieties together with other crops like beans, oats and rye. At that time Ireland was part of the United Kingdom and a major exporter of grains, meat and dairy products. Poor peasants sold these products to England to pay the high land rent to their wealthy British lords. In the late 1830s the formerly high diversity of grown potato varieties was drastically decreased; one variety of potato, the Lumper, with a higher yield per acre became the variety on which they relied. It is estimated that 30% of the Irish population was totally dependent on potatoes for food, especially in western counties. The dependence on potato became visible when in September 1845 potato plants suddenly became sick; leaves turned black, shriveled up and rotted away. It was quite a mystery. Some thought that the potato disease was a result of fog that crossed the land, others proposed that the plants became sick through the static electricity in the air caused by the new invention of locomotive trains, or that it was caused by the devil. The plant disease swarmed through the country. The yield of potato tubers decreased dramatically. Although many tubers rotted away in the ground, still some could be harvested. When it became clear that after harvesting also tubers were rotting in the storage basements, the impact became enormous. In spring, farmers planted the remaining tubers and they saw that the plants once more became sick. Again their harvest failed. Four successive years of potato blight led to the Irish famine. The main food source for the Irish was vanished. Nearly 800,000 people died due to starvation, malnutrition and related diseases such as scurvy, cholera and dysentery. Many had to abandon their houses because they could not pay their rent. One million Irish left their homeland to start a new living in other countries, mainly to the USA and Canada (Donnelly, 2001). It was a plant pathogen that caused one of the largest human migrations in recent history.

In **Section 2** of **this thesis** we focus on the *P*. *infestans* RXLR-dEER effector IPI-O and its role in both virulence and avirulence on potato. We monitored the genetic variation and distribution of *ipiO* in a large set of isolates of *P*. *infestans* and related species, and classified the allelic variants based on their sequence similarity (**Chapter 2.1**). To investigate the correlation between *ipiO* variation and pathogen virulence or avirulence, we determined the compatibility of the isolates on *Rpi-blb1*-containing plants. Gene-for-gene specificity of each class was determined by co-agroinfiltration assays with *Rpi-blb1* in *Nicotiana benthamiana*. In order to determine which part of the effector is responsible for recognition by Rpi-blb1,

IPI-O deletion mutants were constructed and tested in a similar way with co-agroinfiltration assays with *Rpi-blb1*. To investigate the subcellular localization of IPI-O during infection, *P*. *infestans* was transformed with a chimeric construct of *ipiO* fused to a sequence encoding a fluorescent protein (**Chapter 2.2**). *In vivo* localization of *ipiO* was examined by fluorescence microscopy in various life cycle stages of *P*. *infestans in vitro* and during infection of potato.

The *P*. *brassicae*–Arabidopsis interaction

Arabidopsis is a small plant related to mustard and cabbage that has no direct agronomic importance. Nevertheless, it functions as an ideal model plant for basic research aimed at increasing our understanding of biological principles. In the last decade, considerable progress has been made to unravel the complexity of host-pathogen interactions by exploiting Arabidopsis as a model host plant (Box 3). Several plant pathogens have been reported to infect Arabidopsis, either naturally or under laboratory conditions, including some oomycete species. Most *Phytophthora* species trigger specific defense cascades on Arabidopsis, leading to a non-host resistance response. An exception is *P*. *brassicae*, which is capable to infect Arabidopsis (Roetschi *et al*., 2001). So far, around 40 Arabidopsis accessions have been tested with *P*. *brassicae* showing various accession–isolate combinations with distinct incompatible and compatible interactions (Mauch *et al*., 2009). Studies by Roetschi *et al*. (2001) showed that various well-characterized Arabidopsis mutants compromised in salicylic acid (SA), jasmonic acid (JA) or ethylene-dependent defense remained resistant to *P*. *brassicae*. In contrast, the camalexin-deficient mutant *pad2-1* was found to be hypersusceptible to *P*. *brassicae*. However, Parisy *et al*. (2007) demonstrated that this phenotype was not primarily caused by its camalexin deficiency. They showed that the susceptibility of *pad2-1* to *P*. *brassicae* was due to a mutation in a gene encoding a glutamylcysteine synthetase, a key enzyme in the glutathione biosynthesis.

Box 3: Arabidopsis a model plant species and a tool to study plant pathogen effectors

Arabidopsis has multiple advantages; it is well established as a genetic model – including a fully sequenced genome, it has a very rapid life cycle and is highly amenable to molecular analysis. Other advantages of using Arabidopsis as a tool is the availability of T-DNA insertion mutant libraries, whole genome microarrays (Van Baarlen *et al*., 2007) and an extensive collection of accessions representing natural populations (Koornneef *et al*., 2004). Arabidopsis has also been used as a tool to study pathogen effectors by exploiting it as a heterologous expression system. For example, expression of the *P*. *syringae* type III effector *AvrPto* in Arabidopsis showed its role in suppression of salicylic acid-independent cell wall-based defense responses (Hauck *et al*., 2003). A comparable strategy was deployed for the *C*. *fulvum* effectors Avr2 and Avr4, which enhance susceptibility towards various fungal pathogens of Arabidopsis. The finding that these plants were unaltered in their susceptibility to plant pathogenic bacteria and oomycetes supports the idea that some effectors may not contribute to the virulence of all pathogens (Van Esse *et al*., 2007, 2008). Arabidopsis can be exploited to functionally analyze plant pathogen effectors – in particular those of obligate biotrophic pathogens, which are not amendable to DNA transformation.

In **Section 3** of **this thesis** we exploit Arabidopsis as a tool to unravel mechanisms underlying pathogen virulence and plant defense. The rationale to study an Arabidopsis–*Phytophthora* interaction next to the potato–*P*. *infestans* interaction was the finding that Arabidopis has a high affinity site at its plasma membrane for the *P*. *infestans* IPI-O protein (Senchou *et al*., 2004). The reason to search for IPI-O binding sites was the fact that IPI-O contains the cell adhesion motif Arg-Gly-Asp (RGD) that in mammalians is known to bind integrins; membrane receptors that function in 'inside-out' and 'outside-in' signaling and regulate many cellular processes. A follow-up study by Gouget *et al*. (2006) revealed that the RGD site in IPI-O, which overlaps with the second R of the RXLR motif, indeed functions in cell adhesion. Moreover, via phage display an Arabidopsis membrane-spanning protein was identified that interacts with IPI-O. This receptor-like protein is a legume-like lectin receptor kinase (LecRK) that belongs to a family of 45 members. LecRKs are regarded as ideal candidates for monitoring cell wall integrity and are likely functional in adaptive responses to biotic and abiotic stress. In **Chapter 3.1** we present an inventory of the LecRK family in Arabidopsis. Gene structure, gene functionality and gene expression were summarized and by means of phylogenetic analysis the *LecRK* gene family was reclassified. In **Chapter 3.2**, we describe the functional analysis of Arabidopsis *LecRK-I.9*, the LecRK that interacts with IPI-O. We compared the phenotype of *LecRK-I.9* knock-out lines with that of *ipiO*-expressing Arabidopsis lines in their MAMP-responsiveness and resistance to *P*. *brassicae*. **Chapter 3.3** describes the generation and characterization of Arabidopsis and potato plants that constitutively express *LecRK-I.9*.

Section 4 of this thesis describes the development of a novel method for efficient isolation of *P. brassicae* zoospores via infections on an intermediate host plant, which enables large scale pathogenicity tests of *P. brassicae* on a variety of Arabidopsis accessions and mutants.

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1.3 Genome biology cracks enigmas of oomycete plant pathogens

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Abstract

All plant species, wild and cultivated alike, suffer from disease. By far the most devastating plant pathogens are fungi and their look-alikes, the oomycetes. Oomycetes cause severe problems, not only in agriculture but also in natural ecosystems. Best known is *Phytophthora infestans*, the species that caused the Irish potato famine in the mid-nineteenth century. Oomycetes and oomycete diseases have been the subject of numerous investigations but the tactics exploited by these successful plant pathogens are still largely an enigma. In recent years oomycete genomics uncovered a treasure trove of new information and that has enormously stimulated oomycete research. A major discovery was the highly diverse superfamily of secreted RXLR-dEER effectors that play important roles during plant infection. RXLR-dEER is a conserved motif in proteins encoded by oomycete avirulence genes that interact in a gene-for-gene manner with resistance genes. It has similarities to a motif in proteins secreted by malaria pathogens and helps targeting effectors into host cells. In this chapter some of the latest discoveries and insights in oomycete biology and pathology are presented. We describe several oomycete diseases and highlight species that feature as model organisms. We also summarize the genomic resources that are currently available and emphasize the impact of genomics on gene discovery in oomycetes. Finally, we refer to proteins secreted by oomycete pathogens and their potential roles in host–pathogen interactions.

Introduction

Worldwide, plants are attacked by a wide range of pathogenic oomycetes. *Phytophthora infestans*, the pathogen that was responsible for the Irish potato famine in the mid-nineteenth century, is one of many oomycete species that causes severe problems, not only in agriculture but also in natural environments and on indigenous trees and shrubs. In 1861, De Bary (1876) established for the first time that a filamentous organism that he named *P*. *infestans* was the cause of a plant disease. Ever since, *Phytophthora* and other oomycetes have been the subject of numerous investigations but in spite of intense study, the tools and tactics exploited by these successful plant pathogens are still enigmatic. In recent years genomics boosted oomycete research, and genomic data uncovered a treasure trove for plant pathologists, genome biologists and evolutionary biologists alike. A major breakthrough was the discovery of the RXLR-dEER class of effector proteins. These effectors are highly diverse and are thought to play important roles during plant infection. Here we present some of the latest discoveries and insights in oomycete biology and pathology. The first part gives a brief overview of oomycete pathogens of plants and highlights the species that currently feature as model organisms. We then describe the genomic resources available for oomycete research and how genomics has accelerated gene discovery. The last part is devoted to various types of secreted proteins and the potential roles these proteins play in host–pathogen interactions.

Biology and pathology of oomycetes

Branches in the tree of life

Oomycetes, also known as water molds, resemble fungi in many ways. Like fungi, oomycetes have a global distribution and prosper in quite diverse environments. They can live as (hemi-) biotrophic or necrotrophic pathogens in association with plants (Box 1 of **Chapter 1.1**), animals, or other microbes, but also as saprophytes feeding on decaying matter. Currently, at least 800 oomycete species are known, but depending on the definition of a species this number might actually reach 1500 (Dick, 2001). Nevertheless, the species richness seems low when compared to the number of fungal species known to date: 30,000 basidiomycete species have been described and ascomycetes reach a similar number (James *et al*., 2006; Kirk *et al*., 2001). It is, however, likely that there are many oomycetes out there yet to be discovered. In this respect the genus *Phytophthora* is illustrative. In the last ten years multiple new species have been described, expanding the genus to over 100 members (Blair *et al*., 2007; http://www.PhytophthoraDB.org, accessed April 2010).

Due to their shared morphology (filamentous, branched somatic structures that bear spores), oomycetes and fungi were traditionally classified in the same

kingdom, the Fungi (Erwin and Ribeiro, 1996). In the 'Five Kingdom' classification of Margulis and Schwartz (2000), the two groups were separated over two kingdoms: Protoctista and Fungi, the first being a highly diverse group including both unikonts and bikonts. In recent years, new insights based on molecular phylogeny and comparative genomics have again reshaped the tree of life. In 2005, Keeling *et al*. (2005) launched the five supergroups of eukaryotes. In this classification, the ascomycete and basidiomycete fungi are grouped together with animals and Amoebozoa (e.g. slime molds) in the supergroup Unikonts (Figure 1). Oomycetes with their biflagellated zoospores are typical bikonts. They fall within the supergroup Chromalveolates and the lineage Stramenopiles (Cavalier-Smith, 1999). Since the stramenopile lineage also contains photosynthetic organisms like diatoms and brown algae, it is thought that the common ancestor is a photosynthetic life-form that obtained its chloroplast by secondary endosymbiosis, presumably from red algae in the bikont supergroup Plantae (Cavalier-Smith, 2002) (Figure 1). The loss of plastids in the non-photosynthetic stramenopile taxa is supported by whole genome comparisons that revealed numerous photosynthesis related genes shared by *Phytophthora* and diatoms (Tyler *et al*., 2006). Most of these genes encode mitochondrium-targeted proteins with close homology to proteins targeted to chloroplasts in photosynthetic organisms.

Endosymbiosis can explain how genes with high similarity to bacterial genes end up in eukaryotes. In contrast, the processes underlying horizontal gene transfer (HGT) among eukaryotes are not so easy to trace. Nevertheless, as more and more genomes are sequenced evidence for intra-eukaryotic HGT is accumulating. In a thorough study, Richards *et al*. (2006) found strong evidence for HGT from ascomycete fungi to oomycetes. The genes involved presumably have functions related to an osmotrophic lifestyle and could explain convergent evolution of osmotrophy coupled to filamentous growth in two distinct eukaryotic lineages. Convergent evolution probably also shaped the pathogenic behavior of oomycetes and fungi but whether, and to what extent, this involves HGT is still questionable (Latijnhouwers *et al*., 2003).

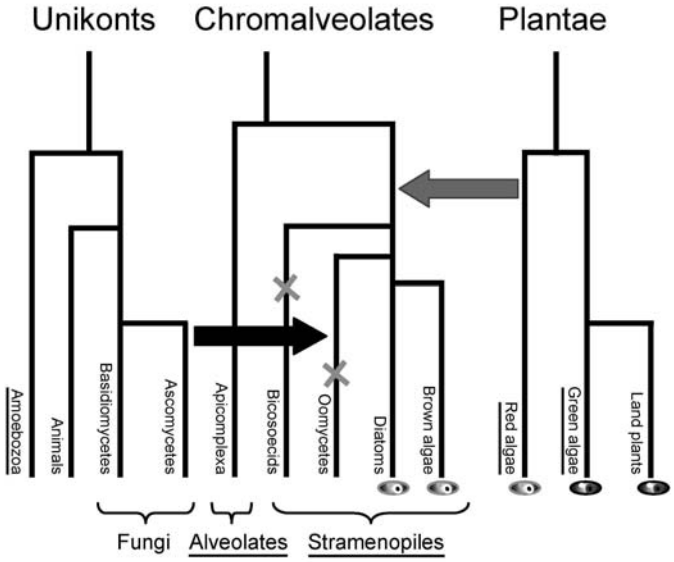

Figure 1. Plastid gain-and-loss and intra-eukaryotic horizontal gene transfer (HGT). This simplified phylogenetic tree shows three of the five eukaryotic supergroups as defined by Keeling *et al*. (2005). Oomycetes and Apicomplexa (a.o. *Plasmodium*) belong to the supergroup Chromalveolates. The supergroup Unikonts comprises, a.o., animals and fungi, and the supergroup Plantae includes red and green algae, and land plants. Two of the four bikont supergroups, Rhizaria and Excavates, are not shown. For simplicity, not all lineages are depicted. Major branches are underlined. During evolution the Chromalveolates acquired a photosynthetic plastid (👁) most likely originating from a red alga (grey arrow). This plastid was lost in the oomycete lineage (**x**) but retained in other chromealveolates that are phototrophic, for example the brown alga *Laminaria digitata* (kelp). *Phytophthora* genomes still carry many genes reminiscent of a phototrophic origin (Tyler *et al*., 2006). The black arrow represents HGT from ascomycetes to oomycetes. Genes found in oomycetes that are thought to have a fungal origin have putative functions in osmotrophy (Richards *et al*., 2006).

Typical features of oomycetes

Molecular phylogeny has now firmly established the distinct taxonomic positions of fungi and oomycetes, but also before the genomics era mycologists recognized several features that are characteristic for oomycetes (Erwin and Ribeiro, 1996). Among these are cell walls that lack chitin but are composed of a mix of cellulosic compounds and glycans, hyphae that lack septa (so-called coenocytic mycelium) and have diploid nuclei (instead of haploid as found in fungi), stacked Golgi cisternae (versus unstacked in fungi), tubular mitochondrial cisternae (versus disc-like in fungi) and sterol auxotrophy. Most characteristic for oomycetes are the

zoospores, the free-swimming asexual spores that are propelled by two unequal flagellae and explain why a moist environment is most favorable for these water molds. One of the flagellae has lateral hair-like structures called mastigonemes that contain the ß-1,3-glucan mycolaminarin, an energy storage molecule that is also found in brown algae and diatoms (Feofilova, 2001). Literally, oomycetes means 'egg fungi', a name based on the egg-shaped resting spores, named oospores. Oomycetes can be either homothallic or heterothallic. Sexual reproduction is initiated upon release of hormones that trigger the formation of gametangia (♀ oogonium and ♂ antheridium), in which meiosis takes place. The diploid oospores are produced as a result of oogamous fertilization when a haploid oosphere fuses to a haploid gamete. Thick-walled oospores are most durable propagules that can survive harsh environmental conditions and are important for the generation and maintenance of genetic variation in a population. For a recent review on sexual reproduction in oomycetes, see Judelson (2007).

Oomycete diseases and model systems

The plant pathogenic oomycetes are remarkably diverse and exhibit lifestyles ranging from obligate biotrophic to necrotrophic (Table 1). Oomycete diseases occur on nearly every agricultural crop across the globe and many of the economically important species were spread unwittingly by humans. The late blight pathogen *P. infestans*, renowned for its impact on history, caused the dreadful Irish potato famine in the 1840s. The journey from its origin to Europe and the subsequent global distribution was undoubtedly supported by potato trading. For a long time Mexico was considered as the center of origin of *P. infestans* but recent findings raise doubts and point to South America (Gómez-Alpizar *et al*., 2007; Grünwald and Flier, 2005). In the late 1870s, another severe oomycete disease appeared in Europe when lice-resistant (*Phylloxera*) grapevine rootstocks were imported from the US to France. Unfortunately, the rootstocks carried with them the downy mildew pathogen *Plasmopara viticola*. The disease was first observed in France in 1878 and in successive years it was found in almost every vineyard in Europe (Gobbin *et al*., 2006). Another oomycete first described in the nineteenth century is *Bremia lactucae*, the causal agent of lettuce downy mildew (Regel, 1843). *B. lactucae* probably originates from Eastern Europe and the Near East, where the majority of the wild lettuce species is found. Nowadays, lettuce downy mildew occurs worldwide and is one of the major problems in lettuce cultivation. During the history of lettuce breeding several resistance genes have been introgressed into commercial cultivars. However, the resistance did not last, probably due to rapid genetic adaptation of the pathogen (Lebeda and Zinkernagel, 2003). Defeat of resistance by oomycete pathogens is not uncommon. Also, *P. infestans* is notorious in that respect (Wastie, 1991).

Table 1. Oomycete diseases and model systems

Species	Host plants	Disease type	Lifestyle	Model species
Phytophthora infestans	Potato and tomato	Late blight	hemibiotrophic	yes
P. sojae	Soybean	Damping-off and root rot	hemibiotrophic	yes
P. ramorum	Several trees and bushes (e.g. oak, rhododendron)	Sudden oak death, canopy dieback	hemibiotrophic	
P. nicotianae syn. *P. parasitica*	Multiple hosts, including citrus, castor bean and tobacco	Leaf and stem blight, root rot	hemibiotrophic	
P. brassicae	Several brassicaceous plants, including *Arabidopsis thaliana*	Leaf blight	hemibiotrophic	potential
P. capsici	Multiple hosts, including cucurbits and *Capsicum* peppers	Leaf blight, fruit, stem and root rot	hemibiotrophic	potential
P. palmivora	Large host range, including cacao and rubber tree	Leaf blight, fruit and root rot	hemibiotrophic	
P. cinnamomi	Extremely large host range, exceeding 3000 species, including several crops	Root rot, dieback	necrotrophic/ saprophytic	
Albugo candida	Several brassicaceous plants, like mustard and *A. thaliana*	White rust	obligate biotrophic	
Bremia lactucae	Lettuce and several close related species	Downy mildew	obligate biotrophic	
Hyaloperonospora arabidopsidis	Several brassicaceous plants, including *A. thaliana*	Downy mildew	obligate biotrophic	yes
Plasmopara viticola	Grapevine	Downy mildew	obligate biotrophic	

Table 1. continued

Species	Host plants	Disease type	Lifestyle	Model species
Plasmopara halstedii	Asteraceae, including sunflower	Downy mildew	obligate biotrophic	
Peronospora destructor	Monocots of the *Allium* genus, like onion and garlic	Downy mildew	obligate biotrophic	
Pseudoperonospora cubensis	Several Curcubitaceae, like cucumbers	Downy mildew	obligate biotrophic	
Sclerospora graminicola	Several monocots, including maize, sorghum and pearl millet	Downy mildew	obligate biotrophic	
Pythium ultimum	Multiple dicots (e.g. potato) and monocots (e.g. turf grass)	Damping-off	necrotrophic	
Aphanomyces euteiches [a]	Several legumes, including peas, alfalfa, *Medicago truncatula* and clover	Root rot	necrotrophic	potential
Aphanomyces cochlioides [a]	Sugar beet	Root rot	necrotrophic	

[a] belongs to the order Saprolegniales

Unintended movement of oomycete plant pathogens is also the cause of severe diseases that disturb complete ecosystems. One illustrative example is Sudden Oak Death (SOD) caused by *Phytophthora ramorum*. This species originates from the Chinese highlands, and most likely made its entry into Europe, Northern America and Australia via trade of ornamental plants (Goheen *et al*., 2006; Werres *et al*., 2001). In California, SOD emerged about a decade ago and killed many of the tanoaks (*Lithocarpus* spp.) and coast live oaks (*Quercus agrifolia*) that dominate in coastal forests. This disease not only harms its host plants but also indirectly changes avian species diversity (Monahan and Koenig, 2006). Another example is dieback in native Australian woods caused by *Phytophthora cinnamomi*. This species has an extremely wide host range (over 3000 plant species) and is believed to have originated near Papua New Guinea but now has a worldwide distribution. It was accidentally introduced into Australia around the 1920s. Dieback is threatening multiple endangered 'red list' plant species (http://www.iucnredlist.org) and also has a severe impact on some agricultural crops (Hardham, 2005; Shearer *et al*., 2007).

Since *Phytophthora* diseases are so important and widespread, it is not surprising that they are intensively studied from different angles, the front runners being *P*. *infestans* (potato blight) and *Phytophthora sojae* (soybean root and stem rot). Also, downy mildews are favorite subjects of study, in particular in the field of molecular plant–microbe interactions. However, their obligate nature make these organisms more difficult to handle. The many destructive *Pythium* species (>125) are mostly soil-borne pathogens and primarily necrotrophs, and hence less attractive as a model for studying the intimate relation between host and pathogen. Table 1 gives an overview of a variety of oomycete pathogens. To choose a pathosystem that fulfills all requirements for a model system for molecular plant-microbe interaction research is nearly impossible. In a recent review, Lamour *et al*. (2007) compared the five species for which the genomes have been sequenced – *P*. *sojae*, *P*. *ramorum*, *P*. *infestans*, *Phytophthora capsici* and *Hyaloperonospora arabidopsidis* – and listed the pros and cons of these five as model organisms for oomycete research. Attractive features include the high potential of *P*. *capsici* for genetic studies, the capability of *H*. *arabidopsidis* to infect the model plant *Arabidopsis* and gene inactivation in *P*. *infestans* by gene silencing. In their comparison, Lamour *et al*. (2007) did not consider *Phytophthora brassicae*. It is true that its genome has yet to be sequenced but its potential as model species lies in the fact that, similar to *H*. *arabidopsidis*, it is a pathogen of *Arabidopsis* and that different strains show differential interactions with various ecotypes (Roetschi *et al*., 2001). The advantage over *H*. *arabidopsidis* is its non-obligate nature and amenability to DNA transformation (Si-Ammour *et al*., 2003). In that respect, the non-obligate *Aphanomyces euteiches* also has the potential to become a model

species because one of its hosts is *Medicago truncatula* which is a model species for legumes and research on beneficial plant–microbe interactions.

Genomic resources

The rise of genomics has had a major impact on oomycete research. In the early 1990s when the discipline of molecular phytopathology saw the light, research on oomycete pathogens lagged behind. The disadvantage of a diploid genome was one reason; homologous recombination, applicable in many fungi to make gene knockouts and mutant libraries for reverse genetics, is out of reach for an oomycete researcher. The large genome sizes of oomycetes and hence, the inability to clearly separate chromosomes on agarose gels (Howlett, 1989; Tooley and Carras, 1992), as well as the lack of easy scorable phenotypic markers for genetic analyses were other barriers that made these organisms less attractive for basic research. Not without reason, Shaw referred to *Phytophthora* and related species as 'a nightmare for the fungal geneticist' (Shaw, 1983). These disadvantages though were a stimulus to search for alternatives and the leap to genomics as an instrument to tackle *Phytophthora* was made in an early stage. As a result, the genomes of five oomycete species have now been sequenced and it is very likely that more will be sequenced in the next few years.

Expressed sequence tags and bacterial artificial chromosome libraries

Genomics activities started off with small-scale expressed sequence tag (EST) projects (Kamoun *et al*., 1999; Qutob *et al*., 2000) that were followed by the release of more extensive EST datasets (Randall *et al*., 2005; Torto-Alalibo *et al*., 2007). The *P*. *infestans* EST resource with over 111.000 ESTs from different isolates, developmental stages and culturing conditions, is still one the largest available for any plant pathogen (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html, accessed April 2010). Table 2 summarizes the genomic resources for oomycete plant pathogens and lists the number of ESTs currently deposited in GenBank.

Bacterial artificial chromosome (BAC) libraries have been constructed for *P*. *infestans* (Randall and Judelson, 1999; Whisson *et al*., 2001), *P*. *sojae* (Zhang *et al*., 2006), *Phytophthora nicotianae* (Shan and Hardham, 2004) and *H*. *rabidopsidis* (Rehmany *et al*., 2003). Zhang *et al*. (2006) obtained the first genome-wide physical map of a *Phytophthora* species by integrating *P*. *sojae* BAC contigs with the genome sequence. BAC libraries have been instrumental for positional cloning, in particular of genes located at avirulence (*Avr*) loci (Allen *et al*., 2004; Armstrong *et al*., 2005; Jiang *et al*., 2006c; Rehmany *et al*., 2005; Shan *et al*., 2004; Van Poppel *et al*., 2008). Also, the mating-type locus of *P*. *infestans* was mapped to a 60-70 kb region by exploiting BAC clones (Randall *et al*., 2003). Several other cloning efforts were aided by the availability of BAC libraries (Laxalt *et al*., 2002; Meijer *et al*., 2005), and restriction and sequence analyses of

individual BACs provided a first glimpse of the complexity and distribution of gene families (Armstrong *et al*., 2005; Jiang *et al*., 2005; Liu *et al*., 2004) and repeat elements such as tRNA-related short interspersed elements (SINEs), retrotransposons and DNA transposons (Ah Fong and Judelson, 2004; Jiang *et al*., 2005; Whisson *et al*., 2005).

Table 2. Genomic resources for plant pathogenic oomycetes

Species	Genome size (Mb)	Gene models	ESTs[a]	Genome sequencing and assembly status	URL[b]
Phytophthora infestans	240	17797	111095 [c]	Completed	http://www.broad.mit.edu/annotation/genome/phytophthora_infestans
P. sojae	95	16988	28467[d]	Completed	http://genome.jgi-sf.org/Physo1_1/
P. ramorum	65	14451	—	Completed	http://genome.jgi-psf.org/Phyra1_1/
P. nicotianae/ *P. parasitica*	n.d.	n.d.	553/ 10525[e]	—	—
P. brassicae	≈ 75[f]	n.d.	12922	—	—
P. capsici	65	n.d.	56457	In progress	http://www.jgi.doe.gov
Hyaloperonospora arabidopsidis	75	14726	46[g]	In progress	http://www.intlgenome.org/
Plasmopara halstedii	n.d.	n.d.	145	—	—
Pythium ultimum	43	15290	100391	Completed	http://pythium.plantbiology.msu.edu/
Aphanomyces euteiches	n.d.	n.d.	18684[h]	—	http://www.polebio.scsv.ups-tlse.fr/aphano/
Aphanomyces cochlioides	n.d.	n.d.	3599	—	—

[a] http://www.ncbi.nlm.nih.gov/dbEST/; [b] see also OMGN website, http://pmgn.vbi.vt.edu; [c] Kamoun *et al*., 1999; Randall *et al*., 2005; Haas *et al*., 2009; [d] Qutob *et al*., 2000; Torto-Alalibo *et al*., 2007; [e] Panabières *et al*., 2005; Le Berre *et al*., 2007; [f] Roetschi *et al*., 2001; [g] Casimiro *et al*., 2006; [h] Gaulin *et al*., 2008; Madoui *et al*., 2007; n.d., not determined

Genome sequencing projects

To date genome sequencing of six oomycetes has been (nearly) completed (Table 2). Four of these are *Phytophthora* species, number five is a downy mildew, and number six a *Pythium* species. Their genome sizes vary from 43 up to 240 Mb and

are overall larger than those of an average fungal genome. The first oomycete genome sequence project actually handled two species in parallel, *P. sojae* and *P. ramorum* (Govers and Gijzen, 2006; Tyler *et al*., 2006), and at an early stage enabled the application of powerful analyses based on comparative genomics. This immediately revealed a high level of conserved synteny between the two species; but interestingly, the synteny appeared to be interrupted at loci harboring genes encoding effectors with putative functions in interactions with host plants (Jiang *et al*., 2006a; Jiang *et al*., 2008). Preliminary analyses including the three other sequenced genomes suggest that large regions of conserved synteny occur throughout the genus *Phytophthora* and often even extend to *H. arabidopsidis* and *Pythium ultimum* (H. Meijer, personal communication). One example of a region of conserved synteny between *P. infestans* and *H. arabidopsidis* that is interrupted by an effector gene (i.e. Avr3a) was published by Armstrong *et al*. (2005).

The phenomenon of conserved synteny also helps the gene-calling process. Over 20 % of the predicted gene models in oomycetes are not known in other species but the existence of orthologs often arranged in similar orders along the scaffolds is an indication that the gene models represent real genes. As shown in Table 2 the predicted number of gene models seems more or less colinear with genome size. Automated gene prediction is mostly based on training sets. The training set of oomycete genes is still relatively small and therefore the current genome browsers often show incorrect models.

Handsaker *et al*. (2007) developed a gene-calling program called Orthosearch (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/GeneFinding.html) that makes use of the DNA conservation between *Phytophthora* species and could help to improve the gene prediction process. To avoid inaccurate models ending up in the literature and being propagated it is important to check the coherence of existing data by comparing genome sequences of related species with ESTs and/or proteome data.

The impact of genomics on gene discovery

One incentive to sequence a genome is to accelerate gene discovery. Before the genomics era, the quest for the mechanisms that underlie pathogenicity in oomycetes already lead to the identification of a number of oomycete genes and gene families with putative roles in the interaction with their host plants. For gene isolation in oomycetes several strategies were used ranging from unbiased approaches, such as differential screening and positional cloning, to more biased approaches aimed at homologs of fungal pathogenicity genes or via reverse genetics starting with the purification of a protein that shows toxic or necrotic activity on a host plant. In this section, we give examples that demonstrate how genomics has influenced gene discovery in oomycetes.

From expression pattern to gene

The first *Phytophthora* genes to be identified were selected based on expression patterns (Pieterse *et al*., 1991; Pieterse *et al*., 1993a). These so called *ipi* (*in planta* induced) genes showed induced or specific expression during growth of the pathogen in association with its host (Pieterse *et al*., 1993b). Many years later, when comparison with the whole gene repertoire of *Phytophthora* became feasible, one of the *ipi* genes, *ipiO*, turned out to be member of the superfamily of RXLR-dEER effectors (see Genomic resources) and to function as an avirulence (*Avr*) gene in a gene-for-gene manner with the resistance (*R*) genes *Rpi-blb1* and *Rpi-sto1* (Vleeshouwers *et al*., 2008).

From protein to gene

Reverse genetics, successfully used to clone several *Avr* genes from fungal plant pathogens (Westerink *et al*., 2004), was applied to clone the first elicitin gene named *para1* (Kamoun *et al*., 1993). Elicitin was initially identified as a highly abundant toxic compound in culture filtrates of *Phytophthora parasitica* that causes necrosis on tobacco (Ricci *et al*., 1989). The elicitin genes *para1* and *inf1*, from *P. parasitica* and *P. infestans* respectively, are single copy genes that encode 10 kDa secreted proteins with a core elicitin domain (Kamoun *et al*., 1993; Kamoun *et al*., 1997). From a small scale EST inventory, it became evident that the 10 kDa elicitins belong to a larger protein family not only comprising soluble extracellular elicitins but also elicitins anchored to the membrane or cell wall (Kamoun *et al*., 1999). Subsequently, whole genome sequencing revealed that elicitins (ELI) and elicitin-like (ELL) genes make up a large, complex and highly conserved family with 17 clades that existed prior to the divergence of *Phytophthora* species from a common ancestor (Jiang *et al*., 2006b). Members of the four ELI clades all have elicitor activity on tobacco comparable to the ELI1 elicitins INF1 and PARA1, whereas ELL proteins are not active as elicitors (A. van 't Slot, P. van de Vondervoort and F. Govers, unpublished). The intrinsic functions of ELI and ELL proteins are still unknown. However, ELIs can bind sterols and since *Phytophthora* is a sterol auxotroph, ELIs possibly function as carrier proteins to acquire sterols from the environment. The whole genome perspective tells us that the simplistic view that a single abundant component secreted in culture filtrate in an artificial environment (i.e. *in vitro* growth) is responsible for elicitin activity in the natural situation should be revisited.

A reverse genetics approach was also used to clone the gene encoding a 42 kDa extracellular glycoprotein from *Phytophthora megasperma* (Sacks *et al*., 1995) that is now identified as a transglutaminase which comprises the pathogen-associated molecular pattern (PAMP) pep-13 as the active site (Brunner *et al*., 2002). Other examples are the elicitors CBEL from *P. parasitica* (Mateos *et al*.,

1997) in which two cellulose-binding domains (CBDs) act as PAMPs (Gaulin *et al*., 2006) and NPP1 from *P*. *parasitica*, another putative PAMP (Fellbrich *et al*., 2002) belonging to the class of Nep1-like proteins or NLPs (Necrosis and ethylene inducing protein-like proteins). NLPs have a wide distribution across taxa and a broad spectrum of activity on plants (Gijzen and Nürnberger, 2006). In bacteria and fungi NLPs are encoded by one or two genes but, in contrast, in *Phytophthora* species large families of NPP genes are found (Tyler *et al*., 2006). As a last example we mention the toxin PcF from *Phytophthora cactorum* (Orsomando *et al*., 2001). Again a single gene was cloned by reverse genetics but genomics data revealed a much more complex situation. *P*. *infestans* homologs of PcF were named Scr74 (secreted cysteine-rich protein). The *scr74* family has at least ten members, some of which are clustered in the genome. They are highly polymorphic and under diversifying selection (Liu *et al*., 2004). Also in *P*. *sojae*, *PcF* is a multicopy family but in *P*. *ramorum* very few *PcF* genes are detected (Tyler *et al*., 2006).

From homolog to gene

The infection strategies of oomycetes and fungi have much in common (Latijnhouwers *et al*., 2003) and therefore it is logical to investigate whether genes involved in host–pathogen interactions are also similar. The approach to isolate homologs of fungal pathogenicity genes by PCR amplification using degenerate primers was, for example, used by Götesson *et al*. (2002) to clone polygalacturonase genes from *P*. *cinnamomi*. Cell wall degrading enzymes are important for the pathogenicity of many plant pathogenic fungi (Ten Have *et al*., 2002) and it is likely that necrotrophic oomycetes also make use of such enzymes. Laxalt *et al*. (2002) used degenerated PCR primers to clone the *P*. *infestans* gene coding for the Gα subunit of the heterotrimeric G-protein (i.e. *gpa1*). G-protein-mediated signal transduction is the most ubiquitous and best studied signaling pathway among eukaryotes and in several plant pathogenic fungi the Gα and Gβ G-protein subunits appear to be crucial for virulence. In *P*. *infestans* silencing of *gpa1* results in non-pathogenic mutants (Latijnhouwers *et al*., 2004) and silencing of *gpb1*, the gene encoding the G-protein β subunit, severely affects sporulation (Latijnhouwers and Govers, 2003). With the emergence of EST databases and genome sequences it is no longer necessary to design the optimal degenerated PCR primers and to face the challenge of finding the perfect PCR conditions to clone the homologs. In the *Phytophthora* EST databases several candidate genes that resemble known fungal pathogenicity genes have been identified and can be readily used for functional analysis (Qutob *et al*., 2002; Randall *et al*., 2005; Torto-Alalibo *et al*., 2007).

From domain to gene

Mining the databases for certain motifs that represent catalytic domains of enzymes is another strategy that can lead to novel candidates. This was nicely demonstrated by Meijer and Govers (2006) who made an inventory of genes involved in phospholipid signaling in *Phytophthora*. They found several novelties including a family of genes encoding secreted proteins with a phospholipase D (PLD) catalytic domain. Similar proteins are present in downy mildews (H. Meijer, personal communication) but have, as yet, not been found in other plant pathogens or other eukaryotes. It is therefore interesting to investigate how widely spread these enzymes are in oomycetes and whether they have a function in pathogenicity. Another novel class of proteins that deserves attention is the G-protein- coupled receptor phosphatidylinositol (phosphate) kinases (GPCR-PIPKs) which are composed of an N-terminal seven transmembrane domain that is typical for GPCRs and a C-terminal PIPK catalytic domain. *Phytophthora* spp. and *H. arabidopsidis* each have twelve GPCR-PIPKs which are slightly different. However, outside oomycetes only one homolog has been identified so far (Bakthavatsalam *et al*., 2006). This is RpkA in *Dictyostelium discoideum* that is essential for cell density sensing (Bakthavatsalam *et al*., 2007). Such a feature might also be important for oomycete pathogens when, for example, zoospores aggregate and prepare to invade the host. Involvement of G-protein signaling in this process was already demonstrated by Latijnhouwers *et al*. (2004) who found that zoospores of *P. infestans* transformants lacking the G-protein α subunit (generated through silencing of *gpa1*), have lost the ability to autoaggregate.

From map position to gene

For organisms that are a 'geneticist's nightmare' (Shaw, 1983), gene discovery based on positional cloning is not the most logical approach to follow. Nevertheless, before the genomics era several groups started to generate molecular genetic linkage maps with the aim to clone genes with a scorable phenotype but no clue about the gene product or stage of expression. Often their genes of interest were *Avr* genes that interact in a gene-for-gene manner with plant *R* genes. In addition, the mating type locus was a target for positional cloning (Randall *et al*., 2003). Species for which molecular genetic linkage maps have been generated are *P. infestans* (Carter *et al*., 1999; Van der Lee *et al*., 2001; Van der Lee *et al*., 2004; Van der Lee *et al*., 1997; Randall *et al*., 2003), *P. sojae* (MacGregor *et al*., 2002; May *et al*., 2002; Whisson *et al*., 2004; Whisson *et al*., 1995), *H. arabidopsidis* (Rehmany *et al*., 2003) and *B. lactucae* (Sicard *et al*., 2003). These long-term investments paid off and at least a handful of *Avr* genes have been isolated based on their map positions. One example where cloning primarily relied on RAPD and RFLP markers, a large number of recombinants and chromosome walking, was *Avr1b*-1 from *P. sojae* (Shan *et al*., 2004). *P. sojae* is

homothallic and once F1 hybrids between two different races have been identified, it is straightforward to obtain F2 mapping populations by selfing the F1 hybrids (May *et al*., 2002; Tyler *et al*., 1995). In most cases, however, the genetically linked markers were not sufficient to locate the gene – the distance was too large or too few recombinants were available. Additional markers obtained by transcriptional profiling such as cDNA-AFLP (Guo *et al*., 2006) or by suppression subtractive hybridization (Bittner-Eddy *et al*., 2003), were needed to nail down the gene of interest and in several cases genomic resources helped to speed up gene isolation. In the case of *P. infestans Avr4* ESTs matching short cDNA-AFLP fragments were instrumental (Van Poppel *et al*., 2008). To find a candidate gene at the *P. infestans Avr3b-Avr10-Avr11* locus, Jiang *et al*. (2006c) made use of a *Phytophthora* GeneChip with over 18,000 unigenes. The chip was used for transcriptional profiling and comparative genomic hybridization (array-CGH) and this provided additional markers that were crucial for identifying the *Pi3.4* gene.

From effector activity to gene

Nearly all the *Avr* genes obtained by positional cloning encode proteins that belong to the superfamily of RXLR-dEER effectors (De Jong and Van den Ackerveken, 2009). This finding stimulated the use of a gene discovery approach based on *in planta* expression of effector genes. The principle of this approach was first described by Torto *et al*. (2003) who mined *P. infestans* ESTs for sequences encoding secreted proteins and cloned these in binary PVX vectors for *in planta* expression in *Nicotiana benthamiana*. This high-throughput approach resulted in the identification of *crn1* and *crn2* (crinkling and necrosis). When expressed *in planta crn1* and *crn2* cause necrosis and *crn2* also induces expression of defense response genes in tomato. The ESTs that were tested by Torto *et al*. (2003) encode a variety of secreted proteins including cell wall degrading enzymes, proteases, a chitinase and elicitins (ELI and ELL proteins). With the prediction that a putative *Avr* gene is likely a member of the RXLR-dEER family Vleeshouwers *et al*. (2008) developed a more dedicated approach concentrating exclusively on RXLR-dEER effectors. The corresponding ESTs were extracted from the *P. infestans* EST database and cloned into a binary PVX vector. In a high-throughput screening a broad range of wild *Solanum* accessions showing resistance to late blight was toothpick-inoculated with the *Agrobacterium* strains carrying recombinant PVX constructs in a binary plasmid. Effectors inducing necrosis were retested on F1 progeny obtained by crossing a responsive, resistant accession with a non-responsive, susceptible potato line. If the progeny showed co-segregation of resistance and response to the effector, the resistant accession was further investigated for the presence of an *R* gene that specifically recognizes the RXLR-dEER effector. This approach, dubbed as 'effector genomics', resulted in the identification of an *R* gene in *S. stoloniferum* that is responsible for recognition

of IPI-O. The *R* gene *Rpi-sto1* turned out to be the ortholog of *Rpi-blb1*, and hence *ipiO* is now known to function as *Avr-blb1* and *Avr-sto1* (Vleeshouwers *et al*., 2008).

Effector genomics is clearly a powerful approach that will soon result in the identification of many more effectors matching the wide range of *R* genes present in the *Solanum* gene pool or in any gene pool that is exploited for crop breeding. It is, however, a very biased approach; only genes that have certain characteristics such as signal peptide sequences and particular domains known to be involved in effector function, are taken into account. Despite the fact that positional cloning is tedious it should not be discarded in the gene discovery process. The *pi3.4* gene located at the *Avr3b-Avr10-Avr11* locus in *P*. *infestans* is an illustrative example of a gene that would have been missed by effector genomics (Jiang *et al*., 2006c; Qutob *et al*., 2006). *Pi3.4* encodes a protein of 1956 amino acids with regulatory domains characteristic for transcription factors. In isolates avirulent on *R3b*, *R10* and *R11* potato plants, truncated versions of *Pi3.4* are amplified with up to 25 copies located in tandem and adjacent to the full length copy of *Pi3.4*. Sequence comparison revealed that the amplification may generate modular diversity and assist in the assembly of novel full length genes via unequal crossing over. Such amplification is not found in virulent isolates. The current hypothesis is that *Pi3.4* is a transcriptional regulator that influences expression of effector genes, for example RXLR-dEER genes. Copy number variation and modular diversity as observed at the *Avr3b-Avr10-Avr11* locus could be a mechanism for pathogens to quickly adapt to the environment.

The secretome

Plant pathogens secrete numerous effector molecules that fulfill important roles in mediating pathogenesis and scouting for targets in the host to deploy their action. The secretome is defined as the sum of all proteins secreted by an organism. Effectors function at the forefront to establish adhesion, penetration of host tissue, and degradation of cell walls. Moreover, effectors interact with plant cell components at the cell wall, plasma membrane or in the cytoplasm, thereby reprogramming the host cell to accommodate the needs of the pathogen. For biotrophic pathogens, the fine-tuning of this reprogramming is very important. They probably have to make compromises: the host cell has to stay alive and should support the pathogen in its urge to survive. Hence, the pathogen has to be able to suppress basal defense responses (PAMP-triggered immunity or PTI) triggered by effectors known as PAMPs (Jones and Dangl, 2006). Once these barriers are overcome, the pathogen may face another barrier, i.e. the effector-triggered immunity (ETI). ETI, however, is only triggered when the plant possesses *R* genes that monitor specific effectors. Figure 2 gives a simplified view of the interplay

between plant and pathogen in a compatible and incompatible interaction. Genome mining expeditions focused on the secretome of *Phytophthora* resulted in numbers ranging from 1188 secreted proteins in *P*. *ramorum* to 1975 in *P*. *infestans* with *P*. *sojae* (1464) as intermediate (Jiang, 2006; Tyler *et al*., 2006; R. Jiang, personal communication). The secretome of the diatom *Thalassiosira pseudonona*, the closest relative of oomycetes that is sequenced and not pathogenic, is much smaller and lacks many of the enzymes found in *Phytophthora* (Tyler *et al*., 2006). More than 80% of the genes encoding secreted proteins (defined as *spe* genes) belong to gene families and they often occur in clusters. The percentage of *spe* orthologs is lower than among non-*spe* genes indicating that the secretome evolved at a faster pace than the average genome. This supports the idea that many of the *spe* genes are involved in molecular 'arms races'. They undergo rapid changes that are driven by diversifying selection. Within the secretome, however, individual families appear to have evolved at different rates. NPPs and elicitins are highly conserved and this could be due to selection pressure exerted on certain domains, for example to maintain enzymatic functions. In contrast, the RXLR-dEER proteins as well as some families containing proteins composed of repeats are highly divergent (Jiang, 2006). In a recent review, Kamoun *et al*. (2006) presented an extensive catalogue of oomycete effectors. In the next sections, we will touch upon a few classes of secreted proteins that were not included in that review. We will also elaborate on the class of RXLR-dEER effectors whose discovery in 2004 has created a flurry of new activity in the research community.

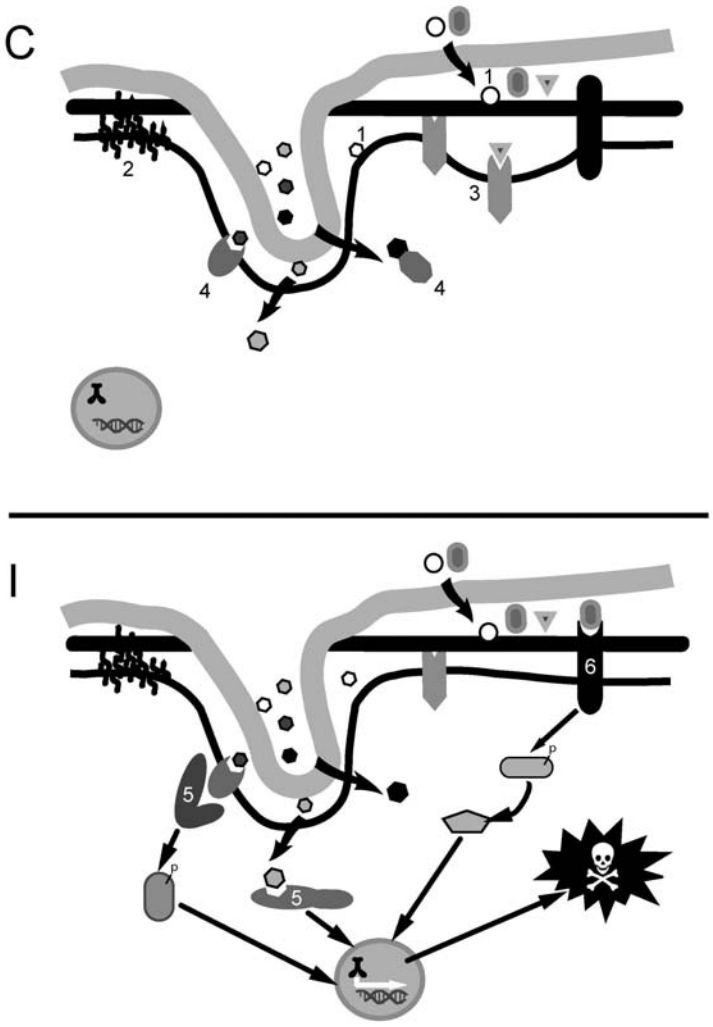

Figure 2. During a host-pathogen interaction effectors (⬡ ○ ▮ ▼) are secreted by the pathogen and directed towards their targets. These targets can be either membrane-spanning or cytoplasmic proteins, or structural molecules such as cell wall components. Effectors are needed to establish a compatible interaction (C) and are active in different steps of the infection process, for example, attachment(1), breakdown of the cell wall by enzymes(2), relaxation of the cell membrane(3) or alteration of host metabolism(4). During an incompatible interaction (I) the actions of effectors are monitored by a warning system in the host. When an effector is detected by an R protein(5), or by a receptor-like kinase (RLK)(6) signaling cascades are initiated leading to transcriptional changes and activation of defense responses, that in many cases result in cell death. The figures are simplified showing only the cross-talks discussed in this chapter. The system is too complex to show all steps known to be involved in host–pathogen interactions.

Adhesins

When zoospores attach to the plant surface they adopt a specific orientation before they encyst. The ventral surface faces the plant. During and after encystment the spore secretes adhesive material via exocytosis of peripheral vesicles (Hardham, 2007). The ventral vesicle contains a high molecular weight adhesive protein named Vsv1 that attaches the cyst to the plant. Cloning of the *Vsv1* gene from *P. cinnamomi* revealed that the protein PcVsv1 carries 47 thrombospondin type 1 repeats (TSR1) (Robold and Hardham, 2005). TSR1 is present in a variety of adhesive molecules secreted by mammalian cells and apicomplexan parasites such as malaria, but is not found in any fungal or plant protein. *PcVsv1* is a single copy gene. Other *Phytophthora* species have a PcVsv1 homolog but in the *H. arabidopsidis* genome sequence no homolog could be detected. The closest homolog outside the genus was found in the apicomplexan parasite *Cryptosporidium parvum* suggesting that apicomplexans and oomycetes have similar types of adhesins.

Other types of adhesins that were identified in *Phytophthora* are mucins. Also these adhesins are composed of variable numbers of repeats. The sizes of the repeat differ per mucin; some have repeats of 45 amino acids, others of only 10 amino acids. Görnhardt *et al*. (2000) cloned two *P. infestans* mucin genes that showed specific expression in germinated cysts. Meijer *et al*. (2006), who used a proteomics approach, identified six different mucins (and mucin-like proteins) associated with the *P. ramorum* cell wall. The gene models showed that some of these are encoded by multigene families.

CBEL is another protein that is involved in the attachment of *Phytophthora* to the host surface (Mateos *et al*., 1997). It was first identified in *P. parasitica* var. *nicotianae*. The two CBDs not only act as PAMPs but also bind to cellulose and tobacco cell walls (Gaulin *et al*., 2006). Silencing of the *CBEL* gene did not impair mycelial growth or virulence but the transformants showed a reduced attachment to cellulose (Gaulin *et al*., 2002).

Phospholipases

Phospholipases are a diverse group of enzymes that break down specific bonds in phospholipids (Shah and Chaturvedi, 2009). Phospholipase C (PLC) and D (PLD) also have functions in signal transduction and are both involved in the production of phosphatidic acid (PA), an important second messenger in many organisms. Biochemical studies by Latijnhouwers *et al*. (2002) showed that PLD and PA play a role in zoospore encystment in *Phytophthora* but no PLC activity was found. The *Phytophthora* genome sequences revealed 18 gene models for PLD but surprisingly no model for PLC (Meijer and Govers, 2006). This is unique since all other eukaryotes sequenced to date have one or more *PLC* genes. On the other hand the diversity in the types of PLDs is remarkably high and possibly one of

these PLDs compensates for the lack of a PLC. Only one of the 18 PLD models matched to a known PLD of the class PXPH-PLD, whereas two others encode novel proteins in which the PLD catalytic domain is combined with transmembrane domains (PXTM-PLD and TM-PLD). Of the remaining fifteen, fourteen encode a PLD with a slightly aberrant catalytic domain and twelve have a N-terminal signal peptide. The secreted ones, named sPLD-likes, belong to the secretome.
Phospholipases in the secretome are not exceptional. Phospholipases A and B are secreted by several organisms and also PLD is described as being secreted by bacteria. It has been postulated that PLDs in bacteria act as virulence factors (Songer, 1997) and the human pathogen *Neisseria gonorrhoeae* was reported to secrete a PLD that enhanced complement receptor-3 mediated invasion of cervical epithelial cells (Edwards and Apicella, 2006). However, PLD secreted by a plant pathogen has not been reported before and none of the sPLD-likes found in *Phytophthora* has homologs in fungi or other eukaryotes. Recent findings in our laboratory show that PLD activity is present in the extracellular fluid of *P. infestans* and that specific phospholipids in plant membrane vesicles are degraded in *in vitro* assays. A function for the sPLDs in degradation of plant tissue is therefore plausible (H. Meijer, personal communication).

RXLR-dEER effectors

When the first few oomycete *Avr* genes were isolated by positional cloning, the first two *Phytophthora* genome sequences were being released. A genome mining and alignment expedition during a genome annotation jamboree in 2004 at the Joint Genome Institute resulted in the discovery of a conserved motif in all Avr's and Avr homologs (Govers and Gijzen, 2006). This motif located adjacent to the signal peptide was christened as RXLR-dEER. Since no conserved motifs have been identified in fungal avirulence proteins the presence of a conserved motif in oomycete Avr proteins came as a surprise. There is now ample evidence that the RXLR-dEER superfamily (collectively called the RXLR-dEER effectome) comprises most, if not all, effectors that interact in a gene-for-gene manner with *R* genes. Table 3 provides a summary of eight oomycete Avr factors, six of which were obtained by positional cloning. Avr3a was characterized by means of association genetics (Armstrong *et al*., 2005) and IPI-O was identified as an Avr protein in an effector genomics screen (Vleeshouwers *et al*., 2008). Several new Avrs are in the pipeline and can soon be added to this list.

The number of RXLR-dEER effectors found in each of the sequenced genomes is astonishing. Tyler *et al*. (2006) reported 385 different RXLR-dEER proteins in *P. sojae* and 370 in *P. ramorum*. With the same mining strategy of reiterated BLAST searches and Hidden Markov Models (HMM) around 563 members were found in *P. infestans* (Haas *et al*., 2009). Lately, several publications have listed different numbers and this is likely due the different

algorithms that are used by the different groups (Lamour *et al*., 2007; Whisson *et al*., 2007; Win *et al*., 2007). Dispute about the best mining strategy continues but there is no doubt that the family is extremely large and diverse. The N-terminal part with the signal peptide and the RXLR-dEER motif is conserved but in the C-terminal regions there is almost no similarity. Avr function and diversity are two characteristics that underscore the role of RXLR-dEER effectors in host specificity. Another characteristic is the distribution of RXLR-dEER genes over the genome. The high level of conserved synteny between two *Phytophthora* species over large segments of the genome is disrupted by indel blocks and nearly all RXLR-dEER genes are located in these blocks (Jiang *et al*., 2006a; Jiang *et al*., 2008). The genomes are highly dynamic and full of transposons and it is remarkable that RXLR-dEER genes are more often flanked by transposon-like sequences than other genes (Haas *et al*., 2009). Apparently transposition is one of the factors that contributed to the rapid evolution of these large effector families.

At the time of discovery the function of the RXLR-dEER motif was a mystery but shortly after the 2004 jamboree Hiller *et al*. (2004) and Marti *et al*. (2004) reported the existence of a conserved motif, named PEXEL/VTS, in secreted effectors of the Alveolate parasite *Plasmodium falciparum*, the causal agent of malaria. RXLR-dEER resembles the PEXEL/VTS motif, both in sequence and location in the protein (Figure 3A). The PEXEL/VTS motif was shown to be involved in host cell targeting of effectors. Via this system, the parasite delivers a secretome consisting of an estimated 400 proteins into the cytoplasm of erythrocytes, presumably to carry out virulence and host remodeling functions (Hiller *et al*., 2004; Marti *et al*., 2004). This led to the hypothesis that RXLR-dEER effectors are also targeted into host cells. Bhattacharjee *et al*. (2006) strengthened this hypothesis by showing that the RXLR motif of *P*. *infestans* Avr3a, and not a mutated motif, can functionally complement a PEXEL/VTS motif in *Plasmodium*. More recently, Whisson *et al*. (2007) were able to demonstrate that *P*. *infestans* transformants carrying an *Avr3a* transgene with a mutated RXLR-dEER motif can not restore the Avr phenotype of a race 3 strain, adding support to the idea that Avr3a has to reach the cytoplasm to be recognized by the intracellular CC-NBS-LRR protein R3a. They also transformed *P*. *infestans* with reporter constructs consisting of GUS fused to wild type and mutated forms of the RXLR-dEER motif and monitored the intracellular location of GUS after infection. GUS is not stable in the apoplast and indeed only in the presence of the wild type motif the infected cells stained blue. Similar results were obtained by Dou *et al*. (2008a, 2008b) with Avr1b-1 from *P*. *sojae*. These experiments strongly support the notion that the RXLR-dEER motif indeed can function as a host cell targeting signal.

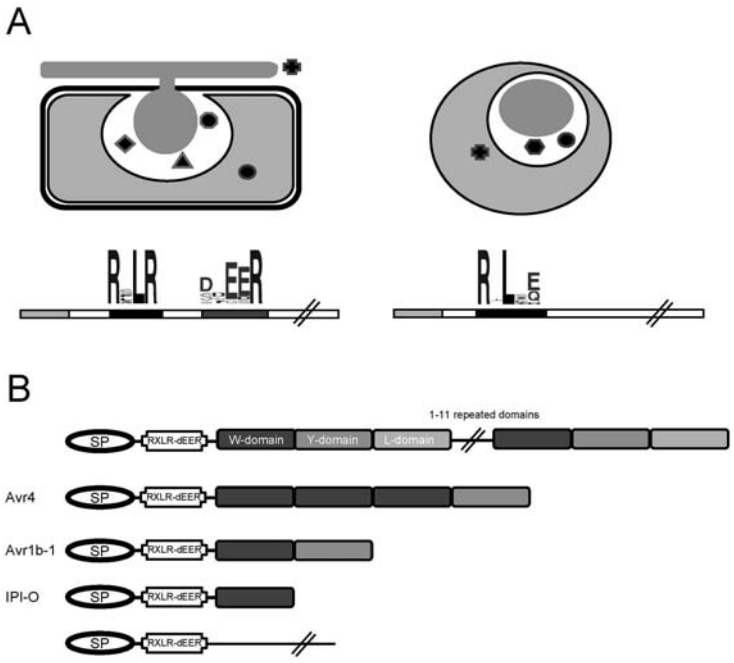

Figure 3. (A) Infection strategies of plant pathogenic oomycetes and the malaria parasite *Plasmodium* show several similarities. Biotrophic oomycetes penetrate plant cells by forming haustoria (left). *Plasmodium* is localized inside erythrocytes within parasitophorous vacuoles (PV) (right). Both the haustorium and the PV are surrounded by a host-derived membrane. The pathogens secrete effectors into the apoplast or extrahaustorial space (oomycetes), or the PV (*Plasmodium*). Effectors equipped with a host cell targeting motif can enter host cells. The host cell targeting motifs, RXLR-dEER in oomycetes and PEXEL/VTS in *Plasmodium*, are located N-terminal and adjacent to the signal peptide (marked in light grey). The dEER motif is conserved in most but not all oomycete RXLR-dEER effectors. The sequence logos show the amino acid conservation in a random set of effectors. The C-terminus in these effectors is highly diverse in both sequence and length. (B) In many of the RXLR-dEER effectors the C-terminus carries a variable number of domains that may occur in a repeated fashion (top row) (Jiang *et al*., 2008). Three domains are defined and named W, Y and L after the most conserved amino acid in that particular domain. Different combinations of domains are found. In a subset of the effectors the domains are lacking (last row). The rows in between show the domain composition of three RXLR-dEER effectors and illustrate the possible variations in the C-terminus. Avr4 and IPI-O from *P*. *infestans* and Avr1b-1 from *P*. *sojae* are recognized as Avr proteins in plants carrying the cognate *R* genes.

What then is the function of these effectors when they end up in the host cell and how do they modulate the host cell machinery? The high diversity of the C-terminus of RXLR-dEER proteins is consistent with the notion that this part is the

effector domain that is active in the cell. For *P*. *infestans* Avr3a, Bos *et al*. (2006) showed that recognition by R3a is based on the last 75 amino acids of the avirulent variant of Avr3a, Avr3a^{KI}, and that the N-terminal region is dispensable for recognition. The C-terminal part also suppresses HR induced by elicitin INF1 in *N*. *benthamiana* suggesting that the presence of Avr3a^{KI} confers a selective advantage to the pathogen when infecting a susceptible host. The virulent variant, Avr3a^{EM}, is not recognized by R3a and is, surprisingly, unable to suppress INF-induced HR. As in Avr3a the RXLR-dEER domains in *P*. *infestans* Avr4 (Van Poppel *et al*., 2008) and *P*. *sojae* Avr1b-1 (Dou *et al*., 2008a, 2008b) are also dispensable for Avr function. Moreover, in several of the Avr proteins the C-terminal domain is under diversifying selection (Table 3). As yet, however, there is no clue about the molecular or biochemical function of the RXLR-dEER effectors, and in the C-termini of the investigated effectors no catalytic domains or ProSite motifs can be distinguished. Interestingly, motif searches and HMM screening revealed three conserved domains (W, Y and L) that are repeated (Jiang *et al*., 2008). W, the most prominent domain, consists of 25 amino acids and occurs in 60% of all RXLR-dEER members. Up to 11 repeats were found (Figure 3B) and the number correlates with the length of RXLR-dEER proteins. The different numbers of repeats may, at least partly, explain the divergence between paralogs and by tracing the evolutionary history of the RXLR-dEER genes it might be possible to find correlations with their specific activities as an Avr factor. In a subset of the RXLR-dEER effectors no W, Y or L domains can be distinguished; an example is ATR13 of *H*. *arabidopsidis* (Table 3). Instead ATR13 has a heptad repeat region and a direct repeat region but, as with W, Y and L domains, the relevance of these repeats is not clear (Allen *et al*., 2004). Also, in the RXLR-dEER domain some peculiarities should be noted. Supposedly this domain interacts with other proteins at the cell membrane to activate an uptake machinery or to induce the formation of channels. Those complexes might involve other pathogen proteins. CRN proteins have a highly conserved motif LXLFLAK and this motif was found to overlap with the RXLR motif of 13 RXLR proteins in *H*. *arabidopsidis*. RXLR proteins with a CRN domain are absent in *Phytophthora*, suggesting that these proteins recently evolved (Win *et al*., 2007). Furthermore, IPI-O has an RGD motif that overlaps with the RXLR motif. Interestingly, RGD in IPI-O has been shown to bind to an *Arabidopsis* lectin receptor kinase (LecRK-I.9) (Gouget *et al*., 2006; **Chapter 3.1**; **Chapter 3.2**) and this LecRK could well be an effector target that mediates uptake of RXLR-dEER effectors into the host cell. LecRK-I.9 plays a role in the cell wall-plasma membrane adhesions and IPI-O was shown to disrupt these adhesions in *Arabidopsis* (Senchou *et al*., 2004). Consistent with the idea that RXLR-dEER effectors have virulence functions, overexpression of *ipiO* in *Arabidopsis* Col-0 leads to gain of susceptibility to *P*. *brassicae* strains that cannot infect wild type Col-0 plants (**Chapter 3.2**).

Table 3. An overview of known oomycete avirulence genes

Effector gene	Origin	*R* gene	Type of R protein	WYL domains	Protein variants	Positive selection	Types of mutations affecting avr phenotype
HpATR1^{NdWsB} [a]	*H. arabidopsidis*	*RPP1*	n.d.	-	8	yes	SNPs, indels
HpATR13 [b]	*H. arabidopsidis*	*RPP13-Nd*	CC-NBS-LRR	-	6	yes	SNPs, indels
PsAvr1a [c]	*P. sojae*	*Rps1a*	n.d.	W	n.d.		n.d.
PsAvr1b-1 [d]	*P. sojae*	*Rps1b*	n.d.	WY	4	yes	Transcript regulation, SNPs
PsAvr3a [e]	*P. sojae*	*Rps3a*	n.d.	-	n.d.		n.d.
PiIpiO [f]	*P. infestans*	*Rpi-blb1*, *Rpi-sto1*	CC-NBS-LRR	W	10	yes	SNPs
PiAvr3a [g]	*P. infestans*	*R3a*	CC-NBS-LRR	W	2		SNPs
PiAvr4 [h]	*P. infestans*	*R4*	n.d.	WWWY	6		SNPs, frameshifts

[a] Rehmany *et al*., 2005; [b] Allen *et al*., 2004; [c] Qutob *et al*., 2007; [d] Shan *et al*., 2004; [e] Tedman-Jones *et al*., 2007; [f] Vleeshouwers *et al*., 2008, **Chapter 2.1**; [g] Armstrong *et al*., 2005; [h] van Poppel *et al*., 2008;
n.d., not determined

Future perspectives

A decade ago molecular genetic research on oomycetes was still in its infancy. Only a few genes were described, there were hardly any resources and the molecular toolbox was limited. Genomics has dramatically changed our way of thinking and has already uncovered many secrets about biology, pathology and evolution of oomycetes. Yet there are still many enigmas that need to be cracked and therefore the various -omics approaches should be further exploited. Oomycete proteomics has already started and the data obtained from expressed peptide tags (EPTs) (Savidor *et al*., 2006) are now being integrated to genome annotations. Peptides that do not fit existing gene models show that the annotation needs further optimization. Also transcriptomics is going strong. Data obtained with the first-generation *P. infestans* DNA chip have already been used for various purposes (Judelson *et al*., 2008; Jiang *et al*., 2006c; Prakob and Judelson, 2007). With the large reservoir of rapidly evolving RXLR-dEER effectors, effectomics is also feasible. The diversity seen in strains that have been sequenced is just the tip of the iceberg. The number of variants of each RXLR-dEER effector in field isolates varies (Table 3). For some just two allelic variants are found in a wide range of isolates. For others already more than ten variants have been found in a limited set of isolates. High-throughput sequencing such as with the 454 or Solexa systems should be exploited to monitor the RXLR-dEER effectome of larger sets of isolates. Also re-sequencing of isolates that originate from different regions or host plants will provide valuable insights to the specialization of such isolates.

To date, oomycete genomics is still largely focused on *Phytophthora*. It will be exciting to unravel the genomes of a wider range of oomycete species, in particular species infecting animals and insects. This will offer many new scientific challenges and provide opportunities to tackle these notorious pathogens.

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2

The *Phytophthora infestans* effector IPI-O and its role in virulence and avirulence on potato

2.1 *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato

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Abstract

A strategy to control the devastating late blight disease is providing potato cultivars with *R gene*s that are effective to a broad spectrum of *Phytophthora infestans* isolates. Thus far, most late blight *R* genes that were introgressed in potato were quickly defeated. In contrast, the *Rpi-blb1* gene originating from *Solanum bulbocastanum* has performed as an exclusive broad-spectrum *R* gene for many years. Recently, the RXLR-dEER effector family *ipiO* was identified to contain *Avr-blb1*. Monitoring the genetic diversity of the *ipiO* family in a large set of isolates of *P. infestans* and related species resulted in 16 *ipiO* variants in three distinct classes. The class I and class II, but not class III, *ipiO* variants induce cell death when co-infiltrated with *Rpi-blb1* in *Nicotiana benthamiana*. Class I is highly diverse and represented in all analyzed *P. infestans* isolates except in two Mexican *P. infestans* isolates, and these were found virulent on *Rpi-blb1* plants. In its C-terminal domain, IPI-O contains a W motif that is essential for triggering Rpi-blb1-mediated cell death and is under positive selection. This study shows that profiling the variation of *Avr-blb1* within a *P. infestans* population is instrumental for predicting the effectiveness of *Rpi-blb1*-mediated resistance in potato.

Introduction

Late blight caused by the oomycete *Phytophthora infestans* is one of the most severe threats to potato production worldwide (Fry, 2008). Despite many efforts, effective methods to control late blight epidemics are still not available. In recent years, however, intensified research on both the pathogen and the host plant has deepened our insight into the molecular basis of virulence and avirulence determinants in *P*. *infestans*, and of host defense responses. This knowledge is instrumental for obtaining genetic resistance in potato (Bryan and Hein, 2008; Govers and Gijzen, 2006; Park *et al*., 2009).

In order to invade plant cells without hindrance, pathogens secrete effector proteins that can manipulate host defense responses thus resulting in effector-triggered susceptibility (ETS) (Jones and Dangl, 2006; Kamoun, 2006, 2007). When an effector is recognized by a resistance (R) protein, effector triggered immunity (ETI) is activated often resulting in a hypersensitive response (HR). Effectors then act as avirulence (Avr) factors and the encoding *Avr* genes interact with *R* genes according to the gene-for-gene model (Flor, 1971). The molecular arms race between the pathogen and its host drives co-evolution of *R–Avr* gene pairs, as has been clearly demonstrated with the *Arabidopsis R* gene *RPP13* and the corresponding *Avr* factor *ATR13* of the oomycete *Hyaloperonospora arabidopsidis* (formerly *H*. *parasitica*) (Allen *et al*., 2004). Both *RPP13* and *ATR13* are highly variable and by examining natural variants of ATR13 key amino acids were identified that are functionally essential for interaction with RPP13 (Allen *et al*., 2008). In *P*. *infestans* only a few *Avr* genes have been studied at the molecular level, so far. *Avr3a*, the counterpart of the *Solanum demissum* gene *R3a*, has two alleles – a virulent and avirulent one – that differ only two amino acids (Armstrong *et al*., 2005). The latter, $Avr3a^{KI}$, not only triggers a *R3a*-dependent HR; it is also capable to suppress a cell death response induced by the elicitin INF1. These two activities, however, are conditioned by distinct amino acids (Bos *et al*., 2009). On the plant side, *R3a* unleashed its evolutionary potential with numerous *R3a*-like genes, which resulted in a major late blight locus on chromosome 11 of *Solanum demissum* (Friedman and Baker, 2007; Huang, 2005; Huang *et al*., 2005). *Avr4*, which interacts with *S*. *demissum R4*, has one predominant avirulent allele in nature but unlike *Avr3a*, the virulent allele has frameshift mutations and can no longer produce an effector protein (Van Poppel *et al*., 2008). Recently, we described the identification of another potential *P*. *infestans Avr* gene, i.e. *ipiO*, the *in planta induced* gene that was postulated as being involved in pathogenicity based on its expression profile (Van West *et al*., 1998). The identification of *ipiO* as *Avr-blb1* resulted from an effector genomics approach that is based on high throughput functional profiling of effector genes in plants carrying *R* genes (Vleeshouwers *et al*., 2008). In the effector screening, two variants of *ipiO*, i.e. *ipiO1* and *ipiO2*, triggered a cell death response in *S*. *bulbocastanum* plants

carrying the late blight *R* gene *Rpi-blb1* (alternatively named RB) (Song *et al*., 2003; Van der Vossen *et al*., 2003; Vleeshouwers *et al*., 2008). Cell death responses to *ipiO1* and *ipiO2* were also noted in *S*. *stoloniferum*, which is the source of the *Rpi-blb1* homologues *Rpi-sto1* and *Rpi-pta1*. Accordingly, agro-coinfiltration of *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* with *ipiO1* and *ipiO2* in *Nicotiana benthamiana* resulted in specific cell death, and provided functional evidence for the *R–Avr* interaction. However, *ipiO4* – a genetically more distant variant – did not elicit cell death when agro-coinfiltrated with *Rpi-blb1*, nor with its homologues. This suggested that alleles or variants of the *ipiO* gene family vary with respect to recognition by *Rpi-blb1* and hence, in avirulence to *Rpi-blb1*.

Like Avr3a and Avr4, IPI-O contains at its N-terminus a signal peptide for type II secretion and a RXLR-dEER motif for host cell internalization whereas the C-terminal domain is required for effector functions (Govers and Bouwmeester, 2008; Rehmany *et al*., 2005; Whisson *et al*., 2007). The RXLR domain of IPI-O partly overlaps with a RGD cell adhesion motif, which has been shown to bind to a lectin receptor kinase in *Arabidopsis* that may function as an effector target (Gouget *et al*., 2006). This lectin receptor kinase participates in protein-protein interactions to mediate cell wall-plasma membrane adhesions and it has been observed that IPI-O can disrupt these adhesions (Senchou *et al*., 2004). In many of the RXLR-dEER effectors the C-terminal domain consists of a variable number of motifs that occur in a repeated fashion (Jiang *et al*., 2008). This allows rapid evolution and diversification within this effector family, which is consistent with a role for *Avr* genes in gene-for-gene interactions with their hosts.

A wealth of resistance (*R*) genes is present in botanical *Solanum* species, but in spite of that, resistance breeding has thus far been unsuccessful. Introgressed *R* genes from e.g. *S*. *demissum* and *S*. *berthaultii* were quickly defeated and virulent *P*. *infestans* isolates were detected in the field sometimes even before introduction into cultivated potato (Flier *et al*., 2003a; Grünwald *et al*., 2001; Rauscher *et al*., 2006; Wastie, 1991). Recently, renewed hope for resistance breeding emerged with the identification of the so-called broad-spectrum *R* genes *Rpi-blb1* and *Rpi-blb2* from *S*. *bulbocastanum* (Song *et al*., 2003; Van der Vossen *et al*., 2003, 2005). Since the introduction in potato, *Rpi-blb1* appears to have remained effective in various geographical areas and over several growing seasons (Colton *et al*., 2006; Helgeson *et al*., 1998; Naess *et al*., 2000). The identification of *ipiO* as the candidate for *Avr-blb1* enables us to address the question how widespread avirulent alleles or variants occur in *P*. *infestans* populations, and related to this, how likely it is that *Rpi-blb1*-mediated resistance will last in the field. In this study, we monitored the genetic variation of *ipiO* in a highly diverse set of *P*. *infestans* isolates and related *Phytophthora* species, and identified 16 naturally occurring *ipiO* variants that could be grouped in three different classes. The *P*. *infestans* isolates lacking one specific class of *ipiO* variants appeared to be virulent on plants

carrying *Rpi-blb1*, thus confirming the gene-for-gene interaction between *ipiO* and *Rpi-blb1*. We also show that IPI-O contains a W motif in the C-terminal domain that is subject to positive selection and that this domain is sufficient to trigger *Rpi-blb1*-dependent cell death.

Results

The *ipiO* gene is highly diverse

For assessing natural genetic variation of *ipiO*, we compiled a set of 29 *P. infestans* isolates that were collected from various geographic regions, including the European potato growing areas and the Central Highlands of Mexico that are both known to harbor genetically highly diverse *P. infestans* populations (Flier, 2001; Flier *et al*., 2003a; Rivera-Peña, 1990a) (Supplementary Table 1). Furthermore, the *P. infestans* isolates originated from diverse host plants, including potato, tomato and botanical *Solanum* species, and they vary for year of collection, mating type, and virulence pattern on the potato *R1-R11* differential set. To examine the genetic diversity we fingerprinted 21 *P. infestans* isolates with SSR markers and showed that nearly all isolates had a unique genotype. Only two isolates, i.e. IPO-0 and UK7824, had identical genotypes, and PRC505705 and PRC506303 have a highly similar genotype. No clear supported branching was observed upon Neighbor Joining in the cluster analysis, illustrating that the assembled set of *P. infestans* isolates is genetically highly diverse (Supplementary Figure 1).

To determine the genetic variation at the *ipiO* loci, we PCR amplified *ipiO* on genomic DNA derived from the 29 *P. infestans* isolates and from five isolates of other clade 1c species, i.e. *P. andina*, *P. ipomoeae*, *P. phaseoli*, and *P. mirabilis* (Blair *et al*., 2008). Sequence analyses revealed 16 variants of *ipiO* with a minimum of one and a maximum of four *ipiO* variants per isolate (Table 1, Figure 1, Supplementary Figure 2). So far, no *ipiO* homologues have been detected in *Phytophthora* species outside clade 1c (data not shown). As described previously, IPI-O1 and IPI-O2 are highly similar with only four different amino acids (Pieterse *et al*., 1994). Most of the newly identified IPI-O variants have amino acid changes due to point mutations, i.e. IPI-O3 to IPI-O9, IPI-O11, IPI-Om1 and IPI-Om2. IPI-O10 is identical to IPI-O2, although this variant contains two nonsynonymous nucleotide polymorphisms. Another variant, IPI-O13 has a C-terminal extension of 10 amino acids compared to IPI-O3. *P. infestans* variant IPI-O12 and *P. phaseoli* IPI-Op1 have frameshift mutations that result in truncated proteins.

Table 1. Occurrence of *ipiO* variants and classes in isolates of *P. infestans* and related species

***Phytophthora* species**	**Isolate**	**I**											**II**		**III**		
		ipiO1	*ipiO2*	*ipiO5*	*ipiO6*	*ipiO7*	*ipiO8*	*ipiO9*	*ipiO10*	*ipiO11*	*ipiO12*	*ipiOm2*	*ipiO3*	*ipiO13*	*ipiO4*	*ipiOm1*	*ipiOp1*
P. infestans	F95573	x	x														
	89148-09	x															
	PIC99177												x				
	88069	x	x										x				
	PIC99189												x		x		
	90128	x	x							x							
	EC1	x	x							x			x				
	H30P04	x								x			x				
	USA618	x	x														
	IPO-0	x													x		
	IPO-C	x											x				
	PIC99183									x	x						
	NL01096	x	x										x				
	VK98014	x	x											x			
	IPO428-2	x	x							x							
	NL00228	x	x											x			
	DDR7704		x														
	UK7824		x												x		
	89094			x			x	x					x				
	91011	x			x	x			x								
	PIC97757	x			x	x			x								
	IPO98014			x			x	x					x				
	NL050105		x										x				
	NL05194		x										x				
	PRC505705		x														
	PRC506303		x														
P. infestans sensu lato	EC3260														x		
	EC3394														x		
	EC3364	x	x										x				
P. andina	EC3414														x		
P. ipomoeae	PIC99193											x					
P. phaseoli	CBS556.88																x
P. mirabilis	PIC99111											x					
	CBS150.88															x	

Phylogenetic analyses on the protein alignment of the 16 IPI-O variants showed a grouping into three classes, which were designated as class I, II and III (Figure 2). Phylogenetic trees based on Neighbor-joining, minimal evolution or maximum parsimony algorithms resulted in similar clustering (not shown) and also analyses based on nucleotide alignments yielded comparable results. Most IPI-O variants group in class I, together with IPI-O1 and IPI-O2. Class II is significantly different from class I and includes IPI-O3 and IPI-O13. Class III comprises IPI-O4, which has 16 amino acid differences compared to the class I and II IPI-O variants and is the most divergent IPI-O variant in *P. infestans*.

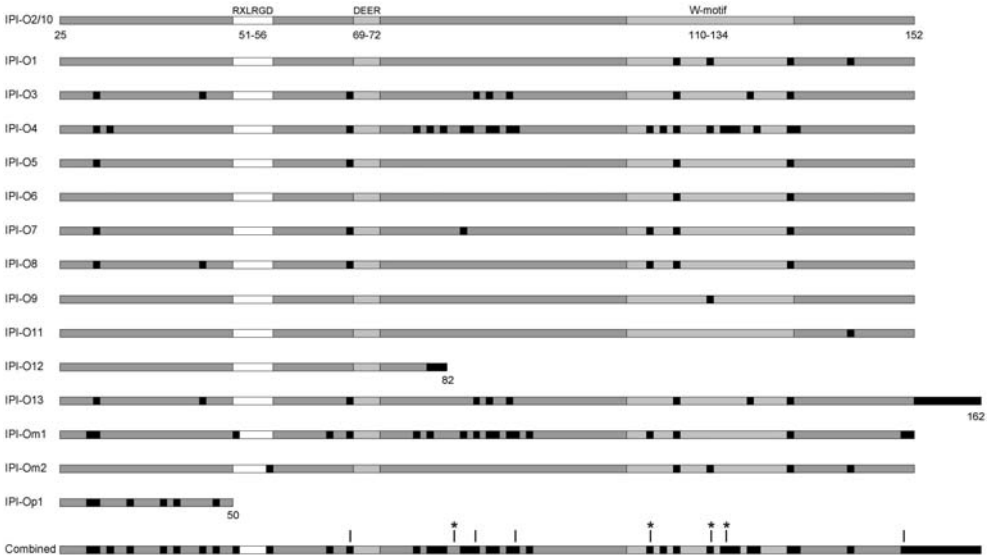

Figure 1. Thirteen variants of the mature IPI-O protein. The motifs RXLR, RGD and DEER and a predicted W motif are indicated. Numbers refer to the amino acid positions. Amino acid polymorphisms in IPI-O variants, as compared to IPI-O2, are depicted in black. In the lower bar (combined) all the amino acids that show polymorphism are indicated. The vertical lines refer to positively selected sites (see Table 2). *= P>99%.

IPI-O has one W motif with several positively selected sites

The majority of the RXLR-dEER effectors contain positively selected amino acid residues in the C-terminus (Jiang *et al*., 2008; Win *et al*., 2007). To investigate whether *ipiO* is also under diversifying selection, we assessed positive selection per residue on two sets of *ipiO* variants. The first set, called Pi, is composed of the 11 full length *ipiO* variants as found in *P. infestans*. The other set, Pi+Pm, includes the variants detected in the sibling species *P. mirabilis*, in addition to the Pi set.

Of the different evolutionary models (Yang *et al*., 2005), model M2a for positive selection fits well on both data sets with ℓ values of -822.55 and -960.76 for Pi and Pi+Pm, respectively. With this model, several positively selected amino acid residues were identified, four of which overlap in the two data sets. Also the selection model M8 gave high log likelihood values for both sets. For the Pi set, M2a and M8 identified the same seven positively selected sites. For the Pi+Pm set, M8 identified the same set of four overlapping positively selected sites and four additional ones, one of which is also selected by the M2a model for the Pi+Pm set (Table 2).

In a recent study that used Hidden Markov Model (HMM) searches to find motifs in RXLR-dEER effectors, it was shown that many of the RXLR-dEER effectors contain conserved C-terminal motifs that may occur in repeated fashion (Jiang *et al*., 2008). These motifs were named W, Y and L after the amino acid at a fixed position in each motif. IPI-O contains a single W motif with moderate to strong HMM scores ranging from 6.5 to 12.3 among the IPI-O variants (Figure 1; Supplementary Figure 3). Interestingly, three of the four positively selected sites that overlap in the M2a and M8 model of the two sets have a high posterior probability (P>99%) and are located within the conserved W motif (Figure 1).

Table 2. Evidence for positively selected sites in *ipiO*

	Model code	Parameters estimates	ℓ [a]	Positively diversified codons [b]
Pi [c]	M0: one ratio	ω=1.089	-839.78	Not allowed
	M1a: nearly neutral	ω0=0, ω1=1, *p*0=0.580, *p*1=0.420	-835.50	Not allowed
	M2a: positive selection	ω0=0, ω1=1, ω2=28.638, *p*0=0.353, *p*1=0.637, *p*2=0.010	**-822.55**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
	M7: beta	*p*=0.012, *q*=0.005	-837.03	Not allowed
	M8: beta & ω	*p*0=0.990, *p*1=0.010, *p*=0.008, *q*=0.005, ω=27.108	**-822.57**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
Pi + Pm [d]	M0: one ratio	ω=0.970	-980.05	Not allowed
	M1a: nearly neutral	ω0=0, ω1=1, *p*0=0.599, *p*1=0.401	-972.94	Not allowed
	M2a: positive selection	ω0=0.476, ω1=1, ω2=12.487, *p*0=0.971, *p*1=0, *p*2=0.028	**-960.76**	85M*, 93G, 113A*, 122R*, 124L
	M7: beta	*p*=0.005, *q*=0.007	-972.94	Not allowed
	M8: beta & ω	*p*0=0.983, *p*1=0.017, *p*=0.005, *q*=0.005, ω=14.665	**-960.34**	68S, 85M*, 87L, 93G, 113A*, 122R*, 124L*, 151P

[a] Log likelihood value; [b] Bayes Empirical Bayes (BEB) analysis (Yang *et al*., 2005); Positively selected sites (P>95%, *=P>99%); [c] based on full length *P. infestans ipiO* variants, n=11; [d] based on full length *P. infestans* and *P. mirabilis ipiO* variants, n=13

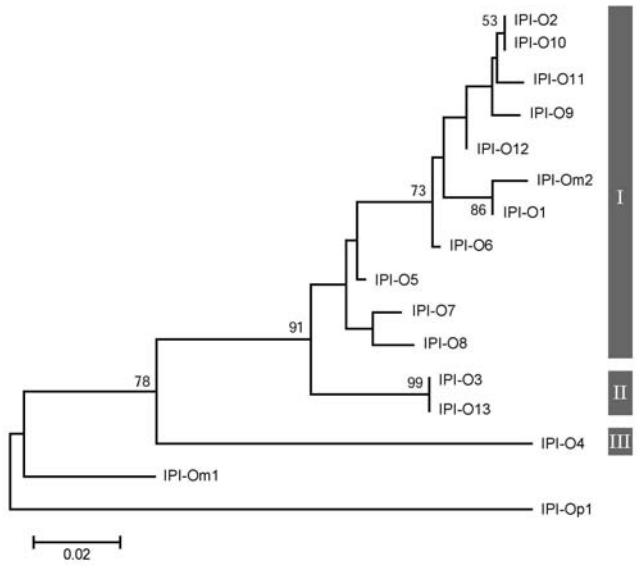

Figure 2. Phylogenetic relationship and clustering of the IPI-O variants. The minimum evolution (ME) tree was rooted with IPI-Op1. Rooting with IPI-Om1, IPI-O4, or *P*. *sojae* Avr1b resulted in similar clustering. Bootstrap values of 1000 replicates are indicated at the nodes*;* values less than 50% are omitted (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Class I, II and III IPI-O variants are indicated.

The region comprising the W motif is sufficient to trigger *Rpi-blb1*-mediated cell death

To investigate whether or not the W motif is involved in triggering *Rpi-blb1*-mediated cell death we analyzed several deletion mutants of *ipiO2* (Figure 3A). In co-agroinfiltration assays in *N*. *benthamiana* IPI-O2 – with or without its signal peptide – triggers cell death in the presence of Rpi-blb1. Deleting the domain comprising the RXLR, RGD and dEER motifs did not abolish recognition and even an additional deletion of the first 26 amino acids of the C-terminal domain did not change the cell death response. Agroinfection and rub-inoculation assays on *S*. *stoloniferum* accession sto17605-4, which harbors the *Rpi-blb1* homologue *Rpi-sto1*, resulted in similar responses and showed that recognition of IPI-O by *Rpi-sto1* follows the same pattern. The results show that the region spanning the last 54 amino acids of IPI-O and comprising the W motif is sufficient for recognition by Rpi-blb1 and Rpi-sto1. Since the only mutations that are consistent between, on the one hand, the class I and II variants and, on the other hand, the class III variant are located within the W motif it is conceivable that this motif plays a role in recognition of IPI-O by Rpi-blb1.

IPI-O variants of class I and II, but not class III, trigger *Rpi-blb1*-mediated cell death

In a previous study, we showed that the class I *ipiO* variants, *ipiO1* and *ipiO2*, trigger *Rpi-blb1*-mediated cell death. To assess whether the newly identified *ipiO* variants are also recognized by *Rpi-blb1* we used agroinfiltration in *Nicotiana benthamiana* to reconstruct the interaction between the *ipiO* variants and *Rpi-blb1*. Coinfiltration of *N*. *benthamiana* leaves with an *A*. *tumefaciens* strain carrying a construct expressing *Rpi-blb1* as well as a strain carrying a construct expressing either class I *ipiO* genes (*ipiO1*, *ipiO2*, *ipiO5*, *ipiO7*, and *ipiO8*), or class II *ipiO* genes (*ipiO3*) resulted in a confluent cell death response (Figure 3B). Also class I *ipiO* variant *ipiOm2* of *P*. *mirabilis* co-infiltrated with *Rpi-blb1* resulted in cell death (data not shown). In contrast, co-expression of *Rpi-blb1* with the class III *ipiO4* gene did not elicit a *Rpi-blb1*-mediated response, as no visible cell death was observed in the infiltrated leaves.

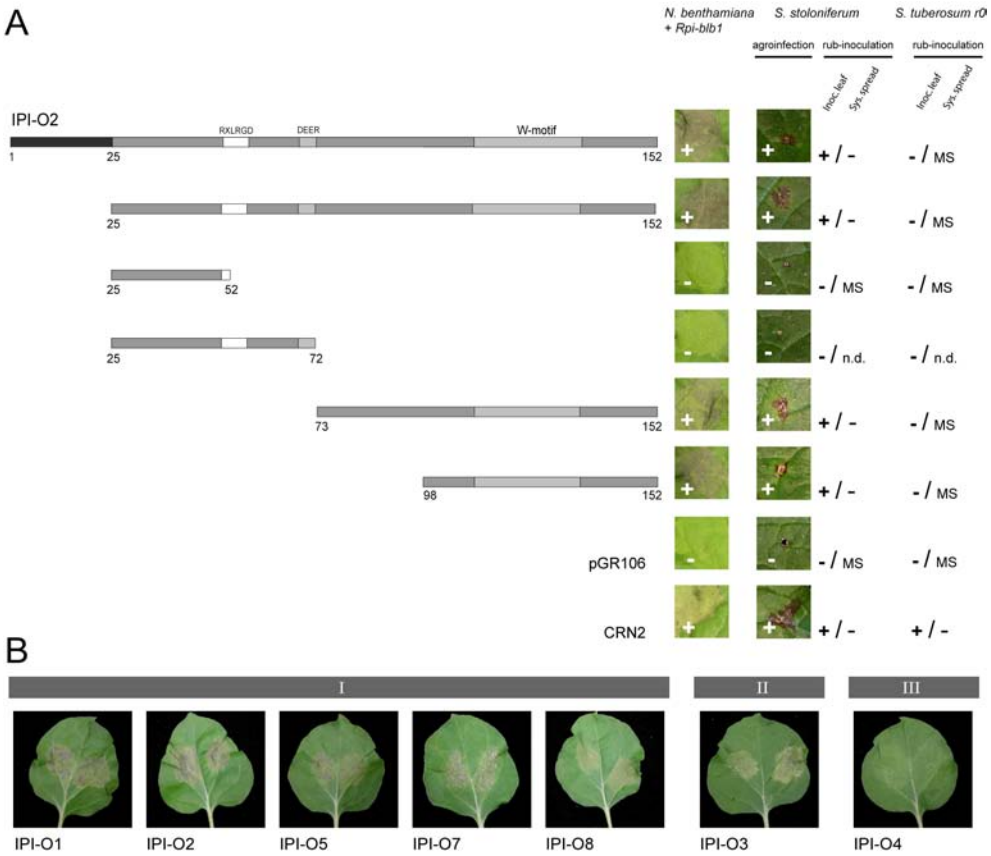

Figure 3. (A) The C-terminus of IPI-O comprising the W motif is sufficient for recognition by Rpi-blb1. Deletion mutants of *ipiO*2 were co-agroinfiltrated with *Rpi-blb1* in *N. benthamiana* or were agroinfected or rub-inoculated with PVX particles carrying the deletion mutants on *S. stoloniferum* accession 17605-4. Potato cultivar Bintje was used as control. Mosaic symptoms (MS) indicate virus spread. Pictures were taken at 5 days after infiltration or agroinfection. (B) Class I and II IPI-O variants are recognized by Rpi-blb1. *N. benthamiana* leaves were agroinfiltrated – at both sides of the leaf midrib – with *Rpi-blb1* in combination with *ipiO* variants of class I, II or III. Pictures were taken at 5 days after infiltration.

IpiO variants are expressed *in planta*

To enable expression analyses of *ipiO* variants belonging to the three classes, class specific primers were designed that were tested for specificity on genomic DNA (Figure 4). Subsequently, RNA isolated from potato leaves infected with *P. infestans* isolates PIC99183, PIC99189 and PIC99177 was analyzed by semi-quantitative RT-PCR. As shown in Figure 4, mRNA derived from class I, II, and III *ipiO* genes is present demonstrating that all three classes comprise functional genes that are expressed during *in planta* growth.

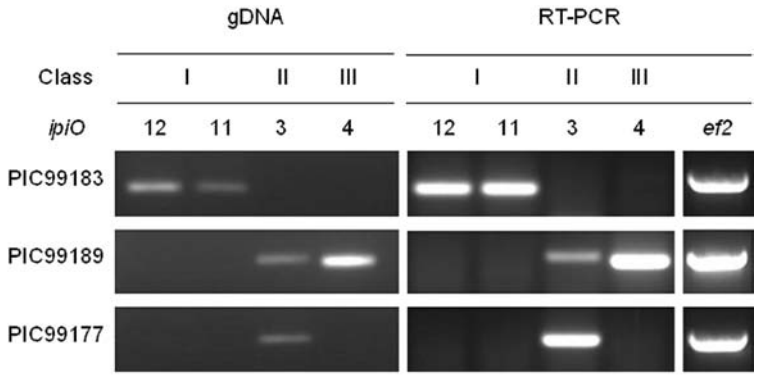

Figure 4. Expression of *ipiO* in infected potato leaves. *IpiO* class specific primers were used for semi-quantitative RT-PCR on RNA isolated from infected leaf tissue of potato cultivar Bintje at 6 dpi. To demonstrate that the primers are specific for each of the three *ipiO* classes, genomic DNA of *P. infestans* isolates PIC99183, PIC99189 and PIC99177 was used as template and detection of specific *ipiO* variants was confirmed. The *P. infestans* elongation factor 2 gene (*ef2*) was used as a control to determine the integrity of the RNA.

Isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* plants

To test whether the identified *ipiO* variants determine *Rpi-blb1*-mediated cell death, we performed infection assays using *P. infestans* isolates that are genetically diverse and carry different classes of *ipiO* variants (Figure 1; Table 1; Supplementary Figure 1). To allow a correct interpretation of the virulence phenotypes of the isolates on *Rpi-blb1* plants we first tested 16 selected isolates for their infection capabilities on potato. Detached leaves of universal susceptible potato cultivar Désirée were inoculated and at 6 days post inoculation (dpi) lesion diameters were measured. Based on the lesion size (LS) the isolates were grouped in three classes of aggressiveness (Supplementary Table 3).

To investigate the specificity spectrum of *Rpi-blb1*, we inoculated the 16 isolates on the *S. bulbocastanum* accession blb8005-8, which is the genotype from which

Rpi-blb1 was isolated, and included Désirée and *S*. *bulbocastanum* blb2002 – containing *Rpi-blb2* – as susceptible and resistant controls, respectively (Table 3). Lesion diameters were measured at 4, 5, and 6 dpi and LS, lesion growth rates (LGR) and infection efficiency (IE) were calculated. Large lesions exceeding 25 mm^2 always coincided with massive sporulation and were scored as compatible interactions. In contrast, smaller lesions typically did not sporulate or showed a 'black-spot' phenotype, indicating an HR. As expected for the so-called 'broad-spectrum' *R* gene *Rpi-blb1*, blb8005-8 was incompatible with nearly all isolates. Two Mexican isolates PIC99177 and PIC99189 however, were clearly compatible with blb8005-8 and both developed sporulating lesions on blb8005-8 leaves.
To investigate the correlation between compatibility or incompatibility, and *ipiO* variants, we compared the virulence phenotypes of the isolates on *Rpi-blb1*-containing host plants with the occurrence or the absence of specific *ipiO* variants. All avirulent isolates contained at least one class I *ipiO* variant (Table 1; Table 3). In contrast, no class I *ipiO* variants were found in the two virulent isolates PIC99189 and PIC99177; only class II and/or III *ipiO* variants were detected. These results suggest that class I *ipiO* variants determine avirulence of *P*. *infestans* isolates on *Rpi-blb1* plants.

To verify these findings, we tested the *P*. *infestans* isolates on transgenic lines of cultivar Impala and cultivar Désirée containing *Rpi-blb1* as transgene. In general, *Rpi-blb1*-meditated resistance levels in the potato transgenic lines were lower than in its wild *Solanum* background, blb8005-8 (Table 3). In accordance with the previous experiment, isolate PIC99189 was able to establish sporulating lesions on transgenic Impala RGC-2A9 expressing *Rpi-blb1*, whereas isolates IPO-C and 90128 displayed an HR (Figure 5). On five Désirée *Rpi-blb1* transformants including A01-20, we quantitatively assessed the resistance levels. The virulent isolates PIC99177 and PIC99189 infected A01-20 (Table 3) and the other four transgenic lines (data not shown) equally well as the Désirée control plants (ANOVA, $P<0.05$). To the other 14 isolates, the *Rpi-blb1* transgene conferred enhanced resistance at various levels, and generally the level of resistance negatively correlated with the aggressiveness of the isolates. The moderately aggressive isolates F95573, 89148-09 and 88069 reached only low levels of IE and LGR on A01-20, and *Rpi-blb1* clearly conferred a high level of resistance. Highly aggressive isolates, however, achieved slightly reduced or similar IE and LGR on A01-20 as compared to Désirée control plants, and were often able to establish high percentages of fast growing lesions on A01-20, despite the fact that these isolates contain a class I *ipiO* variant. Obviously the aggressiveness of the isolates overrules the recognition by *Rpi-blb1* in the transgenic potato background. The observation that the level of resistance conferred by *R* genes is influenced by the genetic background in which they reside, is in line with previous studies.

For example, it was shown that expression of *Rpi-blb1* in *S*. *bulbocastanum* is dramatically higher than in potato lines carrying *Rpi-blb1* as a transgene (Bradeen *et al*., 2009; Kramer *et al*., 2009).

Table 3. *P*. *infestans* isolates virulent on *Solanum* plants containing *Rpi-blb1* lack class I *ipiO* variants

***P. infestans* isolate** [a]	**Plant material**												**Phenotype on *Rpi-blb1* plants** [c]	***ipiO* class**		
	blb8005-8		sto17605-4		pta17831-8		A01-20		Désirée		blb2002					
	IE [b]	LGR [b]	IE	LGR	IE	LGR	IE	LGR	IE	LGR	IE	LGR		I	II	III
F95573	0	0	0	0	0	0	3	1.4	69	4.4	0	0	A	x		
89148-09	0	0	0	0	0	0	16	1.1	69	2.7	0	0	A	x		
88069	0	0	0	0	0	0	22	0.6	100	3.2	0	0	A	x	x	
90128	0	0	0	0	0	0	50	3.7	78	3.4	0	0	A	x		
EC1	0	0	0	0	0	0	31	2.5	84	3.7	0	0	A	x	x	
IPO-0	0	0	0	0	0	0	78	3.4	91	3.8	0	0	A	x		x
PIC99183	0	0	50	0.5	63	1.2	88	3.6	91	3.6	0	0	A	x		
H30P04	0	0	0	0	0	0	9	2.4	100	4.4	0	0	A	x	x	
IPO-C	0	0	0	0	0	0	50	2.9	100	3.9	0	0	A	x	x	
USA618	0	0	0	0	0	0	19	3.5	100	3.4	0	0	A	x		
NL01096	0	0	0	0	0	0	66	3.4	100	3.8	0	0	A	x	x	
VK98014	0	0	0	0	0	0	66	1.8	100	4.7	0	0	A	x	x	
IPO428-2	0	0	0	0	0	0	38	1.9	100	4.2	0	0	A	x		
NL00228	0	0	0	0	0	0	47	4.6	100	4.8	0	0	A	x	x	
PIC99177	72	1.1	75	2.6	81	2.9	50	2.8	88	3.0	0	0	V		x	
PIC99189	34	1.1	75	0.9	100	4.3	91	4.5	91	3.6	0	0	V		x	x

[a] Additional information about the aggressiveness of the *P*. *infestans* isolates on potato cultivar Désirée can be found in Supplementary Table 3. [b] Isolates were inoculated on different *Solanum* plants containing *Rpi-blb1* or its homologues and mean infection efficiency (IE) and lesion growth rate (LGR) were determined. [c] A = avirulent; V = virulent.

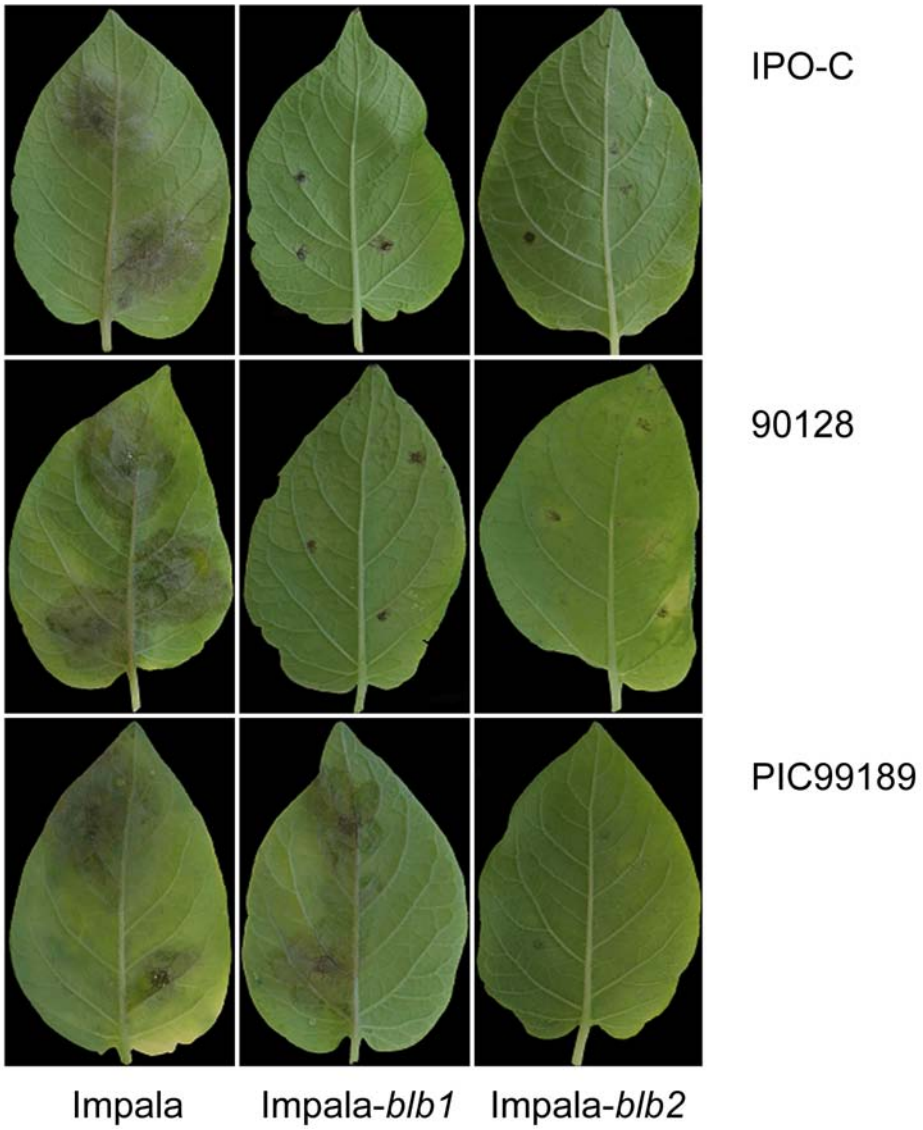

Figure 5. *Rpi-blb1* transgenic potato lines are resistant to *P*. *infestans* isolates carrying *ipiO* class I and/or II variants. Pictures were taken at 6 dpi.

Recognition specificity of *ipiO* variants by *Rpi-blb1* is conserved in *Rpi-sto1* and *Rpi-pta1*

Recently, we described the identification and cloning of functional homologues of *Rpi-blb1* in the distantly related *Solanum* species *S*. *stoloniferum* (Vleeshouwers *et al*., 2008). The homologues *Rpi-sto1* and *Rpi-pta1* are nearly identical to *Rpi-blb1*; they only differ in 3 and 5 non-synonymous nucleotide substitutions, respectively. To test these *R* genes for *ipiO* specificity, we co-infiltrated *N*. *benthamiana* leaves with *A*. *tumefaciens* strains carrying constructs expressing either *Rpi-sto1* or *Rpi-pta1* combined with *A*. *tumefaciens* strains expressing *ipiO* variants of class I, II or III. Leaves co-infiltrated with the *Rpi-blb1* homologues and class I or class II *ipiO* variants showed a confluent cell death response, but leaf panels co-infiltrated with class III *ipiO* did not show cell death (Supplementary Figure 4). These results demonstrate that similar to *Rpi-blb1*, *Rpi-sto1* or *Rpi-pta1* display differential specificity towards the different classes of *ipiO* variants.

We also investigated how *S*. *stoloniferum* sto17605-4 and pta17831-8 responded to infection with the 16 *P*. *infestans* isolates that posses different variants of *ipiO*. In detached leaf assays, infection of sto17605-4 and pta17831-8 with the isolates PIC99189 and PIC99177 resulted in sporulating lesions, in accordance with the results obtained with blb8005-8. Of the other 14 isolates only PIC99183 formed a few lesions on sto17605-4 and pta17831-8. There was, however, no biotrophic growth nor sporulation and the lesions expanded very slowly. Since the LGR and IE parameters were much lower than expected for such highly aggressive isolate we suspect that the necrotic spots are due to a trailing HR following invasion attempts, but not to specific virulence of PIC99183 on *S*. *stoloniferum* sto17605-4 and pta17831-8.

Discussion

Rpi-blb1 is classified as a so-called broad-spectrum *R* gene that confers resistance to a broad range of *P*. *infestans* isolates (Song *et al*., 2003; Van der Vossen *et al*., 2003). In a recent study we identified the RXLR-dEER effector IPI-O as the candidate for the cognate Avr factor of Rpi-blb1 (Vleeshouwers *et al*., 2008). In this study, we detected isolates that are virulent on Rpi-blb1 plants, and showed that *Rpi-blb1 – ipiO* is an *R–Avr* pair that basically interacts according to the gene-for-gene model (Flor, 1971). IPI-O is the effector that triggers ETI in plants carrying *Rpi-blb1* whereas changes in the IPI-O effector repertoire result in loss of ETI.

The *ipiO* gene family is highly diverse but restricted to *Phytophthora* species that belong to clade 1c. Profiling 34 isolates of *P*. *infestans* and its close relatives revealed 16 *ipiO* variants, 14 of which were grouped in three distinct classes. In *P*. *infestans* sensu latu, *P*. *andina*, *P*. *ipomoeae*, *P*. *phaseoli*, and *P*. *mirabilis*,

which all evolved on host species distant from *Solanum* section Petota, a more or less equal distribution of class I, II, and III variants was found and, in addition, two more distant *ipiO* variants were detected. In *P. infestans* however, class I was significantly expanded, with one to four class I variants in most isolates, of which the majority also has one class II and/or one class III variant. Class I includes *ipiO1* and *ipiO2*, the variants that were already isolated in the early 1990s (Pieterse *et al*., 1994), and more recently identified as *Avr-blb1* (Vleeshouwers *et al*., 2008). Disease testing on *Solanum* species showed that all isolates with class I *ipiO* variants were avirulent on *Rpi-blb1* plants, which is in line with the previously reported broad-spectrum character of this *R* gene (Song *et al*., 2003; Van der Vossen *et al*., 2003). In contrast, two Mexican *P. infestans* isolates, PIC99189 and PIC99177, that both lack class I *ipiO* variants appeared to be virulent on *Rpi-blb1* plants. These data suggest that absence of class I *ipiO* genes is correlated with virulence on *Rpi-blb1* plants, and this is supported by the observation that co-infiltration of class I *ipiO* variants and *Rpi-blb1* in *N. benthamiana* leaves results in cell death. Apparently, class I IPI-O variants elicit HR in *Rpi-blb1* plants and this arrests pathogen invasion. Class III *ipiO* appeared unable to induce cell death when co-infiltrated with *Rpi-blb1*, thus strongly suggesting that the presence of class III *ipiO* in *P. infestans* strains is indeed unlikely to confer avirulence. It should be noted, though, that we have not used epitope tagged-constructs in our *in planta* expression assays to monitor the stability of the various IPI-O variants and, hence, can not exclude the possibility that class III IPI-O is less stable than class I or II IPI-O. A more puzzling issue is the finding that class II variants elicit cell death when co-infiltrated with *Rpi-blb1* in *N. benthamiana* leaves. The class II variant *ipiO3* was found to be expressed *in planta* in the virulent strain and one would expect that the presence of a class II variant together with the Rpi-blb1 resistance protein in one cell would lead to HR. This is not the case and raises the question how the numerous RXLR effectors that are predicted to be targeted to the host cell interact with each other. In reconstruction experiments, for example, some RXLR effectors suppress cell death induced by other effectors such as elicitins or BAX (Bos *et al*., 2009; Bouwmeester *et al*., 2009; Dou *et al*., 2008). *In vivo*, there could well be a kind of synergism between class I and class II IPI-O variants or even other RXLR effectors. For example, when class I is lacking the class II variant might not be potent enough to act as Avr factor by itself. Alternatively, the presence of a class I variant could suppress the virulence function of a class II variant by competition for the same virulence target. One can also not rule out the possibility that the class II variants are less stable *in planta* than the class I variants. The finding of a class III variant that is not recognized by *Rpi-blb1* but is expressed *in planta* makes the situation even more complex.

Nevertheless, the clear distinction between the three classes helped us to define which parts of the protein are involved in recognition by Rpi-blb1. Deletion analysis showed that the C-terminal part of the IPI-O effector protein is required for recognition, which is in line with other studies (Bos *et al*., 2006; Dou *et al*., 2008). The smallest fragment that we tested is 54 amino acids in length and comprises the single W motif that is present in IPI-O. For *Phytophthora sojae* Avr1b, specific amino acid residues in the W and Y motifs in Avr1b are responsible for recognition by the RPS1 protein as well as suppressor activity of cell death. In IPI-O, the W motif is the region that is the most divergent in the class III variants and the three amino acids that show positive selection are all located within the W motif. More detailed analysis of the role of each individual amino acid residue in the W motif will reveal the exact determinants of recognition by Rpi-blb1.

In recent years, insights into *R* gene-based resistance in potato and the role of cognate *Avr* genes from *P*. *infestans* have increased. For example, many studies were performed for the *R3a-Avr3a* model system. The *R3a*-harboring species *S*. *demissum* coexists with *P*. *infestans* in the cool and humid mountain forests in Toluca Valley, a perfect condition for a tight co-evolution between *R3a* and *Avr3a*. *R3a* is a typical fast evolving type I *R* gene (Huang, 2005; Kuang *et al*., 2005) resulting in numerous *R3a*-like genes (Friedman and Baker, 2007; Huang, 2005). For Avr3a, only two alleles have been detected; $Avr3a^{EM}$ is present in most *P*. *infestans* isolates world-wide (Armstrong *et al*., 2005; Rivera-Peña, 1990b), whereas $Avr3a^{KI}$ is much less abundant. $Avr3a^{KI}$ but not $Avr3a^{EM}$ is recognized by R3a thus resulting in defeat of *R3a* by most *P*. *infestans* isolates. *R1*, *R2*, and *R4* also originate from *S*. *demissum* and similar to *R3a*, these *R* genes were quickly defeated in the field (Fry, 2008). Similar to *Avr3a*, *Avr4* alleles in field isolates show very little variation and in all virulent strains, the *Avr4* gene is out of frame due to a one base pair deletion (Van Poppel *et al*., 2008). Preliminary analysis of *Avr1* and *Avr2* indicate that also these *Avr* genes are represented by only a few alleles (F. Govers, P. Birch and E. Gilroy, personal communication). A completely different scenario exists for the *Rpi-blb1–ipiO* interaction. *Rpi-blb1* originates from *S*. *bulbocastanum*, which occurs in more arid climates and most likely has less intensive encounters with *P*. *infestans*. Presence of *Rpi-blb1* homologues in other Mexican species such as *S*. *stoloniferum* (Wang *et al*., 2008) that partly grow in *P*. *infestans* conducive climates, creates the opportunity for virulent strains to evolve on these species. Indeed, the virulent strains PIC99189 and PIC99177 described in this study were collected from *S*. *stoloniferum* host plants in Mexico (Flier *et al*., 2002). Both *Rpi-sto1* and *Rpi-pta1* are almost identical to *Rpi-blb1*, and *Rpi-blb1* fulfills the criteria of a type II *R* gene with only little diversifying selection that is typically slow evolving (Kuang *et al*., 2005; Liu and Halterman, 2006). Thus, for the *Rpi-blb1–ipiO* interaction, not the *R* gene, but the *Avr* gene is represented

by a highly diverse and extensive gene family. The notable expansion of class I *ipiO* in *P. infestans*, but not in related *Phytophthora* species infecting other plant species might be due to a certain degree of co-evolution between *Avr-blb1* and *Rpi-blb1* homologues in *Solanum* host plants. Also *Rpi-blb2*, another broad-spectrum *S. bulbocastanum R* gene that is not (yet) defeated, is interacting with a highly diverse *Avr* gene family, and perhaps such *Avr* genes may be less easy to overcome (S. Kamoun, personal communication).

In the wild *Solanum* species that contain *Rpi-blb1* or a functional homologue, full resistance was conferred to all tested avirulent *P. infestans* isolates, with the exception of the two Mexican strains. The transfer of *Rpi-blb1* into potato cultivars resulted in improved protection to *P. infestans* isolates. The enhanced resistance however, could only arrest the growth of mild isolates. Aggressive isolates were not blocked and could still cause high percentages of lesions expanding at high rates. The influence of the genetic background on the performance of *Rpi-blb1* was also reported in other studies, in which the basal *Rpi-blb1* expression levels were found dramatically higher in its wild *S. bulbocastanum* origin compared to transgenic potato (Bradeen *et al*., 2009; Kramer *et al*., 2009). Also the increase in expression after *P. infestans* inoculation was higher. These studies suggest that expression of *Rpi-blb1* as transgene in potato is not high enough to provide satisfactory resistance in the field, and this implies that stacking with other *R* genes, or engineering enhanced transgene expression in cultivars, is recommended.

The future of late blight resistance breeding is controlled application of well-studied *R* genes in high quality potato cultivars, and devising inherent durability predictions based on the interacting *Avr* gene. Potato cultivars engineered with *Rpi-blb1* and *Rpi-blb2* are expected to be the first GM potatoes to be cultivated for consumption purposes in Europe (Application GM field trial 2005). *Rpi-blb1* is still effective to a broad range of isolates; virulent isolates similar to PIC99189 and PIC99177 have not (yet) been detected in the Netherlands and neighboring countries and class I *ipiO* is well-represented in the *P. infestans* isolates analyzed thus far. Future large-scale monitoring aimed at diagnosing the *ipiO* classes in the *P. infestans* population can help answering the question whether virulence to *Rpi-blb1* is evolving in commercial potato growing areas, or whether accidental introduction of potentially virulent *P. infestans* has occurred. When the first virulent isolates are detected, selection pressure towards losing class I *ipiO* might be avoided by omitting cultivars with *Rpi-blb1* for a certain period of time and apply other *R* genes in stead.

Materials and Methods

Phytophthora isolates, culture conditions and inoculum preparation

The *Phytophthora* isolates used in this study were retrieved from our in-house collection or were provided by colleagues. *Phytophthora* isolates were routinely grown in the dark at 15℃ in liquid Plich medium (V an der Lee *et al*., 1997) prior to DNA extraction (Lees *et al*., 2006), or on solid rye sucrose medium (Caten and Jinks, 1968) prior to disease tests. To isolate zoospores for plant inoculations, sporulating mycelium was flooded with cold water and incubated at 4℃ for 1-3 h.

Cloning of *ipiO* variants

Primers (Supplementary Table 2) and *Pfu* DNA polymerase (Promega) were used to amplifiy *ipiO* on genomic DNA. After 30 cycles Super*Taq* polymerase (HT Biotechnology) and its buffer were added followed by 15 minutes at 72℃. The obtained amplicons were cloned into pGEM®-T Easy vector (Promega) and transformed in DH5α competent cells (Invitrogen). Sequencing was performed using universal M13 primers and DNA sequences were analyzed using DNAstar v6, Chromas 2.3 (Technelysium) and Vector NTI software. DNA sequences were deposited in NCBI GenBank under the accession numbers GQ371190-GQ371203.

SSR genotyping

SSR genotyping was performed using two multiplex set of four SSR markers each (SSR1, SSR3, SSR7, SSR11 and SSR2, SSR4, SSR6, SSR8). Experimental details can be found at the Eucablight site (http://www.eucablight.org/EucaBlight.asp).

Phylogenetic data analyses

The SSR data were analyzed by the phylogenetic software package TREECON® for Windows Version 1.3b (Van de Peer and de Wachter, 1994). The evolutionary distance estimation was performed according to Nei and Li (1979) and clustering was performed using the Neighbor Joining algorithm. The tree was rooted using isolate IPO-0. Bootstrap values in percentage (>60) from 1000 replicate trees are shown at the nodes. The scale bar shows genotype divergence in percentage. Phylogenetic analyses of *ipiO* sequences were conducted using the Minimum Evolution (ME) method (Rzhetsky and Nei, 1992) in MEGA version 4 (Tamura *et al*., 2007).

Positive selection analysis

To test for amino acids under purifying or diversifying selection we used codon-based analysis (Codeml) implemented in PAML v.4 package (Yang, 2007). Maximum-likelihood codon substitution models M0, M1a, M2a, M7, and M8 were

used for analysis. Models M2a and M8 are capable to detect sites under positive selection. Bayes Empirical Bayes statistics was used to calculate positively selected sites with high posterior probability (Yang *et al*., 2005).

IpiO expression analysis

The zones surrounding the water-soaked lesions, where *ipiO1* is known to be highly expressed (Van West *et al*., 1998), were cut from infected leaves of cultivar Bintje at 6 dpi. RNA was isolated using the RNeasy Mini Kit (QIAGEN), incubated with DNase and purified with the RNA clean-up protocol. The purity of the RNA was confirmed on gel. Semi-quantitative RT-PCR was performed with the OneStep RT-PCR kit (QIAGEN) using the specific primers RT-ipiO-I-F and RT-ipiO-I12-R for *ipiO12* (class I), RT-ipiO-I-F and RT-ipiO-I11-R for *ipiO11* (class I), RT-ipiO-II-F and RT-ipiO-II-R for *ipiO3* (class II), and RT-ipiO-III-F and RT-ipiO-III-R for *ipiO4* (class III) (Supplementary Table 2). *P*. *infestans* elongation factor 2 gene (*ef2*) was used as a control (Torto *et al*., 2002).

Plant material and generation of *Rpi-blb1* transgenic potato plants

Solanum plant material used is this study is listed in Supplementary Table 4. Potato cultivars and wild *Solanum* accessions were obtained from our-in-house collection and the Center of Genetic Resources CGN, Wageningen, The Netherlands, respectively (http://www.cgn.wur.nl/uk). *Solanum* plants were maintained *in vitro* on Murashige and Skoog medium (Duchefa) supplemented with 20% sucrose (MS20) in climate chambers at 18℃ with a 16h photoperiod. Top shoots were transferred to fresh medium and 1-2 weeks later rooted plantlets were transferred to the soil and grown under greenhouse conditions. *N*. *benthamiana* plants used for agroinfiltration were grown in climate chambers at 22-25℃ and high light intensity. The generation of the *Rpi-blb1* transformant in cultivar Impala has been described previously (Van der Vossen *et al*., 2003), and the transformant A01-20 of cultivar Désirée was generated using similar procedures. Briefly, the binary vector pBINPLUS containing *Rpi-blb1* under control of its native promoter and terminator (Van der Vossen *et al*., 2003) was transformed to *A*. *tumefaciens* strain COR308 and introduced into cultivar Désirée according to standard protocols (Visser *et al*., 1991). Regenerative shoots were transferred on solid selective medium Zcvk (MS20 with zeatine 1 mg l^{-1}, claforan 200 mg l^{-1}, vancomycine 200 mg l^{-1} and kanamycine 100 mg l^{-1}) and transformants to solid MS30 with 100 mg l^{-1} of kanamycine.

Disease tests

Leaves from 6-8 week old plants were detached and placed in water-saturated oasis in trays, according to (Vleeshouwers *et al*., 1999). Leaves were spot-inoculated at the abaxial leaf side with 10 µl droplets containing $5*10^4$ zoospores ml^{-1} and incubated in a climate chamber at 15℃ with a 16h photoperiod. Lesion diameters were measured at 4, 5, and 6 dpi. The area of the lesions (LS), the infection efficiency (IE) representing the percentage of successful infections were calculated, and the lesion growth rate (LGR) was estimated using linear regression in GenStat 10.

In planta expression assays

IpiO variants without signal peptide were introduced in pK7WG2 and pGR106 (Karimi *et al*., 2002). *R3a*, *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* with their native expression elements were introduced into the pBINPLUS binary vector (Van Engelen *et al*., 1995). *Agrobacterium tumefaciens* strain GV3101, and AGL1 (Lazo *et al*., 1991) in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (Van der Fits *et al*., 2000), were used for transformation. For agroinfiltration, *A*. *tumefaciens* strains were grown as described previously (Van der Hoorn *et al*., 2000) to a final OD600 of 0.4. Leaves of 4-5 week old *N*. *benthamiana* plants were infiltrated with the *A*. *tumefaciens* suspensions (at a 1:1 ratio for co-infiltration) in MMA induction buffer (1 l MMA: 5 g MS salts, 1.95 g MES, 20 g sucrose, 200 µM acetosyringone, pH 5.6), and responses were scored from 3-8 days post-infiltration. To obtain PVX particles for PVX agroinfection, *Agrobacterium* strains containing the various recombinant pGR106-*ipiO* plasmids were agroinfected on *N*. *clevelandii* plants. After appearance of mosaic symptoms leaves were grinded in 50 mM potassium phosphate buffer (pH 7.0). Potato plants were rub-inoculated with the obtained homogenate after light dusting with carborundum powder.

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Supplementary material

Supplementary Table 1. *Phytophthora i*solates used in this study

Phytophthora species	Isolate	Origin			MT	Race	Reference
		Country	Year	Source			
P. infestans	F95573	The Netherlands	1995	Potato cull pile	A1	1.3.4.7.10.11	Flier *et al*., 2003b
	89148-09	The Netherlands	1989	Potato crop	A1	0	
	PIC99177	Mexico	1999	*S. stoloniferum*	A2	1.2.3.4.7.9[a].11	Flier *et al*., 2002
	88069	The Netherlands	1988	Tomato crop	A1	1.3.4.7	Van West *et al*., 1998
	PIC99189	Mexico	1999	*S. stoloniferum*	A2	1.2.5.7.10.11	Flier *et al*., 2002
	90128	The Netherlands	1990	Potato crop	A2	1.3.4.7.8.10.11	Vleeshouwers *et al*., 1999
	EC1	Ecuador	n.d. [b]	unknown	n.d.	1.3.4.7.10.11	Armstrong *et al*., 2005
	H30P04	The Netherlands	1995	- [c]	A1	3a.7.10.11	Drenth *et al*., 1995
	USA618	Mexico	n.d.	Potato crop	A2	1.2.3.6.7.10.11	Fabritius *et al*., 1997
	IPO-0	unknown	n.d.	unknown	n.d.	3b.4.7.10.11	
	IPO-C	Belgium	1982	Potato crop	A2	1.2.3.4.5.6.7.10.11	
	PIC99183	Mexico	1999	*S. stoloniferum*	A2	1.2.3.4.5.7.8.10.11	Flier *et al*., 2002
	NL01096	The Netherlands	2001	Potato crop	A2	1.3.4.7.8.10.11	
	VK98014	The Netherlands	1998	Potato crop	A1	1.2.4.11	
	IPO428-2	The Netherlands	1992	Potato crop	A2	1.3.4.7.8.10.11	Flier *et al*., 2003b
	NL00228	The Netherlands	2000	Potato crop	A2	1.2.4.7	
	DDR7704	Germany (fGDR [d])	1977	Potato crop	A1	1.2.4	
	UK7824	United Kingdom	1978	Potato crop	A1	1.2.3.6.7	
	89094	The Netherlands	1989	Potato cull pile	A2	1.2.3.6.7.10.11	Kamoun *et al*., 1998

Supplementary Table 1. continued

Phytophthora species	Isolate	Origin			MT	Race	Reference
		Country	Year	Source			
P. infestans	91011	The Netherlands	1991	Experimental field	A2	3.4.5.10	
	PIC97757	Mexico	1997	*S. demissum*	A1	n.d.	Flier *et al*., 2001
	IPO98014	The Netherlands	1998	Potato crop	A1	1.2.3.4.7.11	Flier *et al*., 2003b
	NL050194	The Netherlands	2005	Potato crop	A2	n.d.	
	NL05105	The Netherlands	2005	Potato crop	A2	n.d.	Gómez-Alpizar
	PRC505705	China	2005	Potato crop	A2	1.2.3.4.5.6.7.9.10.11	
	PRC506303	China	2005	Potato crop	A2	1.2.3.4.5.6.7.9.10.11	
P. infestans s.l. [e]	EC3260	Ecuador	2001	*S. betaceum*	A1	non-host	Adler *et al*., 2004
	EC3394	Ecuador	2001	*S. betaceum*	A1	non-host	Adler *et al*., 2004
	EC3364	Ecuador	2001	*S. betaceum*	A1	non-host	Adler *et al*., 2004
P. andina	EC3414	Ecuador	2001	*Anarrhichomenum* complex	A1	non-host	Gómez-Alpizar *et al*., 2008
P. ipomoeae	PIC99193	Mexico	1999	*Ipomoea longipedunculata*	s.f. [f]	non-host	Flier *et al*., 2002
P. phaseoli	CBS556.88	unknown	1988	unknown	s.f.	non-host	Flier *et al*., 2002
P. mirabilis	PIC99111	Mexico	1999	*Mirabilis jalapa*	A2	non-host	Flier *et al*., 2002
	CBS150.88	Mexico	1988	*Mirabilis jalapa*	A2	non-host	Kamoun *et al*., 1998

[a] not conclusive; [b] n.d., not determined; [c] F1 progeny from a cross between *P. infestans* isolates 80029 and 88133 (Drenth *et al*. 1995); [d] fGDR, the former German Democratic Republic; [e] s.l., sensu lato; [f] s.f., self fertile

Supplementary Table 2. Primers used in this study

Target	Primer[a]	Primer sequence (5'-3')
SSR fingerprinting	SSR1-F	GGCGCCCTACCCACCGTC
	SSR1-R	GTTTGCGCCTCTTCGCGGACGC
	SSR2-F	CGACTTCTACATCAACCGGC
	SSR2-R	GTTTGCTTGGACTGCGTCTTTAGC
	SSR3-F	ACTTGCAGAACTACCGCCC
	SSR3-R	GTTTGACCACTTTCCTCGGTTC
	SSR4-F	TCTTGTTCGAGTATGCGACG
	SSR4-R	GTTTCACTTCGGGAGAAAGGCTTC
	SSR6-R	TCGCCACAAGATTTATTCCG
	SSR6-F	GTTTCATCATGGAGCGTAGGATGG
	SSR7-F	GTCCTCGGCGTTCTATGAC
	SSR7-R	GTTTCCGAGTACCGAATGAGGC
	SSR8-F	AATCTGATCGCAACTGAGGG
	SSR8-R	GTTTACAAGATACACACGTCGCTCC
	SSR11-F	TTAAGCCACGACATGAGCTG
	SSR11-R	GTTTAGACAATTGTTTTGTGGTCGC
ipiO variants	ipiO-F	ATGGTTTCATCCAATCTCAACACC
	ipiO-R	CTATACGATGTCATAGCATGACAC
	F-dT-IpiOwoL	CACCATGGTTTCATCCAATCTCAACACC
	R-dT-IpiOwoL+	CTAGCTAGGGCCAACGTTTTTATC
	PIET10	AAGGCTACGACATGTCC
	ipiO-R-long	GAATTAGAAAAAGACACGTGG
	Cla1-PR1a-ipiOF	CATCGATATGGGATTTGTTCTCTTTTCAC
	Cla1-IPIOF	CATCGATGGTTTCATCCAATCTCAACACCG
	Cla1-IPIO1/3F	CATCGATGGCGTTTTCTATCTCAAA
	Cla1-IPIO2/3F	CATCGATGCGCACTCAGTCCAAGACG
	StopoIPIO1/3R	CACCCTATGAGATAGAAAACGCCCGC
	StopoIPIO2/3R	CACCCTACTTGGACTGAGTGCG
	Not1IPIOR	CGCGGCCGCCTAGCTAGGGCCAACGTTT
Rpi-blb1	AL57-Rpi-blb1	TCGGCTATGACTGGGCACAACAGACA
	AL58- Rpi-blb1	AAGAAGGCGATAGAAGGCGATGCG
RT-PCR	RT-ipiO-I-F	GATGGTACTTTATGGATTCAAAC
	RT-ipiO-I11-R	CTTCTCGGCGTCTCTCCTA
	RT-ipiO-I12-R	CTTCTCGGCGTCTCTCCGG
	RT-ipiO-II-F	GATGGTATTTTATGCATTCAAAA
	RT-ipiO-II-R	GCGGAAGCCTTATCGAGATA
	RT-ipiO-III-F	GTTGGGATTGTATGCATTAAAGA
	RT-ipiO-III-R	ATCGGGATGCTTGTTCTTGTA

[a] F is forward primer, R is reverse primer

Supplementary Table 3. Aggressiveness of *P. infestans* isolates on potato cultivar Désirée

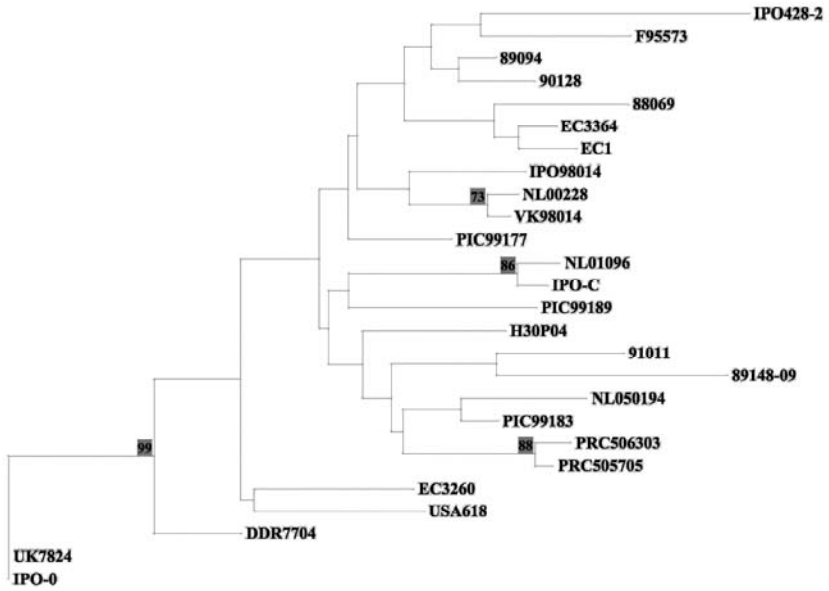
***P. infestans* isolate**	**LS [a]**	**Scale of aggressiveness [b]**
PIC99177	123.0	
F95573	146.2	
89148-09	151.3	moderately aggressive
88069	248.7	
PIC99189	332.6	
90128	370.7	
EC1	391.7	
IPO-0	399.3	
PIC99183	425.7	aggressive
H30P04	431.6	
IPO-C	488.9	
USA618	518.6	
NL01096	572.4	
VK98014	603.8	
IPO428-2	655.2	highly aggressive
NL00228	721.4	

[a] The lesion size (LS) at 6 dpi determined in two independent experiments, the mean LS are presented.
[b] Grouping is based on significant differences between LS (ANOVA, P <0.05).

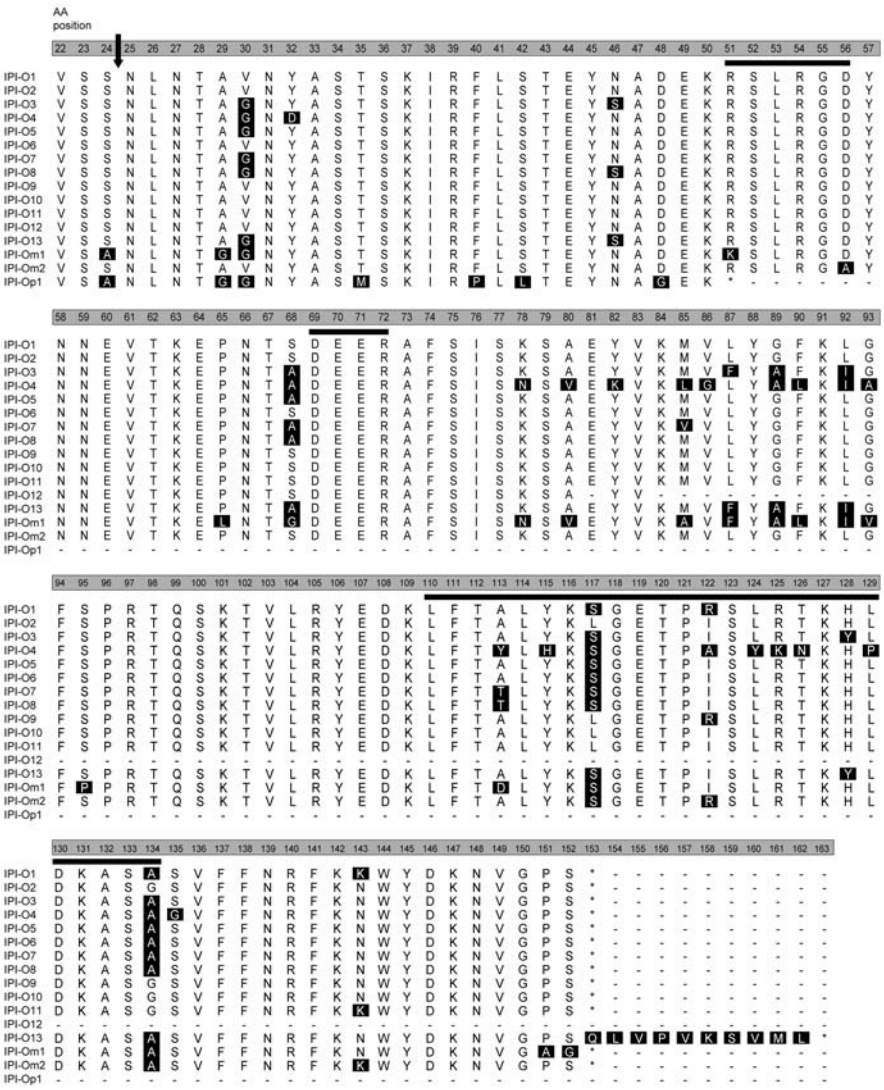
Supplementary Table 4. *Solanum* plant material used in this study

***Solanum* species**	**Abb. [a]**	**Genotype**	***R* gene**	**Background**	**Reference**
S. bulbocastanum	blb	8008-5	*Rpi-blb1*		Van der Vossen *et al*., 2003
S. bulbocastanum	blb	2002	*Rpi-blb2*		Van der Vossen *et al*., 2005
S. stoloniferum	sto	17605-4	*Rpi-sto1*		Vleeshouwers *et al*., 2008
S. stoloniferum[b]	pta	17831-8	*Rpi-pta1*		Vleeshouwers *et al*., 2008
Potato cultivars					
S. tuberosum	tbr	Désirée	-		Van Berloo and Hutten, 2005
S. tuberosum	tbr	Impala	-		Van Berloo and Hutten, 2005
S. tuberosum	tbr	Bintje	-		Van Berloo and Hutten, 2005
Potato transformants					
S. tuberosum	tbr	A01-20	*Rpi-blb1*	Désirée	this study
S. tuberosum	tbr	RGC-2A9	*Rpi-blb1*	Impala	Van der Vossen *et al*., 2003
S. tuberosum	tbr	T5/7	*Rpi-blb2*	Impala	Van der Vossen *et al*., 2005

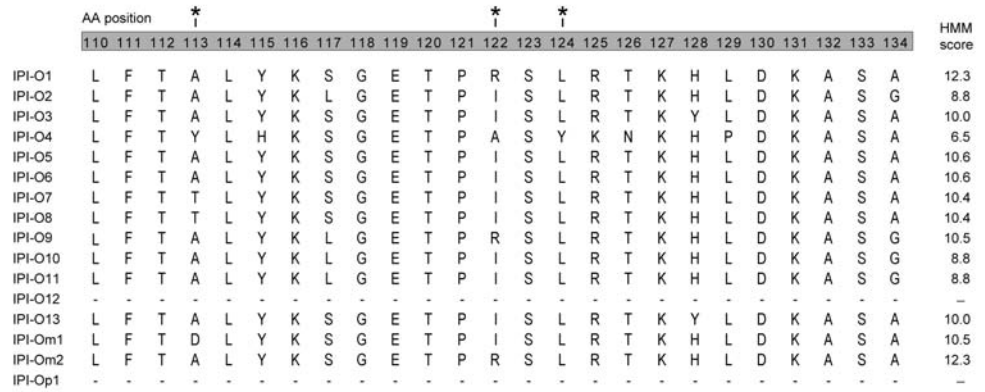
[a] Abbreviation of the *Solanum* species; [b] Previously classified as *S. papita*



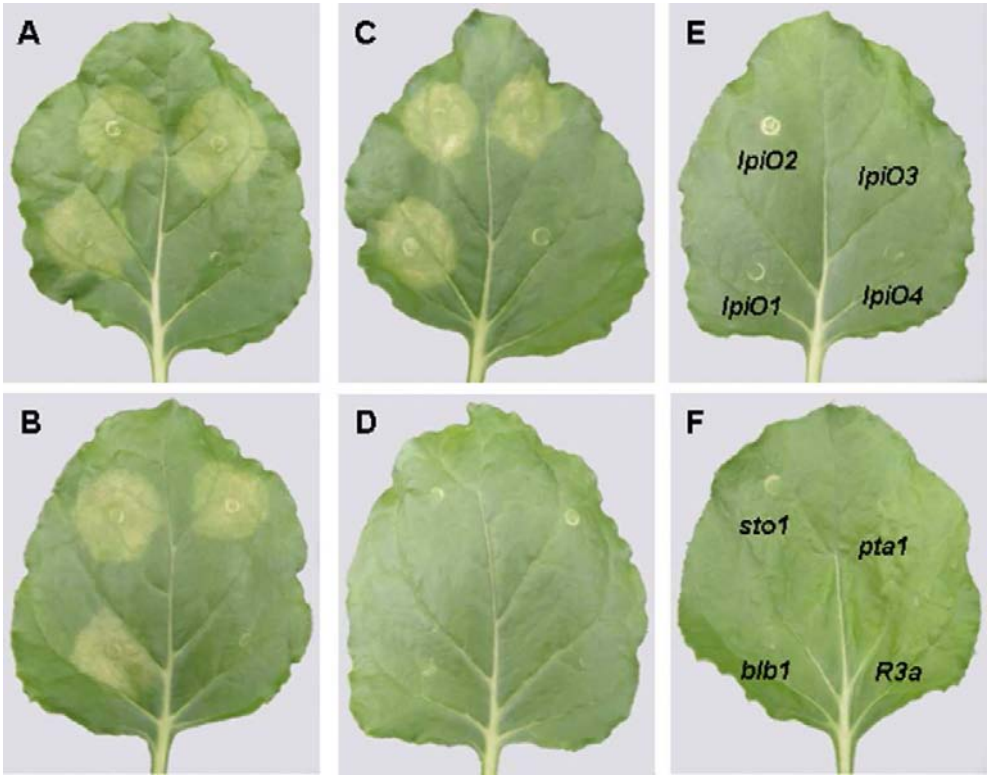
Supplementary Figure 1. Genetic diversity of the *P. infestans* isolates in this study. Bootstrap values in percentage (>60) are shown at the nodes.



Supplementary Figure 2. Amino acid alignment of *ipiO* variants. The motifs RXLR, RGD, DEER and a predicted W motif are indicated. Numbers refer to the amino acid positions. The arrow depicts the predicted signal peptide cleavage site. Amino acid polymorphisms in IPI-O variants, as compared to IPI-O2, are depicted in black background.



Supplementary Figure 3. HMM scores of the conserved W motif in the IPI-O variants. HMM scores >10 are considered as strong. The vertical lines refer to positively selected sites (see Table 2 and Figure 1). *= P>99%.



Supplementary Figure 4. The homologues *Rpi-blb1* (A), *Rpi-sto1* (B) and *Rpi-pta1* (C) induce cell death when co-agroinfiltrated with *ipiO* variants of class I and II. In negative control *R3a* (D), and single infiltration of effectors (E) or *R* genes (F) no cell death is observed. Pictures were taken at 8 days after infiltration. Infiltrated leaf panels in A–D are as indicated in E.

2.2 *In vivo* localization of *Phytophthora infestans* RXLR-dEER effectors IPI-O and Avr4 tagged with red fluorescent protein

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Abstract

IPI-O and Avr4, effectors of the potato late blight pathogen *Phytophthora infestans*, belong to a superfamily of proteins sharing N-terminal RXLR-dEER domains. These domains mediate the translocation of proteins into the host cell. We investigated the *in vivo* targeting of IPI-O and Avr4 in *P. infestans* transformants expressing either IPI-O or Avr4 fused to monomeric red fluorescent protein (mRFP). Fluorescence microscopy showed that fluorescence in young hyphae of a free living, non-sporulating colony accumulates in vesicles that are evenly distributed in these hyphae. In germinating cysts, however, the tips of the germ tubes and the appressoria showed mRFP fluorescence, and during infection of etiolated potato plantlets localized fluorescence was visible at the haustorial neck. Haustoria are highly specialized infection structures that are in close contact with the plant cell and have a putative role in delivering effector proteins into the host cell. The novel experimental set-up, in which etiolated *in vitro* grown potato plantlets are inoculated with *P. infestans*, has the advantage that there is no autofluorescence of chlorophyll that masks the mRFP fluorescence, and disturbs microscopic analysis. The lack of chlorophyll does not seem to interfere with infection; zoospores are capable to encyst and to germinate, and the etiolated tissues are readily colonized by *P. infestans*.

Introduction

Any biotrophic pathogen will produce effectors to facilitate infection of a host plant and to manipulate the host metabolism (Kamoun, 2007). The functions of these effectors include the transcriptional activation of developmental reprogramming (Kay *et al*., 2007), suppression of enzymes such as apoplastic proteases (Tian *et al*., 2005), protection of the pathogen against host defenses (Van Esse *et al*., 2007) and suppression or induction of cell death (Bos *et al*., 2006; Kanneganti *et al*., 2006). Some of the effectors function extracellularly and remain in the plant apoplast after secretion (Rep, 2005). Other effector proteins function inside the plant cell and therefore the pathogen or the host has to facilitate transport of these effectors into the plant cell. Several plant pathogenic bacteria, including *Pseudomonas* and *Xanthomonas* spp., use the type III secretion system to transport effectors into the host (Alfano and Collmer, 2004). As yet, a comparable host cell targeting system has not been described for fungal and oomycete plant pathogens (Ellis *et al*., 2006). Unlike fungal effectors, oomycete effectors share a conserved N-terminal host cell targeting domain, which harbors the RXLR and dEER motifs (Rehmany *et al*., 2005). The putative role of these N-terminal motifs in host cell targeting was deduced from a similar motif ($RXLX^{E}/_{Q}$) in effectors of the malaria parasite *Plasmodium falciparum* named PEXEL/VTS (Charpian and Przyborski, 2008). There is now ample evidence that the RXLR-dEER domain is indeed responsible for targeting effectors into the host cytoplasm (Whisson *et al*., 2007; Dou *et al*., 2008b; Govers and Bouwmeester, 2008).

The oomycete *Phytophthora infestans* is the causal agent of potato late blight. For this pathogen zoospores are important asexual propagules that are spread by wind and rain. When zoospores land on leaves or stems of a host plant they encyst; the cysts then germinate and form appressoria from which penetration pegs emerge that enter epidermal cells. Inside the epidermis an infection vesicle is formed which serves as a starting point for further growth of hyphae invading the extracellular spaces of the mesophyll. These intercellular hyphae form digit-like structures, named haustoria, which penetrate mesophyll cells for feeding. Under optimal conditions sporangiophores will appear on the leaf surface from which new zoospores or sporangia are released (Erwin and Ribeiro, 1996). The *P*. *infestans* effectors IPI-O (Pieterse *et al*., 1994) and Avr4 (Van Poppel *et al*., 2008) are members of the RXLR-dEER effector family. The genes encoding these effectors are expressed in germinating cysts and in early stages of the *P*. *infestans*–potato interaction, and involved in gene-for-gene interactions with potato *R* genes (Van Poppel *et al*., 2008; Vleeshouwers *et al*., 2008). Moreover, IPI-O can bind to an Arabidopsis lectin receptor kinase that has a transmembrane domain and likely spans the plant cell membrane, suggesting targeting of IPI-O to the plant cell membrane (Gouget *et al*., 2006; **Chapter 3.2**). The aim of this study was to

determine the subcellular location of IPI-O and Avr4 in different developmental stages of *P*. *infestans* and during infection of potato. To this end we transformed *P*. *infestans* with chimeric constructs carrying the effector genes *ipiO1* and *PiAvr4* fused to a sequence encoding a monomeric red fluorescent protein (mRFP) (Campbell *et al*., 2002), and used fluorescence microscopy to visualize effector localization. The analysis revealed that in pre-infection stages IPI-O and Avr4 are both targeted to the tips of germ tubes and to appressoria whereas in infected tissues the two effectors accumulate in haustoria.

Results

Phytophthora infestans transformants expressing *mRFP*, *ipiO-mRFP* and *Avr4-mRFP*

To obtain *P*. *infestans* strains that produce mRFP tagged effector proteins we transformed *P*. *infestans* with constructs based on the plasmid pTORmRFP4 (Whisson *et al*., 2007) carrying the open reading frame (ORF) of either *ipiO1* or *PiAvr4*. The stop codons of the *ipiO1* and *PiAvr4* ORFs were removed to create a continuous ORF with the *mRFP* gene that is present in pTORmRFP4 downstream of the multiple cloning site (Figure 1). As recipient strain for transformation we used T35-3, a sibling of strain T30-4 (Drenth *et al*., 1995).

Of the initial colonies that appeared upon transformation six putative IPI-O-mRFP transformants, 13 Avr4-mRFP transformants and five mRFP transformants were still able to grow upon transfer to fresh selective medium. Of these transformants, three IPI-O-mRFP transformants, five Avr4-mRFP transformants, and one mRFP transformant were selected for bioassays (Table 1).

Table 1. *P*. *infestans* transformants used in this study

Transformant	Recipient strain	Insert	Transgene	mRFP mRNA
TR2.1	T35-3	pTORmRFP4-IPIO	+	+
TR12.3	T35-3	pTORmRFP4-IPIO	+	+
TR22.1	T35-3	pTORmRFP4-IPIO	+	+
TR4.2	T35-3	pTORmRFP4-Avr4	+	+
TR14.3	T35-3	pTORmRFP4-Avr4	+	+
TR24.4	T35-3	pTORmRFP4-Avr4	+	+
TR24.6	T35-3	pTORmRFP4-Avr4	+	+
TR24.8	T35-3	pTORmRFP4-Avr4	+	+
211.2b1	T35-3	pTORmRFP4	+	+

For these transformants the presence and expression of the transgenes was confirmed by PCR and RT-PCR respectively. All the selected transformants were able to infect leaves of the susceptible potato cultivar Bintje. To test whether the PiAvr4-mRFP chimeric effector protein is able to elicit a hypersensitive response (HR) on *R4* potato we inoculated the Avr4-mRFP transformants on the *R4* differential potato clone Cebeco44-31-5. Contrary to transformants carrying a *PiAvr4* transgene that are avirulent on *R4* pototo (Van Poppel *et al*., 2008) none of the Avr4-mRFP transformants showed gain of avirulence on R4 potato. In other words, the Avr4-mRFP transformants remained virulent on *R4* plants like the recipient strain. The recipient strain T35-3 carries *ipiO* alleles or variants that confer avirulence on potato clones carrying *Rpi-blb1* or *Rpi-sto1* (Vleeshouwers *et al*., 2008), and therefore it was not possible to determine whether or not IPI-O-mRFP behaved as a functional avirulence protein.

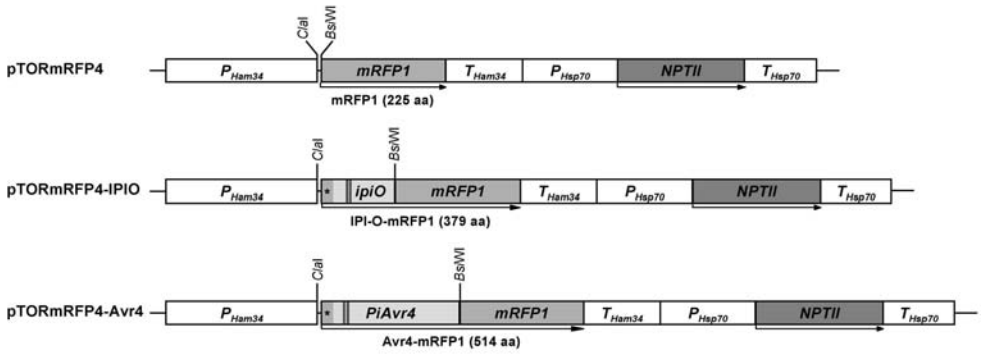

Figure 1. A schematic representation of the constructs used for transformation of *P*. *infestans* strain T35-3. The expression cassette consists of the *Bremia lactucae Ham34* promoter, a *Cla*I/*Sbf*I/*Bsi*WI multiple cloning site, the *mRFP* open reading frame and a *Ham34* terminator. Both the location of the predicted signal peptide (*) and the RXLR (❙) and dEER (▌) motifs in the ORFs of *ipiO* and *PiAvr4* are indicated. The antibiotic selection cassette consists of the *Bremia lactucae Hsp70* promoter, an *NPTII* gene for geneticin resistance and an *Hsp70* terminator. The sizes and positions of the different components in the constructs are on scale.

Transformants carrying chimeric *mRFP* constructs show localized fluorescence in infectious stages

Both *ipiO1* and *PiAvr4* encode RXLR-dEER effector proteins, which are secreted by *P. infestans* and presumably targeted into the host cell. The earliest infectious stage of *Phytophthora* is the germinating cyst, which is formed upon landing of a zoospore on a leaf surface (Hardham, 2001). In all transformants fluorescence was visible (Figure 2A, D, and G), but not in the non-transformed recipient strain T35-3. The fluorescence was not homogenous, but appeared to be concentrated in spheres that moved through the cytoplasm (Figure 2J). Fluorescence was not observed outside the mycelium. By comparing mycelium of different ages it appeared that the intense fluorescence was only visible in young hyphae (Figure 3). Germinating cysts of IPI-O-mRFP and Avr4-mRFP transformants showed specific localization of fluorescence in the tip of the germ tube (Figure 2K). It should be noted, however, that the cytoplasm has moved to the tip of the germ tube and that the cyst itself is devoid of cytoplasm.

In the early stages of infection, encystment and germination is followed by the formation of an appressorium. An appressorium appears like a hyphal swelling at the end of the germ tube and facilitates attachment to and penetration of the host tissue (Figure 2B, E, and H). Formation of appressoria requires a hydrophobic surface, such as the cuticle of a leaf (Hardham, 2001). As substitute for potato leaves we used polypropylene foil as a transparent surface to which germinating cysts can attach and form appressoria *in vitro* (Latijnhouwers *et al*., 2004). We observed that fluorescence in IPI-O-mRFP and Avr4-mRFP transformants was concentrated mainly in the appressoria (Figure 2E, and H). Occasionally fluorescence was observed in the germ tube, but the intensity in germ tubes was always lower than that observed in appressoria. Further development of germinating cysts was halted due to shortage of nutrients as they were germinated in water. Zoospores derived from the mRFP transformant did show fluorescence in appressoria (Figure 2B); however fluorescence was less intense and more diffused throughout the cytoplasm than the fluorescence observed in the IPI-O-mRFP and Avr4-mRFP transformants.

Fluorescence during potato infection is localized in haustoria

In our laboratory we usually perform *P. infestans* infection assays on detached potato leaves (Vleeshouwers *et al*., 1999). Chlorophyll in leaf chloroplasts is red fluorescent with an emission peak at 680 nm (Berg and Beachy, 2008). This autofluorescence interferes with fluorescence of mRFP. Moreover, the low transparency of the potato leaf tissue hampers non-confocal light microscopy. To avoid these interferences we used etiolated *in vitro* grown potato plantlets as tissue for infection (Figure 4). Previous studies have shown that *in vitro* grown potato

plantlets are reliable for *P*. *infestans* infection assays (Huang *et al*., 2005). Here we inoculated the stems of detached etiolated potato plantlets with *P*. *infestans* zoospores and this resulted in growing lesions, high infection efficiency and hyphae clearly visible in the plant tissue.

At 24 hours post-inoculation all tested transformants had formed macroscopic lesions on the etiolated plantlets. The mRFP transformant showed hardly any fluorescence and this fluorescence was randomly distributed (Figure 2C). In contrast, in both the IPI-O-mRFP and Avr4-mRFP transformants the red fluorescence accumulated on sites where haustoria emerged (Figure 2F, I and L). Fluorescence was localized specifically at the haustorial neck and not at the tip of the haustoria. Although these digit-like structures penetrate the plant cell and are in close contact with host tissue, fluorescence was neither found in the extrahaustorial space nor in the infected potato cells.

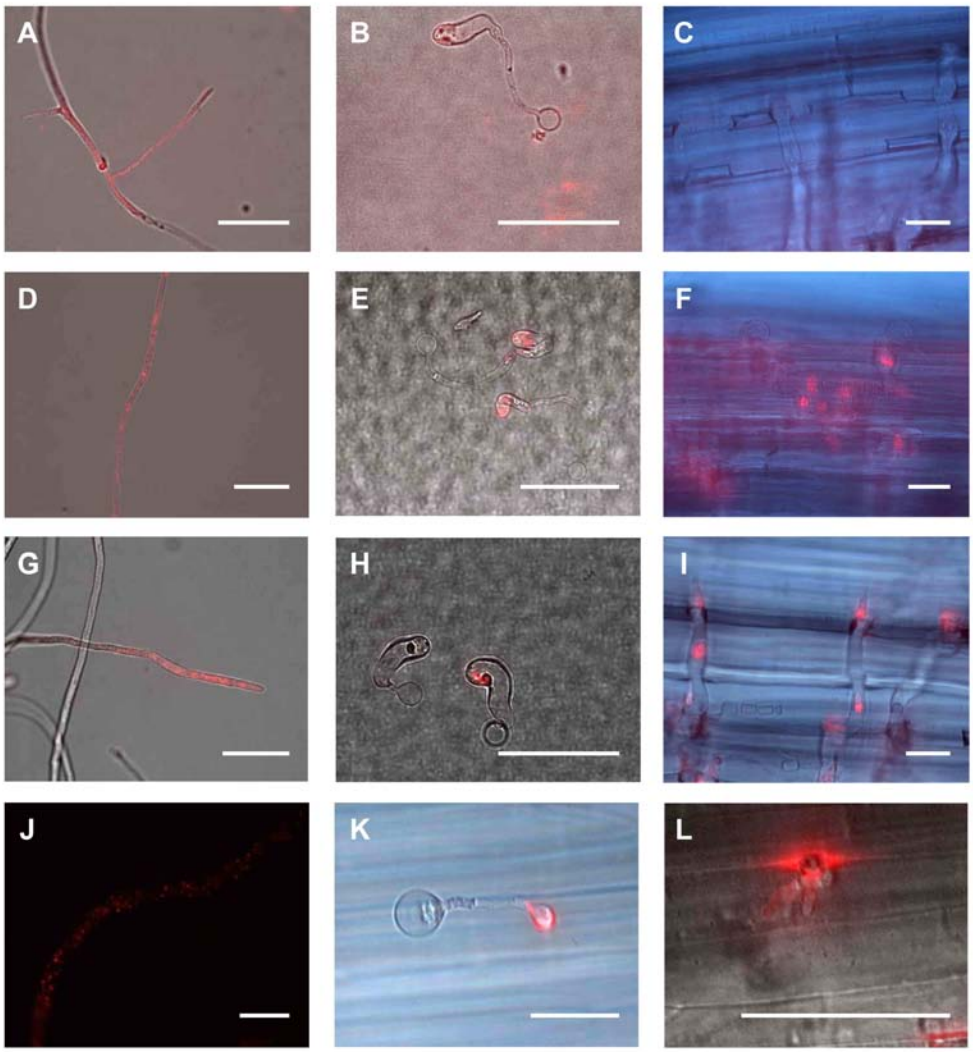

Figure 2. Localized fluorescence observed in *P*. *infestans* mRFP transformant 211.2b1 (A, B and C), IPI-O-mRFP transformant TR2.1 (D, E and F) and Avr4-mRFP transformant TR4.2 (G, H, I, J, K and L). Localized fluorescence observed in mycelium (A, D, G and J), appressoria (B, E and H), a germinating cyst (K) and in hyphal tips invading etiolated potato plantlets (C, F, I). mRFP fluorescence in a haustorium of Avr4-mRFP transformant TR4.2 during infection of etiolated potato plantlets (L). Pictures consist of a brightfield layer and a red fluorescence layer, except J that only shows the red fluorescence layer. The size bars represent 20 µm.

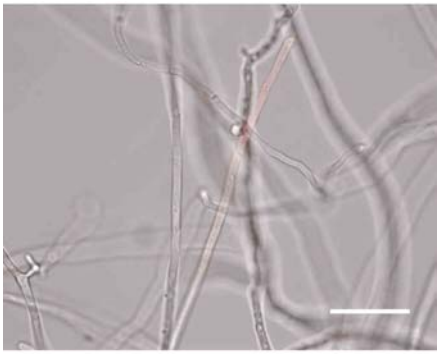

Figure 3. mRFP fluorescence observed in mycelium of the *P. infestans* Avr4-mRFP transformant TR4.2. Mycelium was grown in liquid clarified rye sucrose medium. The picture consists of a brightfield layer and a red fluorescence layer. A young hyphen in the center is surrounded by older hyphae.

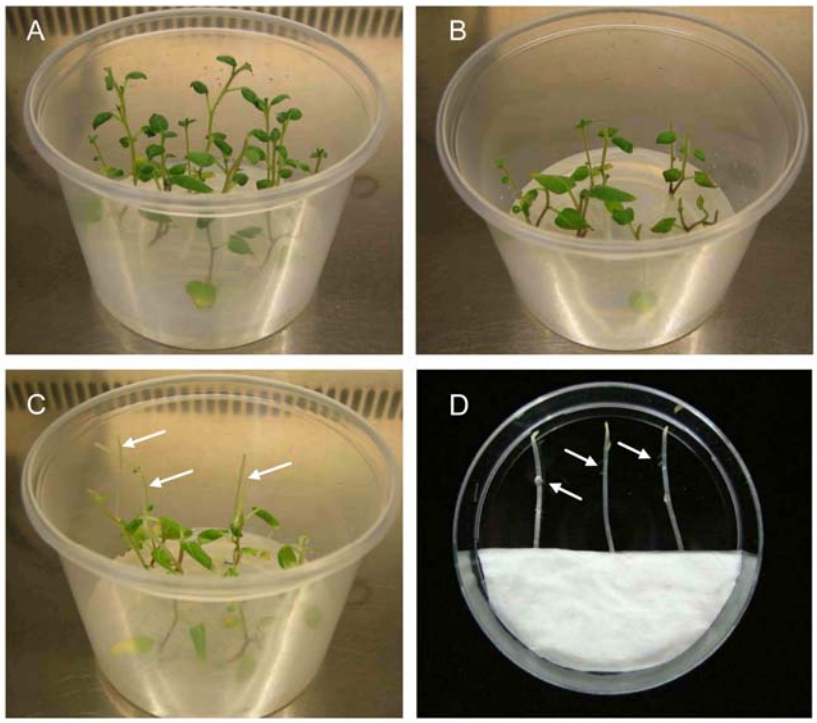

Figure 4. *In vitro* growth of etiolated stems of potato plantlets. The plantlets are grown in transparent jars (A) and decapitated (B). To obtain etiolated shoots the jars are placed in the dark for one week (C). Then the etiolated stems are cut, transferred to a Petri dish and inoculated with *P. infestans* zoospores (D). The inoculation sites are marked by white arrows.

Discussion

Fluorescent tags are convenient tools for *in vivo* localization studies (Berg and Beachy, 2008) and have previously been used to obtain fluorescent *Phytophthora* strains (Bottin *et al*., 1999; Van West *et al*., 1999c). In this study we used fluorophore mRFP (Campbell *et al*., 2002) for *in vivo* detection of *P*. *infestans* effector proteins IPI-O and Avr4. Fusion proteins of mRFP with either IPI-O or Avr4 were localized specifically in pre-infection stages such as the tips of germ tubes, in appressoria, and in haustoria during infection. In the haustoria formed by both IPI-O-mRFP and Avr4-mRFP transformants, mRFP fluorescence was localized at the base, in the haustorial neck, and not in the tip of the haustoria. Similar fluorescence patterns were observed in haustoria formed by *P*. *infestans* transformants carrying the RXLR-dEER effector Avr3a fused to mRFP (Whisson *et al*., 2007). This suggests that the RXLR-dEER effectors accumulate at the haustorial base and this could be the site where RXLR-dEER effectors are released into the extrahaustorial membrane and from there targeted into host cells.

Previous studies showed that both *ipiO1* and *PiAvr4* are highly expressed prior to and early during infection (Van West *et al*., 1998; Van Poppel *et al*., 2008). The promoter that is used to drive the expression of the fusion proteins in the transformants is constitutive and presumably active in free living mycelium. Indeed, in young hyphae we observed fluorescence: the IPI-O-mRFP and Avr4-mRFP fusion proteins were localized in cytoplasmic spheres. These spheres were absent in the control transformant suggesting that the signal peptide targets the fusion proteins into vesicles. However, release of the vesicle content into the extracellular space or into the apoplast of the infected plantlets was not observed. This is in line with the studies by Whisson *et al*. (2007) who were also unable to detect extracellular fluorescence with Avr3a-mRFP transformants.

If the fusion proteins are secreted from the mycelium they either diffuse very quickly, resulting in low local concentrations of fluorescent proteins, or they are unstable. The fact that the proteins accumulate in appressoria and in the haustorial neck suggests some kind of docking mechanism that guides RXLR-dEER effectors to a particular location where the pathogen is in close contact with host tissue. This is likely the site where effectors are released and translocated into the host cell. It is, however, questionable whether the fusion proteins are secreted. The mRFP tag substantially increases the sizes of the effector proteins, from 287 amino acids to 514 for Avr4 and from 152 to 379 for IPI-O, and this may prevent secretion and targeting of the effectors. The C-terminal mRFP tag could also block plant-mediated uptake by changing the tertiary structure of the effector or obstructing host cell targeting domains. The suggestion that the mRFP tag disturbs proper targeting is supported by the observation that the Avr4-mRFP transformants remained virulent on potato plants carrying *R4*. Previously, it was demonstrated

that *P*. *infestans* race 4 transformants carrying a *PiAvr4* transgene under control of its native promoter became avirulent on *R4* plants (Van Poppel *et al*., 2008). A similar gain of avirulence was reported for Avr3a transformants on *R3a* potatoes (Whisson *et al*., 2007), but Avr3a-mRFP transformants remained virulent (S. Whisson, personal communication). This lack of complementation is not necessarily due to improper targeting. It can also be due to confirmation changes caused by the C-terminal mRFP tag that disrupt the effector region of Avr4 and thus prevent recognition by *R4*. As shown for *P*. *infestans* Avr3a, Avr4 and IPI-O, *P*. *sojae* Avr1b and *H*. *parasitica* ATR13 the domains in RXLR-dEER proteins that are recognized by the cognate R proteins are located in the C-terminal part (Bos *et al*., 2006; Van Poppel *et al*., 2008; **Chapter 2.1**, Dou *et al*., 2008a, Allen *et al*., 2008). However, the activity of the TMV-elicitor p50 was not affected by a C-terminal fluorescent tag (Burch-Smith *et al*., 2007). Both p50 and the cognate resistance protein N were produced as cytoplasmic proteins and did not have to cross any membranes in order to interact. To distinguish between improper targeting and disruption of effector activity one could use a biolistics approach to express the Avr4-mRFP construct in *R4* potato plants and monitor HR responses.

In this study we used the stems of etiolated potato plantlets for microscopic studies of the *P*. *infestans*–potato interaction (Figure 4). In nature many *Phytophthora* species infect stems or roots, but typical *P*. *infestans* infections are usually found on leaves. Occasionally infections also occur on the stem of the host plant. In this study we demonstrated that *P*. *infestans* zoospores are capable of infecting etiolated stems at high infection efficiency. The strong reduction of chloroplasts in the etiolated plantlets decrease the autofluorescence otherwise caused by chlorophyll. The etiolated stems are also more transparent which allows the microscopic observation of infection structures below the epidermis. Since this *in vitro* system requires only limited space in a growth chamber it is very suitable for a quick screening of transformants. The plantlets are grown in a sterile environment and can be used multiple times for growing etiolated stems. Altogether, the system presented here accommodates a quick and relatively inexpensive way for microscopic studies of *P*. *infestans*–potato interactions.

Materials and methods

Phytophthora infestans growth conditions and inoculum preparation

Zoospores of *P*. *infestans* were obtained as described by Van Poppel *et al*. (2008). Released zoospores were transferred to glass flasks and encysted by shaking and were subsequently allowed to germinate in water for 2-4 hours at room temperature. To obtain appressoria, cysts were incubated overnight at room temperature on polypropylene foil (Plastibrand catalogue number 759705, Sigma-

Aldrich, St. Louis, MO, USA; Latijnhouwers *et al*., 2004) in Petri dishes with wet paper towels to provide a moist environment. Young mycelium was obtained from sporangia (Latijnhouwers and Govers, 2003) and grown for 3 days at 18˚C in liquid rye sucrose medium.

Nucleic acid manipulations

DNA was isolated using a microprep buffer (0.2 M Tris, pH=8.5, 0.25 M NaCl, 25 mM EDTA, 2 % SDS) that was added to frozen mycelium in 2.2 mL microcentrifuge tubes. Samples were homogenized with glass beads (Ø 3 mm) using a FastPrep instrument (Qbiogene, Carlsbad, CA, USA). A phenol/chloroform extraction was performed, followed by an RNase treatment and DNA precipitation. RNA isolation was performed as described previously (Van Poppel *et al*., 2008).

DNA transformation of *Phytophthora infestans*

For transformation we used plasmids based on pTORmRFP4 (Figure 1; Whisson *et al*., 2007). pTORmRFP4 carries a geneticin resistance gene under control of a *HAM* promoter and a cloning site followed by an *mRFP* sequence (Campbell *et al*., 2002). The plasmids used for transformation are pTORmRFP4, pTORmRFP4-IPIO and pTORmRFP4-Avr4 (Figure 1; Table 1). PCR primers were used to generate different constructs with a 5' *Cla*I and a 3' *Bsi*WI (Table 2) and these constructs were ligated into the pTORmRFP4 plasmid. The fragments lack the 3' stop codon and were cloned into pTORmRFP4.

Table 2. Primers used for cloning

Primer	Sequence
ClaI-IpiO-F	5'-CCATCGATATGCGTTCGCTCCTGTTGACCG-3'
BsiWI-IpiO-R	5'-CGCGTACGGCTAGGGCCAACGTTT-3'
ClaI-PiAvr4-F	5'-CCATCGATATGCGTTCGCTTCACATTTTGC-3'
BSiWI-PiAvr4-R	5'-CGCGTACGAGATATGGGCCGTC-3'
RFP-F	5'-GCAGGCGTACGATGGCCTCC-3'
RFP-R	5'-TCGAACTCGTGGCCGTTCAC-3'

P. infestans isolate T35-3 was stably transformed using the PEG protoplast transformation protocol as described previously (Van Poppel et al., 2008). After transformation protoplasts were resuspended in pea broth with 0.75 % agar, supplemented with 30 µg ml-1 geneticin (G418) and 500 µl aliquots were poured in 24-wells plates. Two and four days after transformation two additional layers of 700 µl pea broth agar supplemented with 30 µg ml-1 G418 and 0.75 and 1.5 % agar respectively were added. Transformants appeared 5-14 days after the second

overlay was added. Expression of the mRFP transcript was confirmed by RT-PCR using the SuperScript III One-Step RT-PCR System (Invitrogen). The virulence phenotypes of the transformants were analyzed on detached leaves as described previously (Van Poppel et al., 2008).

In vitro plant growth conditions and infection assays

In this study potato cv. Bintje (*r0*) was used for infection assays. Plants were maintained *in vitro* in climate chambers (16 hour photoperiod) at 20℃ on MS30 medium. To obtain etiolated plantlets, *in vitro* plants were decapitated after which the lower parts were allowed to form new shoots in the dark. After one week etiolated plantlets of approximately 4 cm long were grown (Figure 4). Infection assays on *in vitro* plantlets were adapted from Huang *et al*. (2005). Several 10 µl droplets of a zoospore suspension with 50 zoospores μl^{-1} were spot inoculated on stems of detached etiolated plantlets (Figure 4D). The plantlets were placed in Petri dishes with wet paper towels to provide a moist environment. Lesions developed overnight in the dark at 18℃.

Microscopy

Microscopic analysis was performed using a Nikon Eclipse 90i epifluorescence microscope (Nikon, Badhoevedorp, The Netherlands). To visualize fluorescence of mRFP (561 nm excitation; emission 600-630 nm) the microscope was equipped with a TRITC filter cube (EX540/25, DM 565, BA 605/55). The NIS-Elements software package was used to analyze and merge digital pictures.

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3

Lectin receptor kinases and their role in disease resistance

3.1 Arabidopsis L-type lectin receptor kinases: phylogeny, classification and expression profiles

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Abstract

In plants, lectin receptor kinases are considered to play crucial roles during development and in the adaptive response to various stimuli. Arabidopsis lectin receptor kinases can be divided in three type-classes based on sequence similarity of their extracellular lectin motifs. The current study focuses on the legume-like lectin receptor kinases (LecRKs), which are regarded as ideal candidates for monitoring cell wall integrity and are likely functional in adaptive responses. An inventory of the Arabidopsis *LecRK* family is presented here. It consists of 45 gene members including three recently identified *LecRK* genes; two encode N-terminal truncated variants one of which has two in tandem kinase domains. Phylogenetic trees derived from full-length amino acid sequence alignments were highly concordant to phylograms that were purely based on lectin motifs or kinase domains. The phylograms allowed reclassification of the *LecRK* genes and hence a new proposal for gene nomenclature was suggested. Additionally, a comprehensive expression analysis was executed by exploring public repositories. This revealed that several *LecRK* genes are differentially expressed during plant growth and development. Moreover, multiple *LecRK*s appear to be induced upon treatment with elicitors and pathogen infection. Variation in gene expression was also analyzed in seedlings of diverse Arabidopsis accessions. Taken together, this study provides a genome-wide overview of the *LecRK* gene family and an up-to-date classification using a novel and systematic gene nomenclature.

Introduction

Plants are facing diverse developmental and environmental stimuli. Perception and transduction of these signals is largely governed by so-called receptor-like kinases (RLKs). The Arabidopsis genome harbors over 600 *RLK* genes, many of which can be grouped into distinct subfamilies based on their extracellular domains (Shiu and Bleecker, 2001a, 2003). The variability in the extracellular domain organization reflects their diverse function and mode of signal perception. For most RLKs, however, neither their ligands nor their downstream targets are known. Examples of elucidated ligand-receptor pairs include the pattern recognition receptors FLS2 and EFR that interact with the microbial associated molecular patterns (MAMPs) flg22 and EF-Tu, respectively, as well as the plant development-associated receptors CLV1, which has the polypeptide CLV3 as ligand, and BRI1 that functions in brassinosteroid perception (Gómez-Gómez and Boller, 2000; Zipfel *et al*., 2006; Kinoshita *et al*., 2005; Ogawa *et al*., 2008).

The adaptive response of plants to extracellular ligands and stimuli is governed by a functional continuum between the plant cell wall (CW) and the plasma membrane (PM) in which RLKs with CW-bound extracellular domains likely function as cell wall integrity sensors (reviewed by Humphrey *et al*., 2007). The Arabidopsis wall-associated kinases (WAKs) are regarded to operate as physical CW-PM linkers (Anderson *et al*., 2001; Verica and He, 2002). By means of various biochemical techniques it was shown that extracellular subdomains of WAK1 interact with a secreted glycine-rich protein (AtGRP3) and with cell wall pectins (Decreux and Messiaen, 2005; Park *et al*., 2001). Arabidopsis plants with impaired *WAK4* expression were found to be affected in cell elongation and morphology (Kohorn *et al*., 2006a; Lally *et al*., 2001). Moreover, inactivation of *WAK2*, one of the Arabidopsis *WAK* genes, reduced the activity of vacuolar invertase that functions in controlling cell turgor, and hence seems to regulate cell expansion (Kohorn *et al*., 2006b). Other Arabidopsis RLKs that may have a role in binding cell wall components are the PERKs and members of the CrRLK1L family closely resembling a RLK from *Catharanthus roseus* (Madagascar periwinkle) (Schulze-Muth *et al*., 1996). PERKs harbor extracellular proline-rich sequences that share similarity with plant extensins, suggesting a direct interaction with cell wall components (Nakhamchik *et al*., 2004), whereas THESEUS1 – a CrRLK1L protein from Arabidopsis – is activated in mutants deficient for cellulose and triggers growth inhibition and ectopic lignin accumulation (Hématy *et al*., 2007).

Another group of RLKs that are regarded as potential CW-PM linkers are the lectin receptor kinases which contain extracellular lectin motifs that are known to bind various carbohydrates. Based on the extracellular lectin motifs three types can be distinguished; G, C and L (Figure 1). Arabidopsis has around 40 *RLK* genes encoding bulb-associated lectin receptor kinases. Previously these were

categorized as B-type lectin receptor kinases because their lectin motif resembles mannose-specific lectins of bulb species. Because these lectins are no longer confined to bulbs Van Damme *et al*. (2007) proposed to rename them as GNA-related lectins, here abbreviated as G-type lectins. Well-studied G-type lectin receptor kinases are the *S*-locus receptor kinases, historically known as *S*-domain RLKs after the functionality of the ectodomain in self-incompatibility (SI) in flowering plants. In Brassicaceae, the *S*-locus receptor kinase SRK acts as the stigmatic determinant of the SI response and likely functions as a receptor for the pollen ligand SCR/SP11. The shift to self-fertility in *Arabidopsis thaliana* is partly due to non-functional SRK sequences (Kusaba *et al*., 2001; Sherman-Broyles *et al*., 2007). G-type lectin receptor kinases were also shown to play roles in plant defense. The rice gene *Pi-d2*, for example, confers resistance to the fungal pathogen *Magnaporthe grisea* whereas NgRLK1 from *Nicotiana glutinosa* was selected in a yeast two-hybrid screen as a putative interactor with capsicein, an elicitin from *Phytophthora capsici* (Chen *et al*., 2006; Kim *et al*., 2010). Besides the G-type lectin motifs the extracellular domains of these proteins contain cysteine-rich EGF-like (epidermal growth factor) and PAN (plasminogen-apple-nematode) motifs that both function in protein homodimerization (Naithani *et al*., 2007). As yet, the role of the G-lectin motif is unknown and there is also no evidence for a function in ligand binding.

C-type (calcium-dependent) lectin motifs can be found in a large number of mammalian proteins that mediate innate immune responses such as antigen uptake and T-cell interaction and play a major role in pathogen recognition (reviewed by Cambi *et al*., 2005). Unlike the omnipresent nature of C-type lectins in mammals, they seem to be scarce in plants. Arabidopsis has only a single gene encoding a protein with a C-type lectin motif but so far its function has not been elucidated.

The third type comprises the legume-like or L-type lectin receptor kinases (LecRKs). Their extracellular domains resemble soluble legume lectins which are ubiquitous in leguminous seeds. For several legume lectins the 3D-structure and their carbohydrate-protein binding specificity has been elucidated. The sequence similarity to legume lectins prompted the hypothesis that the potential ligands of LecRKs are oligosaccharides (André *et al*., 2005). Molecular modeling showed that the sugar-binding residues in LecRKs are poorly conserved and it is therefore unlikely that these receptors bind monosaccharide molecules. There is, however, a rather conserved hydrophobic-binding site, and hence LecRKs may serve in the recognition of small hydrophobic ligands, such as plant hormones or MAMPs (Barre *et al*., 2002; André *et al*., 2005).

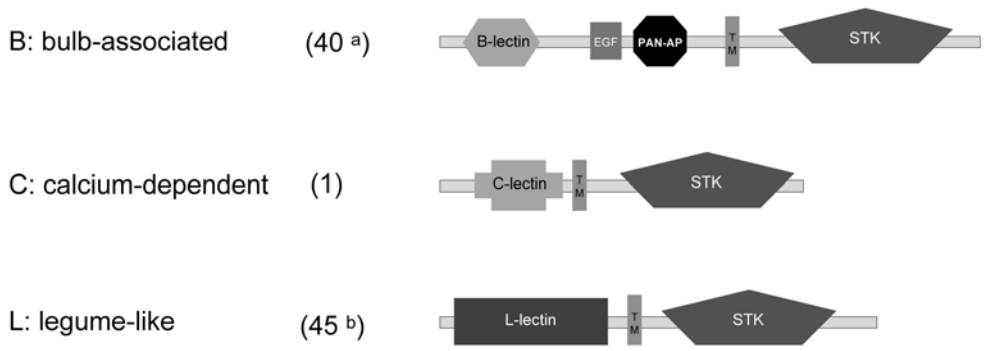

Figure 1. Domain composition and organization of the lectin receptor kinases. Based on the lectin motifs in the extracellular domain lectin receptor kinases are divided in three types, G, C and L. The numbers in brackets refer to the number of genes in Arabidopsis encoding G, C and L-type lectin receptor kinases. G-type lectin receptor kinases, which contain GNA-related lectin motifs, were previously categorized as B-type. [a] Number as listed by Shiu and Bleecker (2001b); [b] Includes the two lectin receptor kinases with a N-truncated terminus. TM, transmembrane; PAN, plasminogen-apple-nematode motif; EGF, epidermal growth factor-like motif; STK, serine/threonine protein kinase domain.

Although LecRKs are implied to function in diverse biological processes, their exact biological role has not yet been clarified. LecRKs that have been described to function during plant development include the SGC lectin RLK of Arabidopsis, which is required for proper pollen development (Wan *et al*., 2008) and MtLecRK1;1 from the nitrogen-fixing legume *Medicago truncatula* that influences nodulation (Navarro-Gochicoa *et al*., 2003). Expression of Arabidopsis lectin receptor kinase *lecRK-a1* was shown to be induced during wounding, senescence of leaves, and in response to oligogalacturonides, which might be released upon disruption of the plant cell wall (Riou *et al*., 2002). More recently, Xin *et al*. (2009) showed that a specific subfamily of LecRKs is responsible for negatively regulating the abscisic acid (ABA) response during seed germination and hypothesized that these genes directly or indirectly affect defense. Lately, several reports have presented data linking ABA-signaling with defense responses (Asselbergh *et al*., 2008). NbLRK1, a legume-like lectin receptor kinase from *Nicotiana benthamiana* was reported to interact intracellularly with the *P*. *infestans* elicitin INF1 and seems to be involved in the subsequent INF1-induced cell death (Kanzaki *et al*., 2008). Another LecRK with a potential link to plant defense and pathogen response is LecRK79 in Arabidopsis. It interacts via its lectin motif with the tripeptide motif RGD (arginine-glycine-aspartic acid) in IPI-O, an RXLR effector from the late blight pathogen *Phytophthora infestans* that can disrupt CW-PM adhesions through the

RGD motif (Senchou *et al*., 2004; Gouget *et al*., 2006). LecRK79 mediates CW-PM adhesions and hence the continuum between the cell wall and the plasma membrane (Gouget *et al*., 2006). Its role in plant defense is, furthermore, supported by the observation that *LecRK79* expression is induced upon inoculation with several non-host and avirulent pathogens (Bouwmeester *et al*., 2008). Taken together, these data suggests that LecRKs play crucial roles in both developmental and adaptive processes.

Over the past years several Arabidopsis *LecRK*s have been investigated. Gene naming, however, has not been uniform and this has sometimes resulted in confusion and miscommunication. Some *LecRK* genes have multiple names whereas the majority has only an AGI gene code. Gene names are often based on protein functionality or molecular mass, but this does not reflect their phylogenetic relationship. Unfortunately, several genes names have been chosen based on incomplete and not always informative gene classification. For example, *LecRK-a4* – named by Hervé *et al*. (1996) – strongly resembles *LecRKA4.1*, the gene name recently assigned by Xin *et al*. (2009) to a LecRK that regulates the ABA response.

In this study, the information available on all members of the Arabidopsis L-type lectin receptor kinase gene family has been collated and summarized and a bioinformatic analysis has been performed to clarify their relationship. Based on the revised phylogenetic classification, a simplified nomenclature for the Arabidopsis *LecRK*s is proposed, which could serve as a basis for gene naming in other plant species and will hopefully improve communication among scientists working on this group of plant receptor kinases. A comprehensive survey of publicly available expression data has also been undertaken. The expression profiles show that *LecRK*s are activated by various biotic and abiotic stimuli and are differentially expressed in various Arabidopsis accessions.

Materials and Methods

Sequence database search and gene analysis

Gene sequences were retrieved using the available servers at the TAIR website (http://www.arabidopsis.org). Additional BLAST searches were conducted on the Arabidopsis genomic sequence and proteome at TAIR and MIPS (http://mips.gsf.de). All genes were checked for annotation mistakes. Structural domain prediction was performed using the SMART (http://smart.embl-heidelberg.de) and Pfam (http://pfam.sanger.ac.uk) databases. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) was used for the prediction of signal peptides. The web servers TMHMM (http://www.cbs.dtu.dk/services/TMHMM), SOSUI (http://bp.nuap.nagoya-u.ac.jp/~sosui/) and PRED-TMR2 (http://athina.biol.uoa.gr/PRED-TMR2/) were used to predict transmembrane regions. Additional domain prediction

and verification was performed using the programs InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) and ProDom (http://prodom.prabi.fr). Homology at protein and nucleotide levels was determined by pairwise sequence comparison with the AlignX module in Vector NTI® (Invitrogen).

Alignment and phylogenetic analysis of *LecRK* genes

Full length protein and domain sequences, as defined in the previous section, were compiled and aligned using ClustalW (Larkin *et al*., 2007). GONNET with a penalty gap opening of 10 and a gap extension of 0.1 was used as protein weight matrix. The obtained sequence alignments were used as input to construct phylogenetic trees with the minimal evolution algorithm within MEGA 4 (Tamura *et al*., 2007). Poisson correction was used to account for multiple substitutions and alignment gaps were removed in a pairwise fashion. The statistical strength was assessed by bootstrap resampling using 10,000 replicates.

Analyses conducted with different algorithms within MEGA 4 generated comparable phylograms. Protein phylogenetic trees were rooted with amino acid sequences of the Arabidopsis RLKs WAK1 (At1g21250), PERK1 (At3g24550), the G-type lectin receptor kinases (SRKs) ARK1 (At1g65790) and CES101 (At3g16030), and the C-type lectin receptor kinase encoded by the single-copy gene At1g52310. Domain-based phylogenetic trees were rooted with the predicted extra- and intracellular domains of these outgroup genes. Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed.

Analysis of expression data

Arabidopsis EST and cDNA data was retrieved from the TAIR website. MPSS signature data was retrieved from the MPSS database (http://mpss.udel.edu/at/) (Lu *et al*., 2005). Sums of abundance of sense strand signatures (classes 1, 2, 5, and 7) were regarded as significant and reliable MPSS expression data. Sequence queries in the 17 bp and 20 bp signature databases showed comparable expression profiles. Expression data was further analyzed by using the Genevestigator V3 web tool (http://www.genevestigator.com) (Hruz *et al*., 2008). Expression analysis was restricted to gene chip experiments based on the sequenced genome of the Arabidopsis accession Col-0. *LecRK-V.7* (At3g58740) and *LecRK-V.8* (At3g59750) share the same probe-set (25148_s_at) and represent equal values. The blue color codes displayed in Figure 3 are based on the concept of expression potential, in which the darkest blue color is representing the maximum expression level that a given gene can reach throughout all arrays available in the database. Because equal blue colors can depict different expression values comparison of expression levels is only justified between one gene in different tissues and not between individual genes.

Natural variation in *LecRK* expression between Arabidopsis accessions was determined by using the Arabidopsis eFP browser of the Bio-Array Resource (BAR) website (http://bar.utoronto.ca/) (Winter *et al*., 2007). Log_2 expression values relative to the control value (Col-0) were tabulated and reformatted to a color-scale with the BAR HeatMapper Plus Tool to easily visualize expression differences. Expression was measured in triplicate samples of aerial parts of 4 day old seedlings of nine geographically separated Arabidopsis accessions; including the reference accession Col-0 (Columbia, USA), Van-0 (Vancouver, USA), Kin-0 (Kindalville, USA), Est-0 (Estonia), Bay-0, (Bayreuth, Germany), Nd-0 (Niederzenz, Germany), L*er*-2 (Landsberg, Germany), Sha (Shahdara, Tajikistan), and Cvi (Cape Verde Islands).

Results and Discussion

Gene identification and protein structure prediction

L-type lectin receptor kinases (LecRKs) were identified by BLAST searches and by exploring previously constructed gene lists (Shiu and Bleecker, 2001b, 2003; Barre *et al*., 2002). In total, 45 *LecRK*s were identified, 42 of which were previously described by Barre *et al*. (2002) (Table 1). The three added in this survey are At5g60310, At3g46760, and At2g32800, which were previously listed as putative lectin receptor kinases in the Arabidopsis RLK inventory by Shiu and Bleecker (2001b, 2003). At5g60310 belongs to a cluster of three closely linked *LecRK* genes, and seems to encodes a typical L-type lectin receptor kinase (Figure 1). Protein sequence comparison revealed, however, that At5g60310 encodes an abnormal protein kinase lacking sub-domains X and XI, thus suggesting impaired kinase activity. The other two, At3g46760 and At2g32800, encode proteins with kinase domains but lacking transmembrane motifs and extracellular domains. Moreover, the kinase encoded by At3g46760 contains a shortened activation loop, which could affect proper kinase functioning. Structure prediction of At2g32800 revealed an peculiar arrangement of two repeated kinase domains (here named AT3G32800A and AT3G32800B) that contain all structural sub-domains. Proteins with two in tandem kinase domains do exist in vertebrates and are known as Janus kinases (JAKs) based on their two-faced function: the first domain exhibits kinase activity while the other downregulates this activity. JAKs appear to play a critical role in diverse processes such as cytokine signaling in mammalian cells (reviewed by Yamaoka *et al*., 2004).

Prediction analyses showed that most LecRKs have all features of typical L-type lectin receptors, i.e. a putative signal sequence, an extracellular lectin motif, a transmembrane region, and a protein kinase domain (Figure 1). In addition to the three exceptions described above, four other *LecRK*s encode structurally different

proteins. The one encoded by At4g28350 was predicted to lack a transmembrane region, and those encoded by At2g29220, At2g29250, and At3g45390 seem to be devoid of a typical kinase structure (Table 1).

LecRK clusters and clades

To categorize the Arabidopsis *LecRK* genes, the *LecRK* gene family was divided into clusters and clades. A gene cluster represents physical proximity on the chromosome and was defined as two or more homologous genes within a maximum of eight adjacent gene models between individual members. Nine distinct *LecRK* gene clusters were found with a maximum of six genes per cluster (i.e. cluster 3A) (Table 1). The gene clusters are dispersed across the five Arabidopsis chromosomes, but the larger gene clusters are predominantly located on chromosome 3 and 5.

A gene clade was defined as a group of at least two homologous genes with a minimum of 50% identity at both the nucleotide and amino acid level (Mondragon-Palomino and Gaut, 2005). By means of pairwise comparison nine major *LecRK* clades were determined. The two largest *LecRK* clades comprise eleven and nine genes, respectively. Two clades contain four genes, and the five smallest ones consist of two *LecRK* genes. Seven *LecRK* genes do not belong to any clade and were assigned as singletons. The 28 *LecRK* genes distributed over clusters all belong to a clade. Hence, physically linked *LecRK* genes also contain a high pairwise identity (>50%). This is probably due to ancient duplication events and it is therefore not surprising that genes in one cluster belong to a single clade (Table 1).

LecRK clade phylogeny

To determine the evolutionary relationship between members of the LecRK protein family the protein sequences were aligned with ClustalW using GONNET as protein weight matrix. Subsequently, phylograms were constructed with MEGA 4 using several algorithms, i.e. Neighbor-Joining, maximum parsimony, and minimal evolution. The robustness of the phylograms was determined by bootstrap analysis with 10,000 replications. The consensus topologies generated by the different algorithms were comparable (data not shown). All phylograms were rooted using the Arabidopsis RLKs WAK1, PERK1, the Arabidopsis C-type lectin receptor kinase (At1g52310), and the G-type lectin receptor kinases ARK1 and CES101 as outgroup representatives and were largely supported by high bootstrap values (Figure 2).

Table 1. *LecRK* classification, nomenclature, and coding sequence information

Proposed clade classification	Proposed gene name	Cluster [a]	Locus [b]	Alternative gene name	References	Former class [c]	Gene alias [b]	Gene models	Protein accession [d]	SP NN/HMM [e]	No. of TM motifs [f]	Protein length (AA)	Remarks
LecRK-I	*LecRK-I.1*		At3g45330			C	F18N11.90	1	Q9M3E5	y/y	2	682	
	LecRK-I.2		At3g45390			C	F18N11.150	1	Q7FK82	y/y	1	604	lacks catalytic kinase sub-domain V, VIa, VIb
	LecRK-I.3		At3g45410	*LecRK2*	1	C	F18N11.170	1	Q9M3D8	y/y	1	664	
	LecRK-I.4		At3g45420			C	F18N11.180	1	Q9M3D7	y/y	1	667	
	LecRK-I.5		At3g45430			C	F9K21.10	1	Q9M1G4	y/y	2	613	annotated as lacking SP
	LecRK-I.6		At3g45440			C	F9K21.20	1	Q9M1G3	y/y	2	669	
	LecRK-I.7		At5g60270			C	F15L12.9	1	Q9LSS0	y/y	1	668	
	LecRK-I.8		At5g60280			C	F15L12.12	1	Q9LSR9	y/y	1	657	
	LecRK-I.9		At5g60300	*LecRK79*	2	C	F15L12.17	3	Q56XH0	y/y	1	718/766	
	LecRK-I.10		At5g60310				F15L12.19	1	Q3E884	y/n	1	616	lacks kinase sub-domain X and XI
	LecRK-I.11		At5g60320			C	K9B18.1	1	Q9FJI4	y/n	2	675	
LecRK-II	*LecRK-II.1*		At5g59260			C	MNC17.17	1	Q9FIF1	y/n	1	674	
	LecRK-II.2		At5g59270			C	MNC17.20	1	N.	y/y	1	668	
LecRK-III	*LecRK-III.1*		At2g29220				F16P2.40	1	Q9ZW09	y/y	1	627	incomplete activation loop
	LecRK-III.2		At2g29250				F16P2.37	1	Q9ZW11	y/y	1	623	incomplete activation loop
LecRK-IV	*LecRK-IV.1*		At2g37710	*LRK1, LecRK-d*	3,4	A2	F13M22.21	1	O80939	y/y	1	675	
	LecRK-IV.2		At3g53810	*SGC lectin RLK*	5	A2	F5K20.110	1	Q9M345	y/y	2	677	*SGC* = small, glued-together and collapsed
	LecRK-IV.3		At4g02410			A2	T14P8.3	1	O81292	y/y	2	674	
	LecRK-IV.4		At4g02420			A2	T14P8.4	1	O81291	y/n	1	669	
LecRK-V	*LecRK-V.1*		At1g70110	*LecRK-b2*	4	A3	F20P5.16	1	O04534	y/y	1	666	
	LecRK-V.2		At1g70130	*LecRK-b1*	4	A3	F20P5.15	1	O04533	y/y	1	656	
	LecRK-V.3		At2g43690	*LecRK-c1*	4	A1	F18O19.20	1	O22834	y/n	2	664	
	LecRK-V.4		At2g43700	*LecRK-c2*	4	A1	F18O19.19	1	O22833	y/y	1	658	
	LecRK-V.5		At3g59700	*Ath-lecRK1, LecRK1, LecRK-a1*	4,6	A1	T16L24.250	1	Q96285	y/n	2	661	
	LecRK-V.6		At3g59730	*LecRK-a2*	6	A1	T16L24.280	1	Q9LEA3	y/n	1	523	
	LecRK-V.7		At3g59740	*LecRK3, LecRK-a3*	4,6	A1	T16L24.290	1	Q9ZR79	y/y	1	659	
	LecRK-V.8		At3g59750	*LecRK-a4*	6	A1	F24G16.20	1	Q9M1Z9	y/n	1	626	has a frameshift mutation
	LecRK-V.9		At4g29050			A3	F19B15.80	1	Q9SZD5	y/y	1	669	
LecRK-VI	*LecRK-VI.1*		At3g08870			A4	T16O11.20	1	Q9SR87	y/n	1	693	
	LecRK-VI.2		At5g01540	*LecRKA4.1*	7	A4	F7A7.60	1	Q9M021	y/y	1	682	
	LecRK-VI.3		At5g01550	*LecRKA4.2*	7	A4	F7A7.70	1	Q9M020	y/y	1	688	
	LecRK-VI.4		At5g01560	*LecRKA4.3*	7	A4	F7A7.80	1	Q66GN2	y/y	1	691	
LecRK-VII	*LecRK-VII.1*		At4g04960			B3	T32N4.9	1	Q9S9U1	y/y	1	686	
	LecRK-VII.2		At4g28350	*LecRK-e*	4	B3	F20O9.40	1	O49445	y/y	0	649	no transmembrane predicted
LecRK-VIII	*LecRK-VIII.1*		At3g53380			B1	F4P12.80	1	Q9LFH9	y/y	1	715	
	LecRK-VIII.2		At5g03140			B1	F15A17.170	1	Q9LYX1	y/n	1	711	
LecRK-IX	*LecRK-IX.1*		At5g10530			B2	F12B17.120	1	Q9LXA5	y/y	1	651	
	LecRK-IX.2		At5g65600			B2	K21L13.11		Q9LSL5	y/n	2	675	
Singletons	*LecRK-S.1*		At1g15530			B3	T16N11.4	1	Q9M9E0	y/y	1	656	
	LecRK-S.2		At2g32800	*AP4.3A*	8		F24L7.6	1	O48837	n/n	0	851	lacks lectin domain and transmembrane, contains two repeated kinase domains
	LecRK-S.3		At3g46760				T6H20.210	1	Q9STF0	n/n	0	337	only kinase part
	LecRK-S.4		At3g55550				T22E16.210	1	Q9M2S4	y/y	2	684	
	LecRK-S.4		At5g06740			B2	MPH15.10	1	Q9FG33	y/n	1	652	
	LecRK-S.6		At5g42120			B1	MJC20.23	1	Q9FHX3	y/n	1	691	
	LecRK-S.7		At5g55830			B1	MDF20.27	1	Q9FHG4	y/y	1	681	

[a] Each level of gray-shading represents a different cluster of *LecRK* genes. A white cell indicates that this gene does not belong to a cluster; [b] Locus and gene alias assigned on the BAC location according to TAIR; [c] gene classification as described by (Barre et al. 2002); [d] protein designation in UniProt KB; [e] signal peptide prediction based on neural networks (NN) and hidden Markov models (HMM), y = predicted, n = not predicted; [f] prediction of transmembrane (TM) motifs based on HMMs. References; 1, He et al. (2004); 2, Gouget et al. (2006); 3, Swarup et al. (1996); 4, Hervé et al. (1996); 5, Wan et al. (2008); 6, Riou et al. (2002); 7, Xin et al. (2009); 8, Cordeiro et al. (1998).

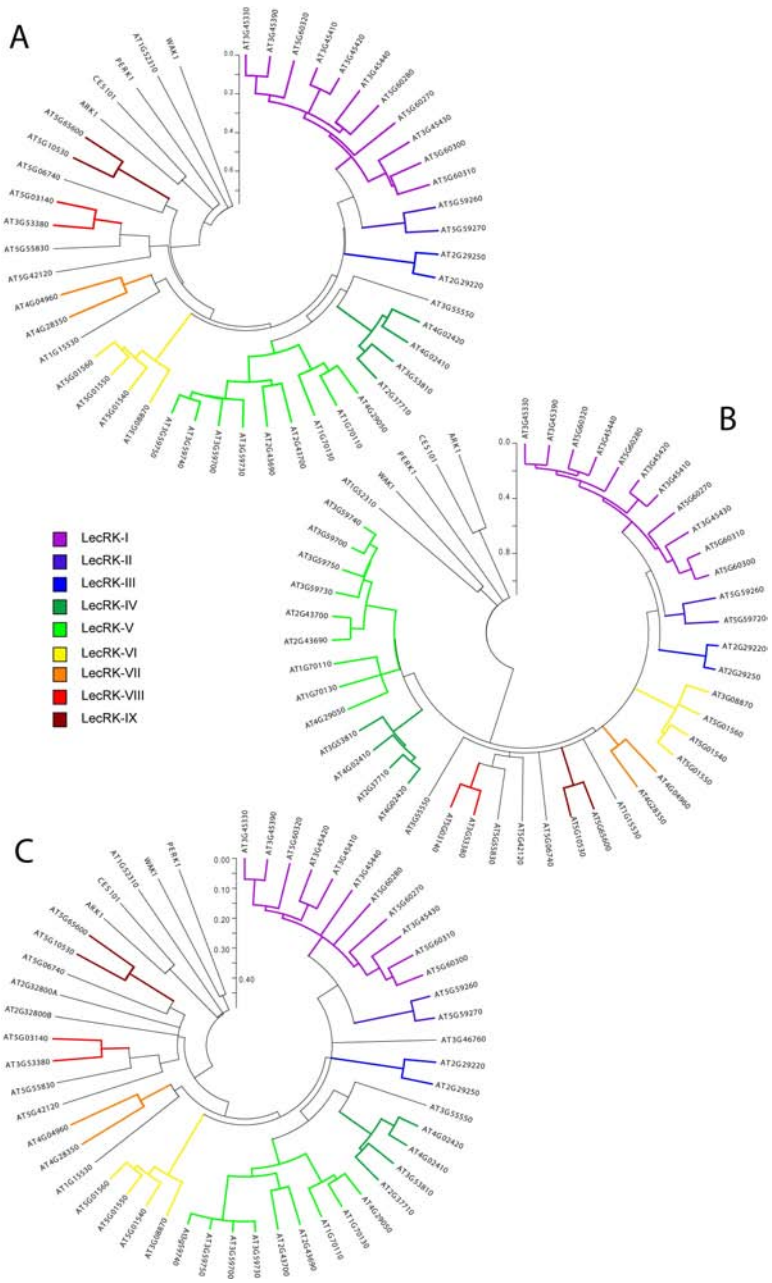

Figure 2. Phylogenetic analysis and classification of Arabidopsis LecRK proteins. (A) Minimal evolution tree of the 43 full-length LecRK amino acid sequences, excluding LecRK-S.2 and LecRK-S.3. Phylograms based on 43 lectin domains (B) and 46 kinase domains, including the kinase domain of LecRK-S.2 and the two kinase domains of LecRK-S.3 (C). Each LecRK clade is depicted by a different color.

Phylogenetic analysis conducted with 43 out of 45 full-length LecRK proteins resulted in 38 LecRKs in nine distinct clades and five singletons, which is in line with the pairwise comparisons (Figure 2A). In this analysis At2g32800 and At3g46760 were excluded because they lack extracellular domains. The phylogram based on the lectin domain shows comparable gene relationships and also similar grouping as depicted in the phylogenetic tree generated with the full length protein sequences (Figure 2B). Similarly, the phylogram based on the kinase domain is comparable. As expected the three kinase domains added in the phylogram At2g32800A, At2g32800B, and At3g46760, group with the LecRKs and not with the outgroups. Moreover, they do not group with any clade and even the two kinase domains within At2g32800 behave as singletons (Figure 2C). Overall these phylograms suggest that the LecRK members of the same clade are highly identical to each other in both extracellular and intracellular domains. This feature can be used for the phylogenetic characterization of full length and truncated LecRK protein sequences of other plant species.

Uniform nomenclature for *LecRK* gene members

The proposed gene nomenclature is based on the grouping of closely related *LecRK* genes within the evolutionary divergent clades. Clades were designated by the Roman numerals I to IX starting clockwise within the phylogram shown in Figure 2A. The individual gene members within the clades were given Arabic numerals based on their physical map position. Singletons were also ordered based on their physical map position and numbered S.1 to S.7. Table 1 lists the revised and more consistent gene nomenclature. For comparison, the previous gene classification (Barre *et al*., 2002) and the alternative *LecRK* gene names are included.

LecRK genes are differentially expressed in various tissues and developmental stages

To obtain more insight in the expression patterns of the individual *LecRK*s during plant development a comprehensive expression analysis was performed by making use of various on-line repositories. Based on the number of full-length cDNAs and ESTs retrieved from the TAIR website, it is apparent that the *LecRK* genes have variable expression profiles. For about one-third of the *LecRK* genes there are no ESTs, for another one-third there are less than 10 ESTs, whereas five of them have over 20 ESTs and thus seem to be highly expressed (Table 2).

Table 2. cDNA, EST and MPSS 20 bp tag expression data of the Arabidopsis *LecRK* genes

Proposed clade name [a]	Proposed gene name [a]	Locus [a]	cDNA [b]		No. ESTs [b, f]	No. MPSS 20 bp tags [c, d]																
			+/-	n.		CAF	CAS	GSE	LEF	LES	ROF	ROS	AP1	AP3	AGM	SAP	INF	INS	SIF	SIS	S04	S52
LecRK-I	*LecRK-I.1*	At3g45330	-		0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0
	LecRK-I.2	At3g45390	-		0	0	0	0	0	0	5	0	0	3	0	0	0	0	0	0	0	0
	LecRK-I.3	At3g45410	+	1	7	6	0	0	8	0	54	0	0	0	0	0	0	0	0	0	0	0
	LecRK-I.4	At3g45420	-		0	0	0	0	0	0	0	0	0	9	0	0	0	2	3	0	0	0
	LecRK-I.5	At3g45430	-		3	0	0	0	2	0	9	0	0	0	0	0	45	0	0	0	0	0
	LecRK-I.6	At3g45440	-		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LecRK-I.7	At5g60270	+	1	14	15	46	16	24	28	3	3	0	0	0	0	0	0	0	0	0	0
	LecRK-I.8	At5g60280	+	2	11	19	2	5	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	LecRK-I.9	At5g60300	+	5	23	64	84	58	31	37	309	82	15	8	11	38	47	15	8	12	7	0
	LecRK-I.10	At5g60310	+		1	0	9	0	0	0	0	13	0	0	0	0	0	0	0	0	0	0
	LecRK-I.11	At5g60320	-		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LecRK-II	*LecRK-II.1*	At5g59260	-		1	0	0	0	1	0	11	0	0	0	0	0	0	0	0	0	0	0
	LecRK-II.2	At5g59270	-		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LecRK-III	*LecRK-III.1*	At2g29220	-		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LecRK-III.2	At2g29250	-		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LecRK-IV	*LecRK-IV.1*	At2g37710	+	2	54	92	92	11	147	49	72	35	72	33	42	145	82	79	125	66	91	1590
	LecRK-IV.2	At3g53810	+	1	12	38	0	2	8	0	28	0	0	5	10	0	16	0	38	0	0	0
	LecRK-IV.3	At4g02410	+	2	13	8	0	0	49	2	12	24	15	3	3	0	11	0	0	9	0	38
	LecRK-IV.4	At4g02420	-		5	0	0	0	29	7	0	0	11	40	5	32	23	20	192	22	0	7
LecRK-V	*LecRK-V.1*	At1g70110	-		0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0
	LecRK-V.2	At1g70130	-		0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
	LecRK-V.3	At2g43690	-		0	4	6	0	0	2	7	9	2	3	0	91	0	0	0	0	0	0
	LecRK-V.4	At2g43700	+	1	3	0	0	0	1	5	0	0	0	0	0	0	4	0	0	0	0	0
	LecRK-V.5	At3g59700	+	1	6	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
	LecRK-V.6	At3g59730	-		0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	LecRK-V.7	At3g59740	-		1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LecRK-V.8	At3g59750	-		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LecRK-V.9	At4g29050	-		5	0	0	19	4	0	0	1	11	14	6	0	0	5	0	0	0	0
LecRK-VI	*LecRK-VI.1*	At3g08870	-		4	0	0	0	30	0	0	0	5	0	0	0	0	0	0	0	0	9
	LecRK-VI.2	At5g01540	+	2	31	0	0	4	5	7	6	18	4	13	13	9	29	5	0	0	0	91
	LecRK-VI.3	At5g01550	-		6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LecRK-VI.4	At5g01560	+	2	36	0	0	13	0	16	0	4	0	7	2	12	12	12	0	8	0	7
LecRK-VII	*LecRK-VII.1*	At4g04960	+	2	16	29	104	3	10	6	12	15	11	13	16	14	7	0	6	0	43	0
	LecRK-VII.2	At4g28350	-		3	7	11	0	0	3	0	17	0	0	0	0	0	0	0	0	0	34
LecRK-VIII	*LecRK-VIII.1*	At3g53380	+	2	12	20	10	231	18	17	11	79	51	82	24	69	130	71	25	56	0	3
	LecRK-VIII.2	At5g03140	+	3	15	28	0	10	60	19	44	0	14	18	5	35	22	11	73	0	0	1
LecRK-IX	*LecRK-IX.1*	At5g10530	+	1	1	0	4	0	0	0	0	41	0	13	2	0	0	4	18	0	0	0
	LecRK-IX.2	At5g65600	+	1	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Singletons	*LecRK-S.1*	At1g15530	+	1	1	0	8	0	0	3	0	3	0	4	0	0	0	0	0	0	2	0
	LecRK-S.2	At2g32800	+	1	50	5	0	24	30	20	8	15	0	5	0	0	8	2	5	0	0	54
	LecRK-S.3	At3g46760	-		0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0
	LecRK-S.4	At3g55550	+	1	0	7	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
	LecRK-S.5	At5g06740	-		2	8	32	23	0	1	18	40	0	0	0	0	0	0	0	0	0	0
	LecRK-S.6	At5g42120	+	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LecRK-S.7	At5g55830	-		1	0	18	0	0	0	2	0	7	9	0	29	4	4	0	0	0	0

[a] see **Table 1**; [b] cDNA and EST expression data as retrieved from TAIR; [c] sum of abundance for sense orientated classes. [d] The MPSS tag abbreviations are: CAF, callus set 1; CAS, callus set 2; GSE, germinating seedlings; LEF, leaves set 1; LES, leaves set 2; ROF, root set 1; ROS, root set 2; AP1, ap1-10 inflorescence mixed stage, immature buds; AP3, ap3-6 inflorescence mixed stage, immature buds; AGM, agamous inflorescence mixed stage, immature buds; SAP, sup/ap1 inflorescence mixed stage, immature buds; INF, inflorescence set 1; INS, inflorescence set 2; SIF, silique set 1; SIS, silique set 2; S04, salicylic acid 4 h after application; S52, salicylic acid 52 h after application. [e] MPSS 17 bp tag data are added as **Supplementary Table 1**.

[f] 0 | < 10 | 10-20 | 21-30 | 31-40 | 41-50 | > 50

0 | < 10 | 10-50 | 51-100 | 101-150 | 151-200 | > 200

Transcript abundance was verified by analyzing expression data generated by massively parallel signature sequencing (MPSS) (Lu *et al*., 2005). This showed that several gene members that lack or have few ESTs do have matching expression signatures (Table 2; Supplementary Table 1). *LecRK-V.3*, for example, has no ESTs and low MPSS values in several Arabidopsis tissues, but displays a specific high MPSS value in the sup/ap1 immature inflorescence stage. Similarly, *LecRK-V.6* lacks ESTs but MPSS signatures revealed a subtle expression in root tissue. Gene expression profiles based on microarray data (Figure 3) also showed some inconsistencies with EST counts or MPSS signatures. Taken together, however, it can be concluded that several *LecRK* genes are not or hardly expressed in any of the Arabidopsis tissues or developmental stages, for example several members of the LecRK-V clade and both *LecRK-III* genes (Figure 3), whereas a few others are broadly expressed in all tissues, in particular *LecRK-I.9*, *LecRK-IV.1*, and *LecRK-VIII.1*.

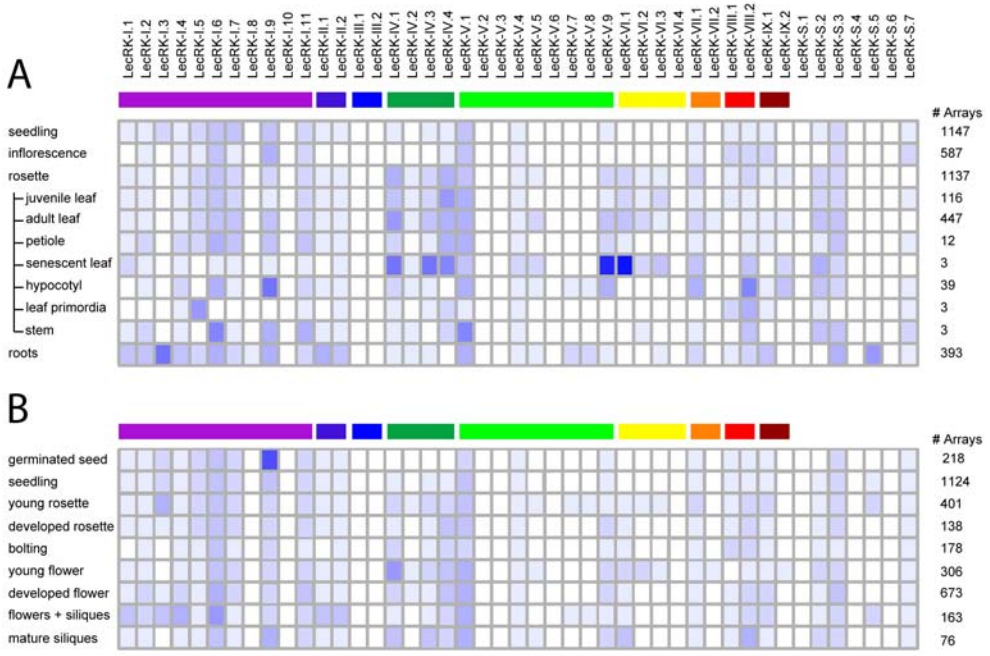

Figure 3. Expression profiles of the *LecRK* gene family in different tissues and developmental stages of Arabidopsis. (A) Heat map showing levels of gene expression across various tissues. (B) Heat map showing levels of gene expression at different stages of development. The expression profile of each individual gene was normalized in such a way that the highest signal intensity is defined as 100% (dark blue) and absence of signal as 0% (white), according to the Meta-profile analysis tool within Genevestigator V3.

LecRK gene expression in response to various stimuli

Microarray data were also queried to reveal changes in *LecRK* expression upon treatment with various hormones, environmental stresses, elicitors and pathogens. As shown in Figure 4 several *LecRK* genes, including some that are not or hardly expressed during plant development, were activated or repressed in response to diverse stimuli.

Treatment of Arabidopsis with the hormones auxin and ethylene did not result in significant changes in expression (Figure 4). By contrast, clear changes in transcript levels of some *LecRK* genes were detected in plants treated with ABA, brassinolide, cytokinin, methyl jasmonate, and salicylic acid (SA). MPSS signature data suggest an extreme high activity of *LecRK-IV.1* at 52 hours after SA application (Table 2; Supplementary Table 1). The same, but to a lesser extent, is true for *LecRK-VI.2*. Both *LecRK-IV.1* and *LecRK-VI.2* showed differential expression in different (untreated) tissues (Figure 3) but it seems that this is not correlated to SA sensitivity; other *LecRK* genes with differential expression during development were not activated by SA.

In addition, several abiotic stresses influenced *LecRK* expression (Figure 4). In response to wounding, potassium deprivation, and salt stress there were hardly any changes. Osmotic stress, anoxia, and cold, resulted in minor overall changes with the exception of one or two genes that strongly responded to one particular treatment. An example is *LecRK-V.6* which was strongly repressed by cold treatment. In the case of drought many of the *LecRK* genes responded, two of which were highly stimulated and two others strongly repressed. Response to treatment with hydrogen peroxide was particularly associated with changes in expression levels of genes in clade VII, VIII, and IX and the same genes, as well as some additional ones, were effected by ozone treatment. The most severe changes were observed in the dark when about half of the *LecRK* genes was activated and showed much higher expression levels than the controls. In the presence of high CO_2 concentrations the reverse was observed, with many of the *LecRK* genes showing a lower expression than the controls.

Upon treatment with a variety of elicitors, rather similar patterns were obtained (Figure 4). Expression of several *LecRK* genes from different clades was strongly induced upon treatment with six out of seven elicitors. The exception is bacterial lipopolysaccharide (LPS) which hardly effected any *LecRK* apart from *LecRK-IX.2*. The genes that were most strongly induced are *LecRK-I.8*, *LecRK-IV.2*, *LecRK-IV.3*, *LecRK-V.2*, *LecRK-V.5*, *LecRK-VI.2*, *LecRK-VI.3*, *LecRK-VII.2* and *LecRK-IX.2*. *LecRK-VI.4* behaved remarkably; a strong repression by syringolin but a strong induction by five other elicitors.

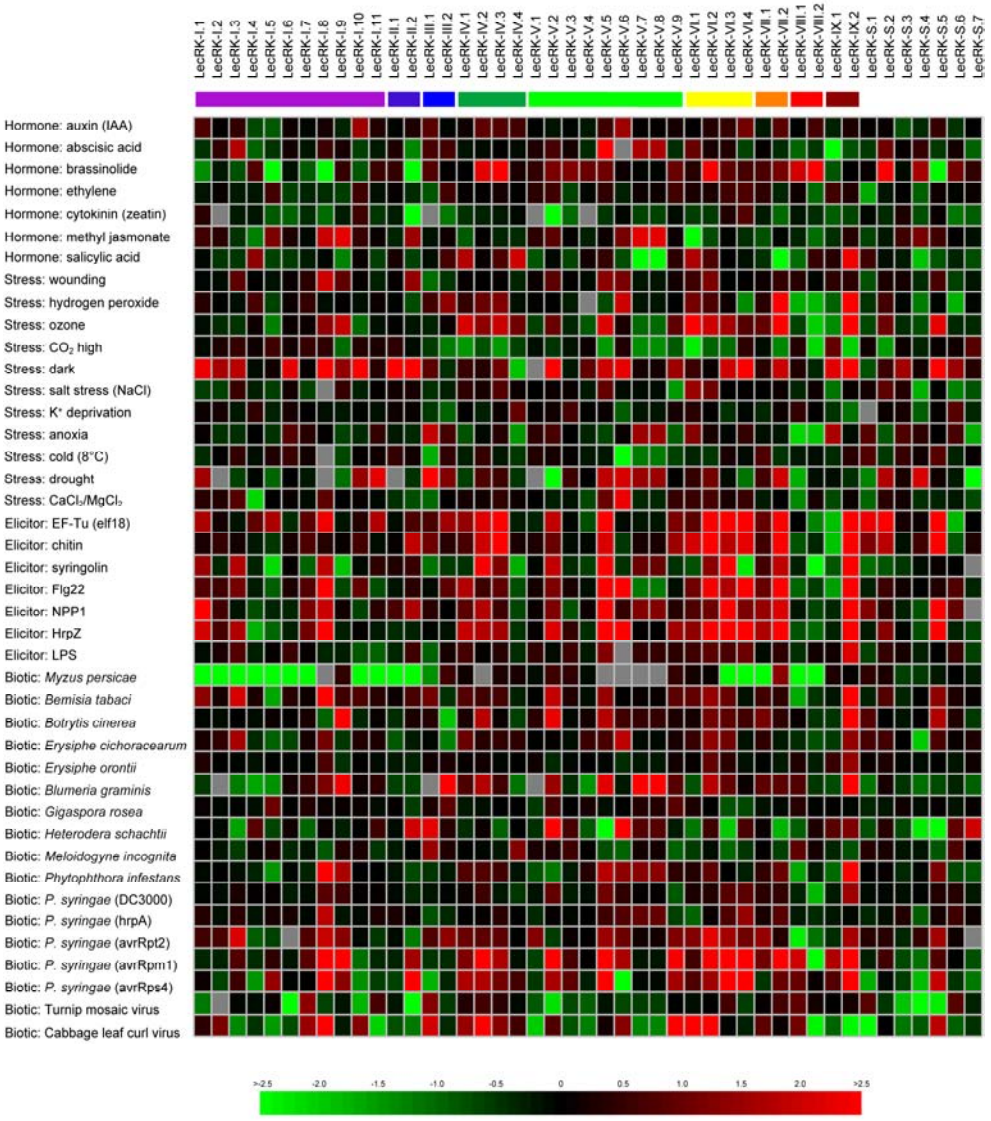

Figure 4. Expression profiles of the *LecRK* gene family in response to hormone treatment, abiotic stress, elicitor treatment and pathogen infection. Signal intensity $\log_2$ ratios (treatment vs. control) are color-coded (red and green for relatively higher or lower expression, respectively) as indicated in the scale bar. Black indicates no change between conditions. Grey depicts missing data points or indicates that both values for treatment and control are in the background range. The highest and lowest $\log_2$ signal value is -7.58 and 11.8, respectively.

Unlike the rather uniform expression pattern that is observed upon elicitor treatment, the patterns observed during interaction with biotic agents are much more diverse. The most strongest repression was measured upon interaction with the aphid *Myzus persicae* with 17 *LecRK* genes strongly down-regulated and none up-regulated. Interaction with another insect, the sweet potato whitefly *Bemisia tabaci*, only effected the activity of a few *LecRK* genes, and resulted in most cases in an increase in expression rather than a decrease. Virus infection influenced the expression of several *LecRK* genes, both positive and negative, and the patterns were specific for each of the two plant–virus interactions included in this analysis. No significant changes in gene expression were observed during interaction with the arbuscular mycorrhizal fungus *Gigaspora rosea* or the nematode *Meloidogyne incognita*. In addition, a compatible interaction with the powdery mildews *Erysiphe cichoracearum* or *E*. *orontii* did not alter *LecRK* expression substantially. By contrast, in an incompatible interaction with the non-host powdery mildew *Blumeria graminis* f. sp. *hordei* (synonym *Erysiphe graminis*) expression of several *LecRK* genes was induced or repressed. Similarly, expression was largely unaffected upon infection with the pathogenic bacterium *Pseudomonas syringae* DC3000, or its non-pathogenic type III secretion-defective mutant hrpA. However, in incompatible interactions resulting from infection with *Pseudomonas* strains secreting the effectors AvrRpt2, AvrRpm1 or AvrRps4, expression of several *LecRK* genes was found to be induced. Four of these, *LecRK-I.8*, *LecRK-I.9*, *LecRK-V.5*, and *LecRK-IX.2*, were also induced upon inoculation of Arabidopsis with the oomycete *Phytophthora infestans*. Arabidopsis is a non-host for *P*. *infestans* and this may explain why these particular *LecRK* genes were also strongly induced upon elicitor treatment. Inoculation of Arabidopsis with the grey mold fungus *Botrytis cinerea* results in necrotic lesions and, as observed in Figure 4, this was accompanied by induction or repression of a few particular *LecRK* genes including some that are affected by other pathogens.

LecRK genes are differentially expressed among Arabidopsis accessions

To examine whether *LecRK* genes are differentially expressed among Arabidopsis accessions, expression data were retrieved from the Bio-Array Resource (BAR) website using the Arabidopsis Natural Variation eFP Browser. Expression values relative to Col-0 were retrieved for each *LecRK* gene member. Only microarray data generated from experiments that made use of triplicate sampling of plant material (aerial parts of four day old seedlings) were analyzed and this restricted the analysis to the Arabidopsis accessions Col-0, Van-0, Kin-0, Est-0, Bay-0, Nd-0, L*er*-2, Sha, and Cvi. Transcript profiles were visualized with the BAR HeatMapper Plus. For most *LecRK* genes relatively similar expression levels were found among the various accessions (Figure 5). Exceptions are *LecRK-II.2*, *LecRK-V.5*, *LecRK-IV.7*, and *LecRK-IV.8*, which seem to have a higher relative expression in all accessions when compared to Col-0. It should be noted that *LecRK-IV.7* and

LecRK-IV.8 hybridize to the same probe set resulting in equal signal intensities. The few genes with a low relative expression among the accessions are mainly in clade LecRK-VIII and LecRK-IX. Only a few *LecRK* genes were found to have a high expression value in one accession but a low expression in another accession. The most striking differences in expression values were found for *LecRK-I.5* and *LecRK-IV.3* both of which have substantially lower relative transcript levels in L*er*-2 and *LecRK-V.5* that has a higher expression in the accession Bay-0.

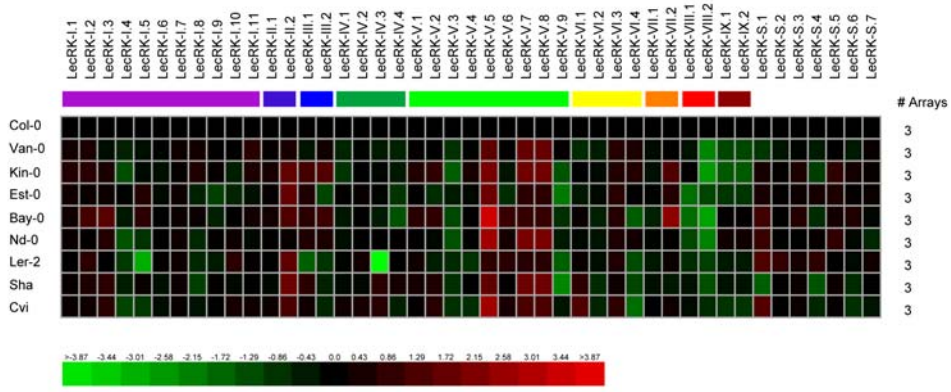

Figure 5. Relative expression difference of *LecRK* genes between Arabidopsis accessions. Col-0, Columbia, USA; Van-0, Vancouver, USA; Kin-0, Kindalville, USA; Est-0, Estonia; Bay-0, Bayreuth, Germany; Nd-0, Niederzenz, Germany; L*er*-2, Landsberg, Germany; Sha, Shahdara, Tajikistan; Cvi, Cape Verde Islands. The $\log_2$ signal intensity ratios are color-coded (red and green for relatively higher or lower expression, respectively) as indicated in the scale bar. The highest absolute value *vis-a-vis* color code is 4.3.

Conclusions

Several membrane-bound receptors in plants are shown to be functional in the perception of external signals and the subsequent triggering of downstream signal cascades to initiate an adaptive response. In this study, the focus was on the LecRK subfamily of membrane-bound receptors in Arabidopsis. The available gene information and expression data of the 45 members in this subfamily have been summarized and a new systematic nomenclature is proposed for the *LecRK* genes based on clades within the subfamily. Previously, Barre and co-workers (2002) conducted a similar analysis for 42 Arabidopsis *LecRK* genes, including structural alignments and molecular modeling of the encoded proteins. In that study, however, three genes that were predicted to encode be LecRKs by Shiu and

Bleeker (2001b, 2003) were not included. Here, the phylogenetic relationship within the *LecRK* gene family has been revised by constructing phylograms with more sophisticated statistical and computational methods. Three phylograms which were rooted with five outgroup RLKs and based on multi-alignments of the full-length proteins, lectin motifs, and kinase domains, respectively, showed clustering in nine different clades and seven singletons. Pairwise comparisons also resulted in the same nine clades and seven singletons and this consensus strengthens our clade division. The subdivision into clades and singletons formed the basis for the new, more systematic nomenclature and provides a helpful standard for gene identification and naming in other plant species.

The currently available expression datasets provide a basis to generate biological hypotheses as specific patterns of gene expression may give hints about their functionality. EST, MPSS, and microarray data indicate that the *LecRK* genes have variable expression patterns in different Arabidopsis tissues, developmental stages, in response to stimuli, and in various accessions. Several of the *LecRK*s have low transcript levels during plant development, but are induced by hormone or elicitor treatment or during interaction with pathogens. For example, a striking difference is found for the two genes in the LecRK-III clade. They are not expressed during plant development, but are expressed in response to biotic and abiotic stimuli. They do respond to different stimuli, though and, thus, their expression pattern is not clade specific. Overall, there was no correlation between the expression pattern of a particular gene and its position in the phylogenetic tree. For all 45 *LecRK* genes, including the ones encoding unusual protein structures, expression was observed in at least one of the conditions that was analyzed, hence, demonstrating that none of them represents a non-functional dead copy. So far, functional analysis of the Arabidopsis *LecRK* genes based on knock-out mutants has been limited to 5 of the 45 family members, i.e. *LecRK-I.9*, *LecRK-IV.2* and three members of the LecRK-VI clade (**Chapter 3.2**; Wan *et al*., 2008; Xin *et al*., 2009). The phylogenetic relationship and the expression analysis presented in this study may help to select other candidates for more in depth studies aimed at unraveling the role of LecRKs in various biological processes, in stress responses or in interaction with pathogens.

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Supplementary material

Supplementary Table 1. MPSS 17 bp tag expression data of the Arabidopsis *LecRK* genes

Proposed clade name[a]	Proposed gene name[a]	Locus[a]	No. MPSS 17 bp tags[b,c]																	
			CAF	CAS	GSE	LEF	LES	ROF	ROS	AP1	AP3	AGM	SAP	INF	INS	SIF	SIS	S04	S52	
LecRK-I	*LecRK-I.1*	At3g45330	0	0	0	0	0	0	17	0	0	0	0	0	0	0	0	0	0	
	LecRK-I.2	At3g45390	0	0	0	0	0	4	0	0	2	0	0	0	0	0	0	0	0	
	LecRK-I.3	At3g45410	3	0	0	4	0	23	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-I.4	At3g45420	0	0	0	0	0	0	0	0	8	0	0	4	2	2	0	0	0	
	LecRK-I.5	At3g45430	0	0	0	3	0	9	0	4	0	0	0	40	0	1	0	0	0	
	LecRK-I.6	At3g45440	0	0	0	0	0	0	8	0	0	0	0	0	3	1	0	0	0	
	LecRK-I.7	At5g60270	14	32	8	24	1	3	3	0	0	0	0	0	0	0	0	0	0	
	LecRK-I.8	At5g60280	48	13	4	0	0	4	0	2	3	3	14	24	0	2	0	0	0	
	LecRK-I.9	At5g60300	57	77	43	28	34	174	76	14	3	10	32	41	13	7	15	7	0	
	LecRK-I.10	At5g60310	0	8	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	
	LecRK-I.11	At5g60320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LecRK-II	*LecRK-II.1*	At5g59260	6	0	0	1	0	10	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-II.2	At5g59270	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LecRK-III	*LecRK-III.1*	At2g29220	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-III.2	At2g29250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LecRK-IV	*LecRK-IV.1*	At2g37710	79	62	17	127	49	59	29	62	24	37	118	71	70	106	65	85	1470	
	LecRK-IV.2	At3g53810	18	0	2	4	0	12	0	0	6	8	0	13	0	17	0	0	0	
	LecRK-IV.3	At4g02410	7	8	0	53	2	11	20	11	2	3	0	15	3	0	9	0	33	
	LecRK-IV.4	At4g02420	0	0	8	25	25	0	0	14	34	4	28	19	17	161	18	0	6	
LecRK-V	*LecRK-V.1*	At1g70110	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	
	LecRK-V.2	At1g70130	0	0	0	0	2	0	0	0	0	0	0	0	7	0	0	8	0	
	LecRK-V.3	At2g43690	3	6	0	0	2	6	8	2	2	0	76	0	0	0	0	0	0	
	LecRK-V.4	At2g43700	2	0	0	1	6	5	0	0	0	0	0	3	0	0	0	0	0	
	LecRK-V.5	At3g59700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-V.6	At3g59730	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	
	LecRK-V.7	At3g59740	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-V.8	At3g59750	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-V.9	At4g29050	0	0	16	0	4	0	1	10	12	6	2	0	14	0	0	0	0	
LecRK-VI	*LecRK-VI.1*	At3g08870	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	21	
	LecRK-VI.2	At5g01540	0	0	4	4	8	5	15	4	11	11	11	24	5	0	0	0	80	
	LecRK-VI.3	At5g01550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-VI.4	At5g01560	0	0	17	0	15	0	4	0	6	2	11	11	10	0	21	0	7	
LecRK-VII	*LecRK-VII.1*	At4g04960	25	88	4	15	5	11	15	11	13	20	12	7	0	5	0	40	0	
	LecRK-VII.2	At4g28350	5	9	0	0	2	0	16	0	0	0	0	0	0	0	0	0	30	
LecRK-VIII	*LecRK-VIII.1*	At3g53380	16	7	150	19	28	10	81	51	71	22	59	116	63	25	46	0	3	
	LecRK-VIII.2	At5g03140	23	0	8	52	16	38	0	12	17	11	30	19	9	73	0	0	1	
LecRK-IX	*LecRK-IX.1*	At5g10530	0	6	0	0	0	0	33	0	11	2	0	0	4	16	3	0	0	
	LecRK-IX.2	At5g65600	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Singletons	*LecRK-S.1*	At1g15530	9	6	0	8	3	6	11	5	21	14	25	30	9	0	0	2	0	
	LecRK-S.2	At2g32800	9	0	20	42	20	8	15	0	5	3	0	16	2	5	0	0	57	
	LecRK-S.3	At3g46760	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	
	LecRK-S.4	At3g55550	6	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	
	LecRK-S.5	At5g06740	6	21	16	0	1	15	35	0	0	0	0	0	0	0	0	0	0	
	LecRK-S.6	At5g42120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	LecRK-S.7	At5g55830	0	14	0	0	0	2	0	7	8	0	24	3	4	0	0	0	0	

[a] see **Table 1**. [b] sum of abundance for sense orientated classes. [c] The MPSS tag abbreviations are: CAF, callus set 1; CAS, callus set 2; GSE, germinating seedlings; LEF, leaves set 1; LES, leaves set 2; ROF, root set 1; ROS, root set 2; AP1, ap1-10 inflorescence mixed stage, immature buds; AP3, ap3-6 inflorescence mixed stage, immature buds; AGM, agamous inflorescence mixed stage, immature buds; SAP, sup/ap1 inflorescence mixed stage, immature buds; INF, inflorescence set 1; INS, inflorescence set 2; SIF, silique set 1; SIS, silique set 2; S04, salicylic acid 4 h after application; S52, salicylic acid 52 h after application.

[d] 0 | < 10 | 10-50 | 51-100 | 101-150 | 151-200 | > 200

3.2 The lectin receptor kinase LecRK-I.9 is a novel *Phytophthora* resistance component and a RXLR effector target

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Abstract

In plants, an active defense against biotrophic pathogens is dependent on a functional continuum between the cell wall (CW) and the plasma membrane (PM). It is thus anticipated that proteins maintaining this continuum also function in defense. The legume-like lectin receptor kinase LecRK-I.9 is a putative mediator of CW-PM adhesions in Arabidopsis and is known to bind *in vitro* to the *Phytophthora infestans* RXLR-dEER effector IPI-O via a RGD cell attachment motif present in IPI-O. Here we report that two T-DNA insertions lines deficient in LecRK-I.9 (*lecrk-I.9*) show a 'gain-of-susceptibility' phenotype towards the oomycete *Phytophthora brassicae*. Accordingly, overexpression of *LecRK-I.9* leads to enhanced resistance to *P. brassicae* and not to other pathogens tested. A similar 'gain-of-susceptibility' phenotype was observed in transgenic Arabidopsis lines expressing *ipiO* (35S-*ipiO1*). This phenocopy behavior was also observed with respect to other defense related functions; *lecrk-I.9* and 35S-*ipiO1* were both disturbed in pathogen- and MAMP-triggered callose deposition and they both had weakened CW-PM adhesions. By site directed mutagenesis we demonstrated that the RGD cell attachment motif in IPI-O is not only essential for disrupting the CW-PM adhesions, but also for disease suppression. These results suggest that destabilizing the CW-PM continuum is an obvious strategy for *Phytophthora* to promote infection. As countermeasure the host may want to strengthen CW-PM adhesions and the novel *Phytophthora* resistance component LecRK-I.9 seems to function in this process.

Introduction

Plants deploy multiple strategies to defend themselves against pathogen attack. A key step is the perception of pathogen molecules in order to activate various defense responses. During infection pathogens produce microbe-associated molecular patterns (MAMPs) or elicit the production of host-derived damage-associated molecular patterns (DAMPs). These signals are recognized by the plant via so-called pattern recognition receptors (PRRs) and trigger cell wall-associated defenses, such as the production of antimicrobial compounds and cell wall strengthening (Boller and Felix, 2009; Hückelhoven, 2008). An active defense response is, however, dependent on a functional continuum between the cell wall (CW) and the plasma membrane (PM) (Mellersh and Heath, 2001). When this continuum is disturbed the effectiveness of defense is lost. Destabilizing the CW-PM continuum to circumvent recognition could therefore be an obvious strategy for a pathogen to promote infection.

In animals, cell adhesion is mediated by integrins, membrane-spanning receptors whose ligands are extracellular matrix (ECM) proteins carrying the cell-attachment motif Arg-Gly-Asp (RGD) (Ruoslahti, 1996). Other proteins or peptides comprising the motif RGD can act as integrin antagonists and as such interfere with integrin-related functions, including cell adhesion and proliferation (Watson *et al*., 2006; Olfa *et al*., 2005). Examples are certain viral, bacterial and fungal proteins (Garrigues *et al*., 2008, Stockbauer *et al*., 1999; Hostetter, 2000), and various snake venom disintegrins (Calvete *et al*., 2005).

Also plant pathogens produce proteins carrying a RGD motif. Well-studied is PtrToxA, a secreted proteinaceous toxin of the foliar wheat pathogen *Pyrenophora tritici-repentis*, which induces cell death in toxin-sensitive wheat mesophyll cells (Sarma *et al*., 2005). Internalization of PtrToxA is dependent on its RGD-containing solvent loop, which is largely identical to the integrin-binding RGD loop of the mammalian ECM glycoprotein vitronectin (Manning and Ciuffetti, 2005; Manning *et al*., 2008). PtrToxA proteins with mutations in the RGD-loop were found to be less toxic due to impaired internalization. Toxicity of PtrToxA was also reduced when RGD peptides were added as competitor and this supports the hypothesis of a RGD-dependent receptor-mediated endocytosis (Manning *et al*., 2008).

Several studies have shown that exogenously applied RGD peptides can have a strong disrupting effect on plant cells. For example, in cell suspensions of soybean and Arabidopsis, and in onion epidermal cells RGD peptides caused loss of CW-PM adhesions in a concentration dependent manner, whereas RGE or DGR peptides did not show this effect (Schindler *et al*., 1989; Canut *et al*., 1998). Moreover, RGD peptides added to pea epicotyls reduced the production of the phytoalexin pisatin (Kiba *et al*.,1998), and in shear-stressed *Taxus* cells these peptides negatively affected the alkalization response, as well as the accumulation

of both, phenolics and reactive oxygen species (ROS) (Gao *et al*., 2007). Accordingly, cowpea and pea cells treated with RGD peptides display a disturbed CW-PM continuum and decreased expression of cell wall-associated defense responses upon fungal penetration, whereas these effects were not seen when RGE peptides were added. Hence, the RGD-mediated reduction of defense response stimulated fungal penetration and intracellular hyphal growth (Mellersh and Heath, 2001).

IPI-O, an RXLR-dEER effector of the oomycete pathogen *Phytophthora infestans*, disrupts CW-PM adhesions in Arabidopsis through its RGD motif (Senchou *et al*., 2004). When the RGD motif in IPI-O is mutated to RGE or RGA the adhesions remain intact (Senchou *et al*., 2004). Plant plasma membranes possess high affinity RGD-binding sites, and binding was shown to be saturable, reversible and RGD-specific (Canut *et al*., 1998). IPI-O interacts with these sites in a similar manner, and hence competes with other proteins possessing the RGD motif. A phage display, set up to find proteins interacting with the RGD motif of IPI-O, resulted in two peptides that act as RGD-binding antagonists and lead to the identification of an Arabidopsis legume-like lectin receptor kinase named LecRK-I.9 that binds IPI-O via its RGD motif (Gouget *et al*., 2006). Since LecRK-I.9 functions in CW-PM adhesions it is conceivable that LecRK-I.9 is involved in protein-protein interactions with RGD-containing proteins as potential ligands and plays a structural and signaling role at the plant cell surface. As yet, little is known about the function of LecRK-I.9 and other legume-like lectin receptor kinases (**Chapter 3.1**) nor about their natural ligands.

Besides the RGD motif, IPI-O contains another characteristic motif called RXLR, that partially overlaps with RGD (i.e. RSLRGD). This motif is shared by numerous secreted oomycete effector proteins several of which are known to function as race-specific avirulence factor (**Chapter 1.3**). For two other RXLR effectors – i.e. *P. infestans* Avr3a and *P. sojae* Avr1b – it has demonstrated that the RXLR motif is crucial for transfer of the effector to the host cell (Whisson *et al*., 2005; Dou *et al*., 2008; Govers and Bouwmeester, 2008). The effector function of IPI-O is further supported by expression data; *ipiO* expression is not detectable in *in vitro* grown mycelium but induced in germ tubes invading host tissue. In susceptible potato lines expression is highest in the periphery of the lesion where the plant cells are being colonized, but is lacking in the necrotic center of the lesion and in the sporulating zone (Van West *et al*., 1998). Recently we have shown that *ipiO* is the avirulence (*Avr*) gene *Avr-blb1* that acts in a gene-for-gene manner with *Rpi-blb1*, a late blight resistance (*R*) gene from *Solanum bulbocastanum* which encodes a cytoplasmic NBS-LRR protein (Vleeshouwers *et al*., 2008). In potato plants carrying *Rpi-blb1*, colonization by *P. infestans* isolates that contain class I variants of IPI-O is blocked (**Chapter 2.1**).

The observation of binding between a *Phytophthora* effector and an Arabidopsis legume-like lectin receptor kinase via the RGD cell attachment motif (Gouget *et al*. 2006) raised questions about the role of LecRK-I.9 in the interaction with *Phytophthora*. Since, Arabidopsis is a non-host plant for *P*. *infestans* (Vleeshouwers *et al*., 2000), we made use of another *Phytophthora* species, *P*. *brassicae*. For the interaction between *P*. *brassicae* and Arabidopsis various distinct incompatible and compatible isolate-accession combinations have been described, and this pathosystem can be regarded as an attractive model for the concurrent analysis of both host and pathogen (Roetschi *et al*., 2001; Mauch *et al*., 2009; **Section 4**). In this study, we first analyzed expression of *LecRK-I.9* under biotic and abiotic stress. Subsequently, we analyzed the response of Arabidopsis *LecRK-I.9* T-DNA insertion mutants (*lecrk-I.9*) to infection with *P*. *brassicae* and investigated the role of IPI-O in the infection process by making use of transgenic Arabidopsis lines expressing *ipiO1*. Interestingly, the *lecrk-I.9* mutants and the *ipiO1*-expressing lines showed strikingly similar phenotypes. In both cases, we not only observed gain of susceptibility but also comparable patterns of pathogen- and MAMP-triggered callose deposition. Overall, our observations strongly suggest that LecRK-I.9 plays a role in disease resistance and point toward involvement of the RXLR effector IPI-O in infection processes.

Results

LecRK-I.9 expression is induced in incompatible interactions

Analysis of endogenous *LecRK-I.9* expression in Arabidopsis Col-0 by RT-PCR revealed *LecRK-I.9* transcripts in all tested tissues, i.e. in flowers, siliques, stems, rosette leaves and roots (data not shown). To analyze *LecRK-I.9* expression in more detail we used a GUS reporter-aided approach. The reporter construct $P_{LecRK\text{-}I.9}$-GUS that contains a genomic DNA fragment covering the promoter region (-1486 to +9 bp) of *LecRK-I.9* fused to the coding sequence of the ß-glucuronidase (GUS) reporter gene was transformed into the Arabidopsis accession Col-0 through *Agrobacterium*-mediated gene transfer. All transformants that were subjected to histochemical GUS staining showed comparable GUS staining patterns. One transformant possessing a single T-DNA insertion was selected for further analysis. In hypocotyls of 2-week old plants, GUS activity was detected in all cell layers, including the vascular tissue, and epidermal and cortex cells (data not shown). GUS staining of 4-week old plants resulted in a deep blue color in the petiole, and in the primary veins of rosette leaves but, in contrast, no GUS activity was found in leaf epidermal and mesophyll cells (Figure 1A). GUS activity was also detected in the steles of primary and secondary roots, especially during lateral and adventive root setting (data not shown). These results show that *LecRK-I.9* is

differentially expressed during organ differentiation with very low or no expression in fully differentiated tissues.

We then analyzed *LecRK-I.9* expression in plants exposed to various biotic and abiotic stresses. We inoculated the $P_{LecRK\text{-}I.9}$-GUS reporter line with two *P. brassicae* isolates, isolate CBS686.95 that is compatible with Col-0 and isolate HH that is incompatible with Col-0. GUS staining upon inoculation with isolate CBS686.95 was comparable to the staining observed in un-inoculated or mock-treated leaves (Figure 1B). In contrast, inoculation with HH resulted in a significant increase in GUS activity as early as 1 day post-inoculation (dpi). Figure 1C shows the GUS activity at 6 dpi. A similar increase, that also appeared within 1 dpi was observed in the non-host interaction of Col-0 with *P. infestans* (Figure 1D), and upon inoculation with isolate IMI169558 of the necrotrophic grey mold fungus *Botrytis cinerea*, which can infect and colonize Col-0 (Figure 1E). An increase in GUS activity was also found within 6 hours after inoculation with the avirulent *Pseudomonas syringae* strain DC3000(*avrRpm1*), while in a compatible interaction with *P. syringae* DC3000 GUS activity did not change (Supplementary Figure 1).
Both jasmonate and salicylate are well-know pathogen-induced plant signals that activate various defense responses. Hence, we treated rosette leaves of the $P_{LecRK\text{-}I.9}$-GUS reporter line with methyl jasmonate (MeJA) and salicylic acid (SA). GUS activity was strongly enhanced 20 hours after treatment with MeJA (Supplementary Figure 2B). Treatment with SA though, resulted in a GUS-staining pattern comparable to that of mock-treated leaves (Supplementary Figure 2A, and C). Upon exposure to abiotic stress such as wounding by toothpicks (Figure 1F), heat or cold treatment (data not shown) or high salinity up to 0.2 M (Supplementary Figure 2D) there was no increase in GUS activity. In summary, these results reveal induction of expression of *LecRK-I.9* upon infection with *B. cinerea* and in incompatible interactions with biotrophic *Phytophthora* and *Pseudomonas* pathogens.

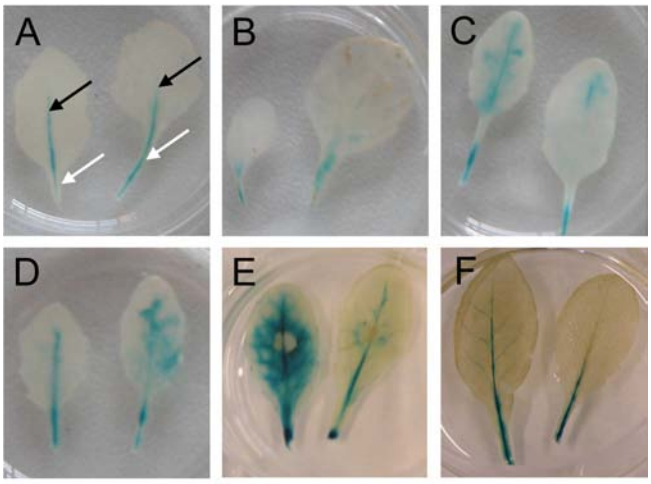

Figure 1. Expression of *LecRK-I.9* in Arabidopsis is induced in an incompatible but not in a compatible interaction with *Phytophthora brassicae*. GUS activity in leaves of a transgenic Arabidopsis line carrying promoter-GUS construct $P_{LecRK\text{-}I.9}$-GUS. (A) In mature non-inoculated leaves GUS activity is localized in the petiole (white arrow) and in veins (black arrows). GUS activity in leaves, 6 days post-inoculation (dpi) with the virulent *P. brassicae* isolate CBS686.95 (B), the avirulent *P. brassicae* isolate HH (C), *P. infestans* IPO-0 (D), 4 dpi with *Botrytis cinerea* IMI169558 (E), and 2 days after wounding (F).

Arabidopsis mutants disrupted in *LecRK-I.9* show gain of susceptibility towards *Phytophthora brassicae*

To be able to study the potential function of LecRK-I.9, we generated two mutant lines with T-DNA insertions just downstream of the translation start site (Figure 2A). Growth behavior and morphology of the two homozygous *lecrk-I.9* mutant lines (i.e. *lecrk-I.9-1* and *lecrk-I.9-2*) and the recipient line Col-0 were comparable (data not shown). Also generation time, seed setting and seed germination were not affected in LecRK-I.9 deficient plants. We then compared the response of the *lecrk-I.9* mutants and Col-0 to inoculation with *P. brassicae*. Isolate HH is incompatible with Col-0 and unable to colonize Col-0 plants. In contrast, plug-inoculation of *lecrk-I.9* plants with isolate HH resulted in lesions that first appeared 2 days after inoculation and expanded further until complete leaf collapse after 7 days (Figure 2B). Similar results were obtained when Col-0 and *lecrk-I.9* plants were inoculated with zoospores of isolate HH (data not shown). This gain of disease susceptibility was observed in several assays on both *lecrk-I.9-1* and *lecrk-I.9-2*. It is a stable phenotype and the infection efficiency (IE) of isolate HH on the two *lecrk-I.9* lines is on average over 80 percent. Trypan blue staining of *lecrk-I.9* leaves infected with

HH revealed heavy tissue colonization and sporulation (Figure 2C). This was further confirmed by UV fluorescence microscopy in *lecrk-I.9* leaves infected with a GFP-tagged transformant of isolate HH (Figure 2D). Comparable disease symptoms and IE were found when *lecrk-I.9* plants were inoculated with *P. brassicae* isolate II, which is – like isolate HH – incompatible with Col-0 (Supplementary Figure 2). To determine the specificity of this gain of disease susceptibility, we performed infection assays with other pathogens. Despite the fact that expression of *LecRK-I.9* is induced in response to infection with *B. cinerea* (Figure 1E), *lecrk-I.9* did not show stronger grey mold disease symptoms compared to Col-0. Moreover, inoculation of *lecrk-I.9* with the non-host pathogens *P. infestans* and *Alternaria brassicicola*, a necrotrophic fungus, did not result in lesion formation and no obvious changes in disease susceptibility were found with respect to the hemibiotrophic fungus *Colletotrichum destructivum*, which forms a compatible interaction with Col-0, nor in an incompatible and compatible interaction with the bacterium *P. syringae* (Table 1).

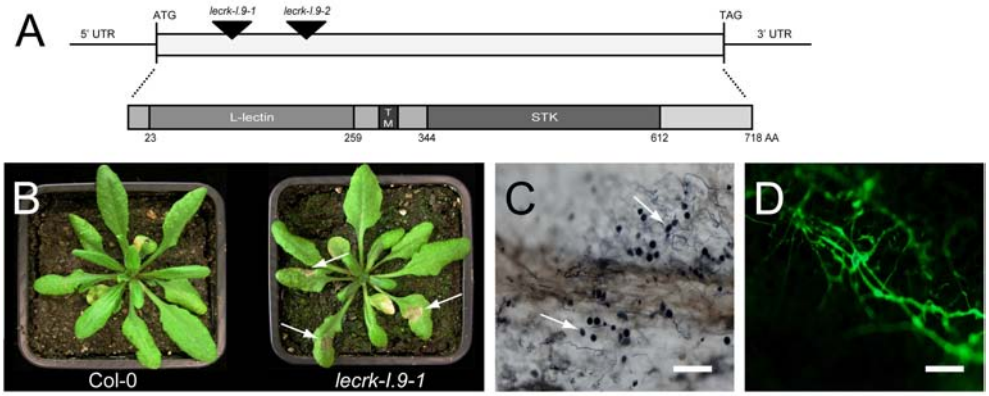

Figure 2. Arabidopsis mutants disrupted in *LecRK-I.9* are impaired in resistance to *P. brassicae*. (A) Schematic representation of *LecRK-I.9* and its gene product. The coding sequence is 2154 nt in length and has one continuous open reading frame. The two mutant lines *lecrk-I.9-1* and *lecrk-I.9-2* have a T-DNA insertion (black arrowheads) at position 282 and 566, respectively, relative to the translational start. The LecRK-I.9 protein contains an extracellular legume-like lectin domain (L-lectin), a transmembrane motif (TM), and an intracellular serine/threonine protein kinase domain (STK). (B) Col-0 and *lecrk-I.9-1* plants 4 days post-inoculation with *P. brassicae* isolate HH. Arrows point at lesions. Colonization of *lecrk-I.9-1* leaves by *P. brassicae* isolate HH visualized by trypan blue staining (C) and *P. brassicae* GFP transformant 155m revealed by UV epifluorescence (D). Arrows in (C) point at sporangia. Scale bar in (E) represents 100 µm and in (F) 200 µm.

Overexpression of *LecRK-I.9* leads to developmental effects and enhanced resistance to *P. brassicae*

To further investigate the role of *LecRK-I.9* in the defense response, transgenic Arabidopsis plants were generated that constitutively express *LecRK-I.9*. A construct containing the full-length coding sequence of *LecRK-I.9* under the control of the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter was transferred to Arabidopsis accession Col-0 via flower-dip transformation. Multiple independent lines were obtained, two of which were selected for further analysis (i.e., C-0123 and C-0126). In comparison to the recipient line Col-0, both 35S-*LecRK-I.9* lines had more compact rosettes with smaller and slightly wrinkled leaves (Figure 3A), and were shorter in height (data not shown). Moreover, the transgenic lines displayed a substantially higher accumulation of anthocyanin and lignin, a phenomenon not observed in Col-0 or the *lecrk-I.9* lines (Figure 3B, and C). Anthocyanin pigmentation and lignin deposition was most intense in mature cotyledons, and along the petioles and midribs of young rosette leaves (Figure 3B, and C).

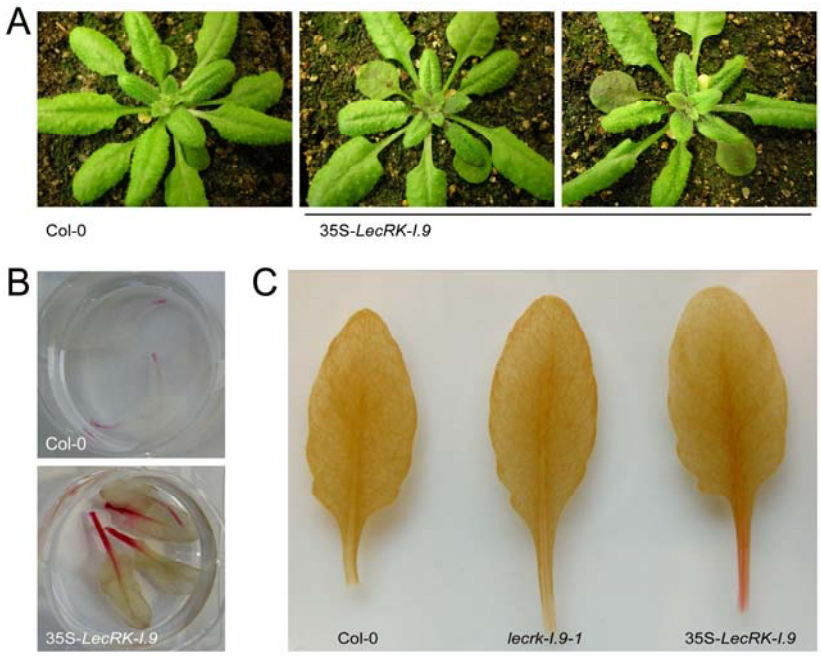

Figure 3. Overexpression of *LecRK-I.9* in Arabidopsis leads to changes in morphology and accumulation of anthocyanin and lignin. In comparison to the recipient line Col-0, 35S-*LecRK-I.9* lines have more compact rosettes with smaller and slightly wrinkled leaves (A). Anthocyanin (B) and lignin (C) staining in rosette leaves of Col-0 and 35S-*LecRK-I.9* lines.

When we challenged the 35S-*LecRK-I.9* lines and the recipient line Col-0 with *P. brassicae* isolate HH we observed no differences. Col-0 is incompatible with HH and this incompatibility with HH is maintained in the 35S-*LecRK-I.9* lines (data not shown). Col-0 is compatible though, with *P. brassicae* isolate CBS686.95 and upon inoculation with this isolate, both with mycelium plugs and by drop inoculation with a zoospore suspension, Col-0 leaves became completely colonized. In contrast, such colonization was not observed in the transgenic 35S-*LecRK-I.9* lines. These plants showed an elevated level of resistance towards this *P. brassicae* isolate (Figure 4A, and B); there were no or only minor disease symptoms but instead there was a hypersensitive response (HR), including an increase in callose deposition, which was not found in Col-0 (Figure 4C). The experiment was repeated several times with comparable results. No differences in disease progression were observed between Col-0 and the 35S-*LecRK-I.9* lines upon inoculation with *Botrytis cinerea* (data not shown).

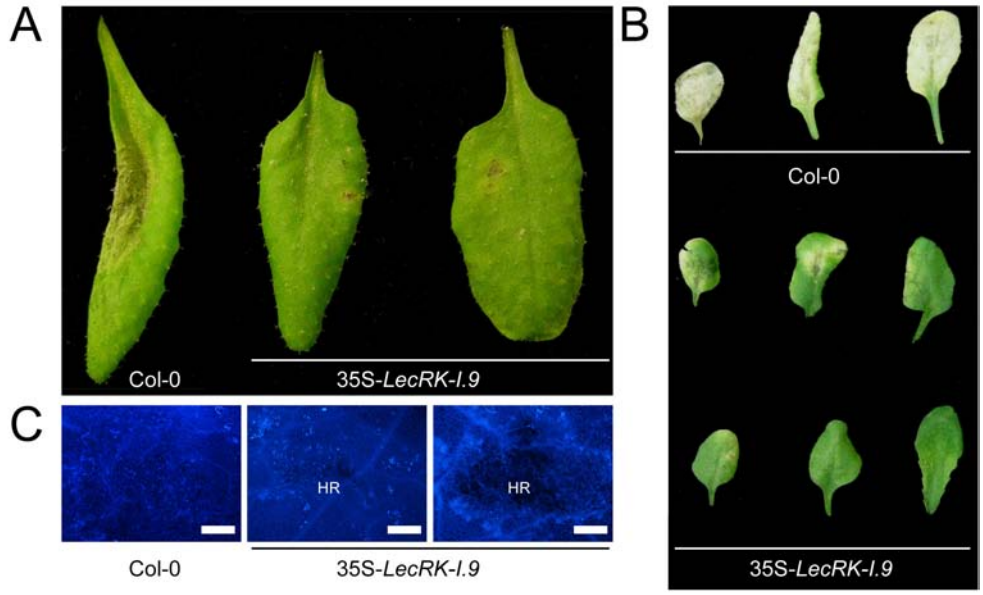

Figure 4. *LecRK-I.9* overexpression in Arabidopsis results in enhanced resistance to *Phytophthora brassicae*. Arabidopsis leaves inoculated with plugs (A) or zoospores (B and C) of *P. brassicae* isolate CBS686.95. Pictures were taken 4 days post-inoculation (dpi) (A), 8 dpi (B), and 5 dpi (C). Lesion expansion was observed on Col-0 leaves, but not on leaves from two independent 35S-*LecRK-I.9* lines. In (C) leaves were stained for callose (light blue fluorescence). HR; hypersensitive response. Scale bars in (C) represent 50 µm.

lecrk-I.9 and 35S-*ipiO1* lines are gain of susceptibility phenocopies

To examine whether *ipiO* functions as an effector that manipulates host defense responses, we generated Arabidopsis transformants carrying a transgene composed of the coding sequence of the *P. infestans ipiO1* gene fused to the constitutive CaMV 35S promoter in a Col-0 background. The transformants developed normal and with respect to morphology and size they could not be distinguished from Col-0 plants. Multiple independent transformants (35S-*ipiO1*) were tested for their response to *P. brassicae*. In contrast to Col-0, which, as expected, exhibited full resistance to *P. brassicae* isolate HH, the 35S-*ipiO1* plants displayed clear disease symptoms 3 days after inoculation with HH (Figure 5A). The foliar lesions developed gradually over time, and leaves were completely wilted after 7 days. Trypan blue staining of infected leaf material revealed massively intercellular hyphal growth and sporulation similar to what was observed in the infected *lecrk-I.9* lines (data not shown). Comparable disease symptoms and IE were found when the 35S-*ipiO1* plants were inoculated with *P. brassicae* isolate II (Supplementary Figure 3). No clear changes in disease progression were observed upon inoculation of the 35S-*ipiO1* plants with the fungal and bacterial pathogens (Table 1). Since this gain of susceptibility, specifically to the *P. brassicae* isolates HH and II, was also observed in *lecrk-I.9* lines, we can conclude that the *lecrk-I.9* and 35S-*ipiO1* lines behave as phenocopies.

Table 1. Gain of susceptibility to *Phytophthora brassicae* in Arabidopsis *LecRK-I.9* knock-out lines (*lecrk-I.9* in Col-0) and Col-0 lines expressing *ipiO1* (35S-*ipiO1*).

Pathogen	**Observed disease phenotype** [a]		
	Col-0	***lecrk-I.9***	**35S-*ipiO1***
Botrytis cinerea IMI169558	S	S	S
Colletotrichum destructivum IMI349061	S	S	S
Alternaria brassicicola MUCL20297	R	R	R
Phytophthora infestans IPO-0	R	R	R
Phytophthora brassicae HH	R	S	S
Phytophthora brassicae II	R	S	S
Phytophthora brassicae CBS686.95	S	S	S
Pseudomonas syringae DC3000(*avrRpm1*)	R	R	R
Pseudomonas syringae DC3000	S	S	S

[a] based on at least three independent experiments; R, resistant; S, susceptible.

lecrk-I.9 and 35S-*ipiO1* lines show changes in cell wall integrity

Previously Senchou *et al*. (2004) showed that exposing Arabidopsis cells to IPI-O protein, obtained by heterologous *ipiO* expression in *E*. *coli*, results in disruption of the CW-PM continuum. To investigate if ectopic expression of *ipiO* in Arabidopsis causes the same effect we examined the CW-PM continuum in 35S-*ipiO1* plants. Plasmolysis was induced by soaking the hypocotyls in 0.4 M $CaCl_2$. Upon staining the cytoplasm with neutral red we observed that in Col-0 the PM readily separated from the CW, but at several points the adhesions between CW and PM were maintained, resulting in pockets that are concave with respect to cells. In contrast, convex forms of plasmolysis were observed in 35S-*ipiO1* plants; the PM quickly separated from the CW reaching a near-spherical shape (Figure 5B), similar to what Senchou *et al*. (2004) observed upon adding IPI-O protein to Arabidopsis cell suspension cultures. To exclude that constitutive expression of *ipiO* leads to secondary effects, indirectly causing disruption of CW-PM continuum, we generated stable transformants in which *ipiO1* expression is under control of the alcohol-inducible *alcA* promoter. Similar to the 35S-*ipiO1* transformants, the *alcA-ipiO1* transformants were morphological comparable to the parental line Col-0. After exposure of the *alcA-ipiO1* plants to 0.01% (v/v) ethanol for 30 min, plasmolyzed hypocotyls displayed a phenotype identical that of 35S-*ipiO1* plants: i.e., convex forms of plasmolysis (Table 2; Supplementary Figure 4). Hence, we can conclude that IPI-O causes the loss of CW-PM adhesions. A logic next step was to examine the CW-PM adhesions in the phenocopy lecrk-I.9 lines. Interestingly, the convex forms of plasmolysis that were observed in 35S-*ipiO1* and *alcA-ipiO1* plants were also found in both knock-out lines, *lecrk-I.9-1* and *lecrk-I.9-2* (Figure 5B; Table 2). Although the relative number of cells that showed a convex shape of plasmolysis was slightly less than in the 35S-*ipiO1* and *alcA-ipiO1* plants, the absence of LecRK-I.9 clearly caused a reduction in the strength of the CW-PM adhesions.

Table 2. The RGD motif in IPI-O is required to disrupt CW-PM adhesions *in planta*

Transgene [b]	Observed type of plasmolysis [a]	
	concave	convex
none	x	
35S-*ipiO1*		x
35S-*ipiO1*^{RGE}	x	
alcA-ipiO1		x
alcA-ipiO1^{RGE}	x	
alcA-ipiO1^{RGA}	x	

[a] observation based on three independent experiments, each including at least 4 hypocotyls; n.d.= not determined; [b] in Col-0 background

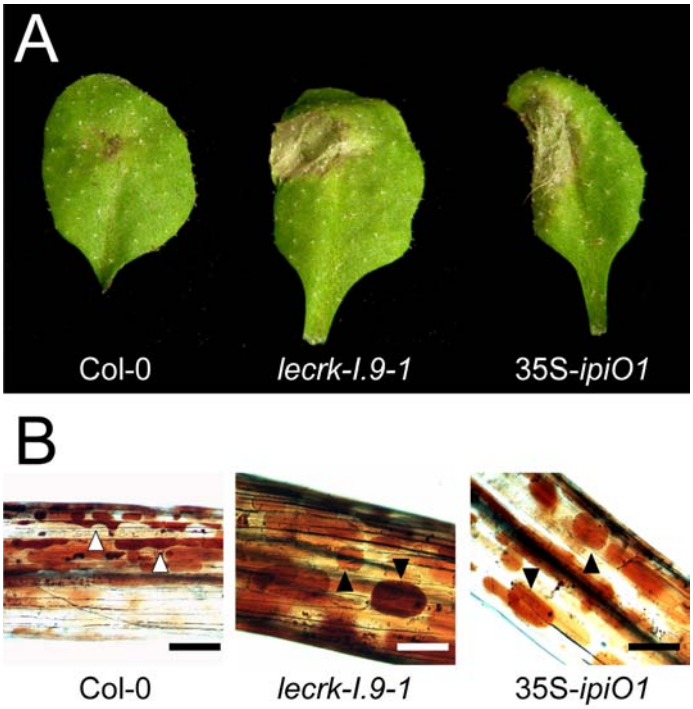

Figure 5. Arabidopsis *lecrk-I.9* and 35S-*ipiO1* lines are phenocopies; both show gain of susceptibility to *Phytophthora brassicae* and defects in cell wall integrity. (A) Leaves inoculated with *P. brassicae* isolate HH 3 days post-inoculation. (B) Elicitation of plasmolysis in etiolated hypocotyls by treatment with 0.4 M $CaCl_2$ reveals concave forms of plasmolysis in Col-0 (white arrowheads) and convex forms of plasmolysis in *lecrk-I.9* and 35S-*ipiO1* (black arrowheads). Scale bars represent 100 µm.

lecrk-I.9 and 35S-*ipiO1* lines are impaired in callose deposition

The gain of susceptibility and impairment of cell wall integrity suggests that cell wall associated defense response could be affected in the *lecrk-I.9* and 35S-*ipiO1* lines. To investigate this, we analyzed the level of callose deposition in the phenocopy lines after infiltration with *P. syringae*. Strain DC3000 produces effectors that suppress defense in Col-0 and, as a result, callose deposition is decreased (Hauck *et al*., 2003). The *P. syringae* hrcC mutant can no longer suppress defense resulting in callose accumulation. Microscopic analysis revealed that DC3000-infiltrated leaves did not (or hardly) display callose deposition in Col-0, and neither in *lecrk-I.9* nor in 35S-*ipiO1* lines (Figure 6A, and B). As expected, Col-0 leaves infiltrated with the *P. syringae* hrcC mutant displayed a significant increase of callose deposition but in contrast, this increase was not observed in hrcC infiltrated leaves of *lecrk-I.9* and 35S-*ipiO1* plants (Figure 6A, and B). Similarly, infiltration with the oligopeptide flg22 resulted in extensive callose deposition in Col-0, but hardly any callose deposition was found in *lecrk-I.9* and 35S-*ipiO1* lines (Figure 6C, and D). This points at a defect in MAMP-triggered callose deposition in both, the *lecrk-I.9* and 35S-*ipiO1* lines.

The RGD motif in IPI-O is a determinant of the phenotypic changes

We then addressed the role of the RGD motif in IPI-O and questioned if RGD is a determinant of the phenotypic changes observed in Arabidopsis upon ectopic *ipiO* expression. We therefore generated transgenic lines carrying *ipiO1* constructs with site-directed mutations changing RGD to RGE or RGA. Two independent 35S-*ipiO1*^{RGE} lines, one *alcA-ipiO1*^{RGE} line and two *alcA-ipiO1*^{RGA} lines were tested in a plasmolysis assay. In none of these lines a convex type of plasmolysis was observed, and as in Col-0 the CW-PM adhesions remained intact (Table 2). This is in agreement with the results of Senchou *et al*. (2004) who showed that in cell suspension cultures exogenously added mutant forms of IPI-O (IPI-O^{RGE} and IPI-O^{RGA}) had no disrupting effect. Subsequently, we examined how a RGD-to-RGE mutation in IPI-O affects susceptibility to *P. brassicae*. As described above Col-0 exhibited full resistance to *P. brassicae* isolate HH whereas the leaves of 35S-*ipiO1* plants were completely wilted leaves 5 days post-inoculation. On the two independent 35S-*ipiO1*^{RGE} lines no lesions appeared and the phenotype was comparable to the incompatible interaction between Col-0 and HH (Figure 7). Taken together, these results show that the RGD cell adhesion motif in IPI-O is crucial for disruption of the CW-PM continuum, as well as the gain of susceptibility to *P. brassicae*.

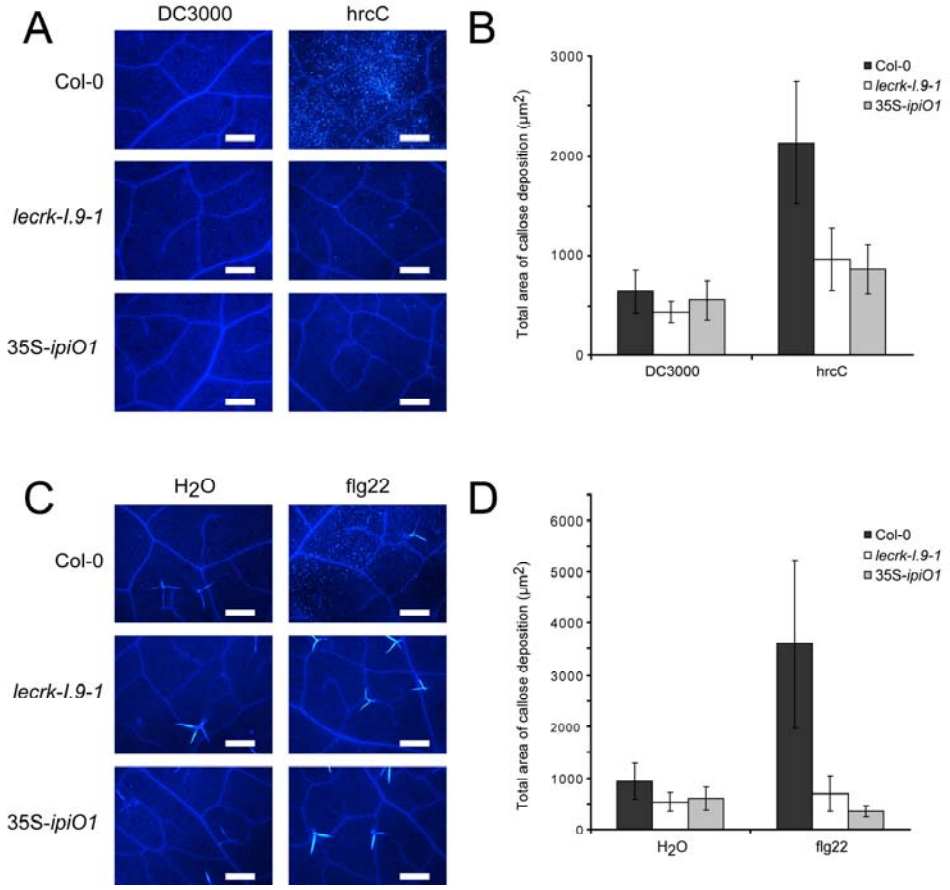

Figure 6. Pathogen- and MAMP-triggered callose deposition is reduced in *lecrk-I.9* and 35S-*ipiO1* lines. (A) Arabidopsis leaves stained with aniline blue after infiltration with *Pseudomonas syringae* DC3000 and hrcC. (B) Average totals of callose deposition (μm^2) and associated 95% confidence intervals (CIs) after infiltration with DC3000 and hrcC (n= >16) (C). Callose deposition after infiltration with flg22. (D) Average totals of callose deposition after treatment with flg22 (μm^2) + 95% CIs (n= >10). Scale bars in (A) and (C) represent 50 µm.

Figure 7. Arabidopsis transgenic lines expressing *ipiO1* with a RGD-to-RGE targeted show no gain of susceptibility to *P. brassicae* HH. Arabidopsis leaves inoculated with *P. brassicae* strain HH 5 days post-inoculation.

Discussion

Membrane-spanning receptor proteins are supposed to play important roles in sensing alterations at the plant cell wall and, subsequently, to mediate response reactions (Humphrey *et al*., 2009). Legume-like lectin receptor kinases (LecRKs) are regarded as candidates for monitoring cell wall integrity, and are likely functional in responses to various stresses. Up-till now only few reports have addressed the role of LecRKs in plant–pathogen interactions. Arabidopsis has a family of 45 LecRKs divided over nine clades and a few singletons (**Chapter 3.1**). This study revealed that one of these LecRKs, i.e., LecRK-I.9, is a novel *Phytophthora* resistance component. In incompatible interactions LecRK-I.9 plays a crucial role in arresting growth of the pathogen. In compatible interactions the pathogen exploits an effector containing a RGD cell-attachment motif to disturb the cell wall-plasma membrane continuum, possibly by targeting the anchor protein LecRK-I.9.

LecRK-I.9 was initially identified in a phage display as a protein interacting with the *P. infestans* RXLR-dEER effector IPI-O via the RGD motif included in IPI-O. In this study we first analyzed *LecRK-I.9* gene expression in Arabidopsis by making use of transformants carrying a promoter-GUS fusion construct ($P_{LecRK\text{-}I.9}$-GUS) as transgene. Under normal growth conditions *LecRK-I.9* expression is observed throughout the plant at a low level with higher levels of expression during organ differentiation. Exposure to abiotic stresses, such as wounding, heat, cold and

salinity, or treatment with salicylic acid did not change the basal expression levels. Treatment with methyl jasmonate, however, resulted in a strong increase in expression. The observation that wounding, which is known to elicit a jasmonate dependent systemic defense response, does not induce expression while methyl jasmonate does suggests that expression of *LecRK-I.9* is regulated via a separate signal transduction route. Upon colonization of the $P_{LecRK\text{-}I.9}$-GUS plants with biotrophic pathogens *LecRK-I.9* expression did not change. Infected and mock-treated leaves showed the same low basal level of *LecRK-I.9* expression. In contrast, the *LecRK-I.9* expression was much higher when these biotrophic pathogens encountered a strong hypersensitive response (HR) and were thus unable to colonize the leaves. Such clear differences in *LecRK-I.9* expression between compatible and incompatible interactions were observed in leaves infected with *P. syringae* (DC3000 versus DC3000 expressing *avrRPM1*) and *P. brassicae* (HH versus CBS686.95). *LecRK-I.9* expression was also strongly increased in a non-host interaction with *P. infestans*, which shows an HR reminiscent of an incompatible interaction, and in lesions resulting from infection with the necrotrophic pathogen *B. cinerea*, in which cells are committed to programmed cell death (Van Kan, 2006). Taken together, these expression patterns suggest that LecRK-I.9 has a role in pathways leading to HR or programmed cell death. Strong evidence for a role for LecRK-I.9 in defense was obtained by making use of *LecRK-I.9* T-DNA insertion mutants (*lecrk-I.9*) and *LecRK-I.9* overexpressing lines. Their response to infection with various pathogens demonstrated that LecRK-I.9 plays is crucial for resistance of Arabidopsis to *P. brassicae*. The gain of susceptibility in *lecrk-I.9* lines and the enhanced resistance of the 35S-*LecRK-I.9* lines is specific for isolates of *P. brassicae* and there is no obvious effect of the mutation or the overexpression on other pathogens tested in this study. However, both the mutation and the overexpression of LecRK-I.9 cause certain development effects. In the 35S-*LecRK-I.9* lines this is visible with the naked eye. In comparison to the parental Col-0 line, 35S-*LecRK-I.9* plants are smaller in size, have wrinkled leaves and display accumulation of anthocyanins and lignin suggesting that these lines are some-how stressed. As yet, we have no clue if there is a correlation between the increase in anthocyanin and lignin and the enhanced resistance towards *P. brassicae*. Both anthocyanin and lignin are known to be induced after elicitation of basal defense. An increase in anthocyanin was shown to inhibit susceptibility of potato tubers to *Erwinia carotovora* ssp. *carotovora* (Lorenc-Kukuła *et al*., 2005), whereas Arabidopsis plants with an impaired monolignon synthesis exhibit enhanced susceptibility towards various bacterial and fungal pathogens (Quentin *et al*., 2009). The 35S-*LecRK-I.9* plants also showed an increase in callose deposition upon inoculation with *P. brassicae*, whereas this increase was not found in Col-0. This

suggests that LecRK-I.9 directly or indirectly triggers the enhancement of callose deposition, which is regarded as an early cellular marker of response upon cell wall damage, and recognition of MAMPs and pathogen elicitors.

The developmental effect that we observed in the *lecrk-I.9* plants is more subtle. The morphology of the plants is not affected but at the cellular level we do find a phenotype that is in line with what we expected based on previous studies. Gouget *et al*. (2006) who selected LecRK-I.9 in a phage display that was aimed at identifying proteins interacting with the RGD cell attachment motif in the *P. infestans* effector IPI-O, showed that the seven amino acid peptides resulting from the phage display disrupted the CW-PM adhesions in Arabidopsis hypocotyls as visualized by convex forms of plasmolysis. Since these peptides correspond to sequences in the extracellular domain of LecRK-I.9 we assumed that this disruption was due to competition with the natural ligands of LecRK-I.9 thereby disabling the function of endogenous LecRK-I.9. Also in *lecrk-I.9* lines the function of LecRK-I.9 is disabled and indeed, the CW-PM adhesions are strongly reduced resulting in convex forms of plasmolysis. Our observations thus provide evidence for the hypothesis of Gouget *et al*. (2006) that LecRK-I.9 functions in protein-protein interactions to mediate adhesions between the CW and PM.

LecRK-I.9 was identified as a protein potentially interacting with IPI-O (Gouget *et al*. 2006). The fact that, similar to LecRK-I.9 peptides, endogenously added IPI-O has the capacity to disrupt CW-PM adhesions (Senchou *et al*., 2004) urged us to investigate the effect of ectopic expression of *ipiO* on CW-PM adhesions. As shown in Figure 5B *in planta* expression of *ipiO* results in disturbance of the CW-PM continuum in an RGD dependent manner. In the 35S-*ipiO* lines the convex form of plasmolysis was even stronger and more frequently observed than in the *lecrk-I.9* lines. Expression analysis in *lecrk-I.9* showed that in some tissues other clade I *LecRK* genes have increased expression levels compared to Col-0 suggesting that the lack of LecRK-I.9 is compensated by other LecRKs. This redundancy likely explains the difference between 35S-*ipiO1* lines and *lecrk-I.9* in the plasmolysis assays. Most interestingly, the 35S-*ipiO1* lines were also found to display enhanced disease susceptibility to *P. brassicae* and thus behaved as phenocopies of *lecrk-I.9*. This phenocopy behaviour was also observed with respect to callose deposition: neither *lecrk-I.9* nor 35S-*ipiO1* lines accumulate callose upon pathogen- and MAMP treatment. Similarly, infiltration with *P. syringae* hrcC, resulted in callose deposition in Col-0 but not in the *lecrk-I.9* and 35S-*ipiO1* lines. Based on these results one would expect that the *lecrk-I.9* and 35S-*ipiO1* would also be more susceptible or would even gain susceptibility to an avirulent *P. syringae* strain. Remarkably, in our infection assays we did not observe an obvious change in phenotype with regard to *Pseudomonas* infection. However, since we have not yet performed quantitative measurements of bacterial growth it

can not be excluded that the phenocopy lines are more susceptible. These quantitative assays are in progress.

In recent years evidence that pathogen effectors function by manipulating the host is accumulating. Suppression of defense could be an effective mechanism that often requires entry of the effector into the host cells. The RXLR-dEER motif that is shared by numerous oomycete effectors, including IPI-O, functions as a host cell targeting motif (Whisson *et al*., 2007; Dou *et al*., 2008). To gain entry animal pathogens often make use of receptors present in the plasma membrane and there are several examples where integrin receptors that bind RGD-containing proteins produced by the pathogen play a role in this process. Obviously, the role of these integrins is not to help pathogens to enter the host cell, but to regulate normal developmental processes that require 'inside-out' and 'outside-in' signaling over the plasma membrane. We hypothesize that LecRK-I.9 is an RGD-binding protein that interacts with extracellular ligands. This interaction then results in activation or repression of kinase activity – mediated by the intracellular domain of LecRK-I.9 – leading to regulation of normal plant development. *Phytophthora* uses LecRK-I.9 as an effector target (Figure 8A). Based on our results we propose that IPI-O by itself can loosen the CW-PM adhesions and, in addition, bind to LecRK-I.9 via the RGD motif to further disrupt these adhesions. This model explains why *lecrk-I.9* and 35S-*ipiO1* lines show gain of susceptibility and behave as phenocopies (Figure 8B). The model also explains why overexpression of *LecRK-I.9* enhances resistance (Figure 8C).

The effector gene *ipiO* was isolated from *P. infestans*, a pathogen that infects potato and tomato, but can not infect Arabidopsis. IPI-O belongs to the enormous reservoir of RXLR-dEER effectors present in all *Phytophthora* species analyzed so far (Haas *et al*., 2009). The diversity among RXLR-dEER effectors is extremely high; not only between species but also within a species. For *P. infestans* and *P. sojae* many races are described that show differential interactions with cultivars of potato and soybean, respectively. We now know that the molecular basis of cultivar specificity can be traced back to the polymorphisms and copy number variations in RXLR-dEER effector genes. Based on our current knowledge on the diversity in RXLR-dEER effectors it seems unlikely that *P. brassicae*, the species that infects Arabidopsis, contains an *ipiO* homologue. Nevertheless, *P. brassicae* may have a RGD-containing effector that targets LecRK-I.9 in Arabidopsis, but this should be revealed by genome sequencing. Resistance in Arabidopsis to *P. brassicae* was reported to be independent from SA- JA- and ET-defense signaling pathways; i.e. disease resistance was maintained in mutants deficient in these pathways (Roetschi *et al*., 2001). It was, however, reported that the *Pad2* gene, is crucial for full-resistance to *P. brassicae* (Roetschi *et al*., 2001). *Pad2* encodes a γ-glutamylcysteine synthetase (GSH1), which is the first enzyme in the

glutathione biosynthesis pathway (Parisy *et al*., 2007). Lower gluthatione (GSH) levels in the *pad2* mutant were found to be correlated with a reduced accumulation of glucosinolates; toxic plant compounds which could have an negative effect on *P*. *brassicae* (K. Schläppi, personal communication; Schläppi *et al*., 2008, 2010). A link between GSH and lectin receptor kinases is not reported. Intriguingly, however, it was reported that integrin-mediated cell adhesion in human cells is regulated by a glutathione redox potential (Ball *et al*., 2008).

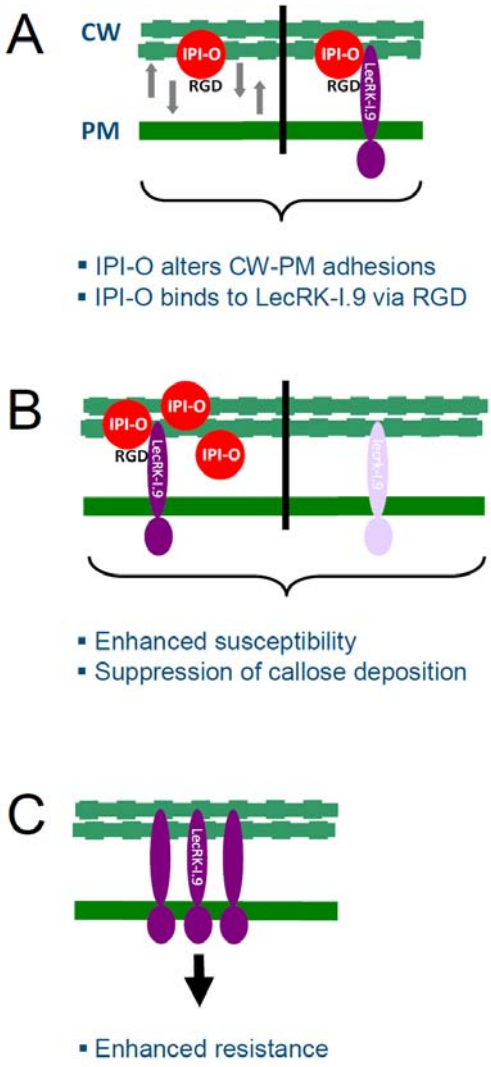

Figure 8. Models depicting the activities of the RXLR-dEER effector IPI-O and its effector target LecRK-I.9. (A) IPI-O alters the continuum between the cell wall (CW) and the plasma membrane (PM) by interfering with LecRK-I.9. (B) Arabidopsis plants expressing *ipiO* or lacking *LecRK-I.9* are phenocopies in respect to *P. brassicae* susceptibility and callose deposition. (C) Overexpression of *LecRK-I.9* in Arabidopsis results in an enhanced resistance to *P. brassicae*.

Materials and methods

Plant material and growth conditions

Arabidopsis plants were grown in soil or *in vitro* on solid MS medium (4.4 g/l Murashige and Skoog salts (Duchefa), 0.5% (w/v) sucrose and 1% (w/v) plant agar). Arabidopsis was grown in a conditioned growth chamber at 21-22℃ with a 16 h photoperiod and at a relative humidity of 75-80%. Transgenic plants and T-DNA insertion lines were in Col-0 background. The Arabidopsis T-DNA insertion mutants *lecrk-I.9-1* (SALK_042209), and *lecrk-I.9-2* (SALK_024581) were obtained from the European Arabidopsis stock centre NASC (http://arabidopsis.info). T-DNA insertion lines were tested for resistance on MS plates with 50 mg/l kanamycin and genotyped by PCR using T-DNA border primers LBb1 and Npt-II-as and primers mlecat5g60300-s and mlecat5g60300-as on the genomic DNA flanking the T-DNA insertion. Homozygosity of was verified by PCR genotyping and absence of *LecRK-I.9* transcript was verified by RT-PCR with primers At5g60300-s and At5g60300-as. Primer sequences are listed in Supplementary Table 1.

Abiotic stress treatments

Arabidopsis leaves were wounded with wooden toothpicks, and used in GUS histochemical staining assays 4, 8, 24 and 48 hours after wounding. For high salt stress, leaves were infiltrated with 0.2 M NaCl, and harvested after 14, 20, and 44 hours. Heat- and cold stress was achieved by exposing Arabidopsis plants for 6 hours to 37℃ and 4℃, respectively. SA, and MeJA treatments were performed by spraying or infiltrating leaves with concentrations of 0.1 M and 0.3 M, and leaves were harvested at 14, 20, and 44 hours after treatment.

Pathogen growth and infection assays

Botrytis cinerea strain IMI169558 and *Alternaria brassicicola* strain MUCL20297 were grown and maintained at 22℃ on malt agar and potato dextrose agar plates, respectively. Infection assays of Arabidopsis with *B. cinerea* and *A. brassicola* were performed as described by Van Esse *et al*. (2007). *Colletotrichum destructivum* strain IMI349061 was propagated at 22℃ on Mathur's agar plates and inoculum was prepared as described by O'Connell *et al*. (2004). Arabidopsis leaves were drop-inoculated with a conidial suspension ($1*10^6$ spores ml^{-1}), and subsequently incubated at 22℃ in trays covered with lids to maintain high humidity. *Phytophthora infestans* isolate IPO-0 was grown on rye agar medium supplemented with 20 g l^{-1} sucrose at 18℃ (Caten and Jinks, 1968), and zoospores were isolated as described in **Chapter 2.1**. *P. brassicae* strains HH, II, CBS686.95 and the HH GFP-transformant 155m were grown at 18℃ on 10% V8-juice agar plates (Erwin and Ribeiro, 1996), and zoospores were obtained as described in **Section 4**. Inoculation was performed by placing plugs of young

mycelium (Ø 5mm) or 10 µl drops of a zoospore suspension ($1*10^5$ zoospores ml^{-1}) on the abaxial leaf surface. Inoculated plants were kept in trays covered with lids to maintain a high humidity and placed in the dark, and placed in a growth chamber with a 16 h photoperiod at 18°C and a RH of 75%. The first day the trays were kept in the dark. After two days the mycelial plugs were removed from plants to stop the facilitation of nutrition from the agar medium. *Pseudomonas syringae* pv. *tomato* strains were grown on King B agar supplemented with the appropriate antibiotics at 28°C. Arabidopsis leaves were infiltrated with bacterial suspensions of $1*10^6$ colony-forming units (cfu) ml^{-1}, and incubated as described by Jambunathan and McNellis (2003).

Plasmid construction and Arabidopsis transformation

For the construction of $P_{LecRK\text{-}I.9}$-GUS the DNA fragment covering the promoter region (-1486 to +9 bp with position 1 at A of the start codon) of *LecRK-I.9* (GenBank accession number AY056317.1, At5g60300) was amplified from Arabidopsis BAC F15L12.17 (ABRC, Ohio State University) by polymerase chain reaction (PCR) with primers pro60300s1.5 and pro60300as (Supplementary Table 1). A *Sal*I site and *BamH*I-site were included in pro60300s1.5 and pro60300as, respectively, to facilitate cloning of the promoter fragment in binary vector pBI101.1 (Clontech). For cloning *ipiO* in binary vectors *ipiO1* fragments were PCR amplified from the plasmids pPIN18-c, pMBP-IPIO1D56A and pMBP-IPIO1D56E, respectively, using primers IPIOXHO-F and MAL-R (Supplementary Table 1; Senchou *et al*., 2004). PCR fragments were purified and digested with *Xho*I and *Pst*I to release a 550 bp fragment containing the coding region of *ipiO1*, starting immediately after the signal peptide cleavage site, and ligated into the *Xho*I/*Pst*I digested vector pRH80 (Van der Hoorn *et al*., 2000). Fragments containing the *ipiO1* coding region fused to the TPI-II terminator sequence were released by digesting with *Xho*I and *Eco*RI, purified and ligated into *Xho*I/*Eco*RI-digested binary vector pRH90 (Van der Hoorn *et al*., 2000), thereby fusing the *ipiO1* coding sequence to the tobacco *PR1a* signal peptide sequence. The resulting binary plasmids were named pRW100, pRW101 and pRW102 with the latter two carrying RGD-tripeptide mutations E56A and D56E, respectively. Amplicons obtained by PCR amplification on pRW100, pRW101 and pRW102 with the primers PstPR1a and PR1 were digested by *Pst*I and ligated into the *Pst*I-digested vector pACN (Supplementary Table 1; Caddick *et al*., 1998). These plasmids were digested with *Hin*dIII, and the fragments were ligated into the *Hin*dIII-digested vector binSRNACatN (Caddick *et al*., 1998). In the resulting binary plasmids pRW110, pRW111 and pRW112, the *ipiO1* coding sequence is fused to the ethanol-inducible promoter of the *alcA* gene. For cloning the full-length coding sequence of *LecRK-I.9* driven by the 35S CaMV promoter in a binary vector, a PCR was

performed on BAC clone F15L12.17 with primers pK60300s and pK60300as (Supplementary Table 1). PCR fragments were cloned in the binary Gateway vector pENTR/D-TOPO. LR recombination enabled cloning into binary vector pK2GW7 (http://www.psb.ugent.be/gateway; Karimi *et al*., 2002), resulting in plasmid pK-35S-LecRK-I.9. Cloning steps were verified by sequencing. Binary vectors were transformed to *Agrobacterium tumefaciens* strain GV3101 and cultured on medium containing the appropriate antibiotics. *Arabidopsis thaliana* accession Col-0 was transformed by the floral dip method (Clough and Bent, 1998). Transformed plants were selected on MS agar with 50 mg/l kanamycin. Rooted seedlings were subsequently transferred to potting soil. Multiple independent lines were selected.

Staining techniques and microscopy

For GUS histochemical staining, Arabidopsis tissues were immersed and vacuum-infiltrated in X-gluc staining buffer [50 mM phosphate buffer pH 7.0, 0.1% (v/v) Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucorunide (X-gluc, Biosynth, Staad, Switzerland), 1 mM $K_3Fe(CN)_6*3H_2O$), 1 mM $K_4Fe(CN)_6*3H_2O$ and 10 mM EDTA] and incubated overnight at 37℃. Chlorophyll was removed by incubation in a 50-96% ethanol series. GUS stained samples were examined at low magnification under brightfield illumination. Pictures of GUS-stained tissues shown in this paper are representative results from at least three independent experiments. Neutral red staining and plasmolysis of etiolated Arabidopsis seedlings was performed as described by Gouget *et al*. (2005) with minor modifications. Etiolated seedlings were incubated in a 0.05% neutral red solution for 30 min and rinsed afterwards in distilled water. Plasmolysis was induced by the addition of 0.4 M $CaCl_2$. Pictures were taken after 5 min of plasmolysis. Trypan blue staining was performed according to Keogh *et al*., (1980). Lignification was visualized as described by Mohr and Cahill (2007). In brief, leaves were cleared in ethanol and incubated overnight in a phloroglucinol-ethanol mixture, and subsequently placed for 5 min in 20% HCl and washed with water. To visualize anthocyanin pigmentation, leaves were incubated overnight in the dark at 4℃ in 80% methanol containing 1% HCl. Brightfield and fluorescence microscopy was performed with a Nikon 90i epifluorescence microscope (Nikon, Badhoevedorp, The Netherlands). GFP fluorescence was visualized using a GFP-B filter cube (EX 460-500, DM 505, BA 510-560). To visualize callose deposition, Arabidopsis leaves were harvested approximately 18 h after inoculation with bacteria or infiltration with 10 µM flg22, cleared overnight in an ethanol series (70-96%) and stained with 1% (w/v) aniline blue in 150 mM K_2HPO_4 (pH 9.5) for 1 h. Stained leaves were mounted in 50% glycerol, and fluorescent callose deposits were viewed using epifluorescence microscopy (DAPI filter; EX340-380, DM 400, BA 435-4850). Images of randomly selected fields were captured using a

Nikon DS-5Mc digital camera and processed with ImageJ software (http://rsb.info.nih.gov/ij) to calculate the total area (μm^2) of callose deposits. Values in Figure 6B and D are the average of at least 16 and 10 microscopic fields, respectively. Error bars represent 95% confidence intervals (CIs).

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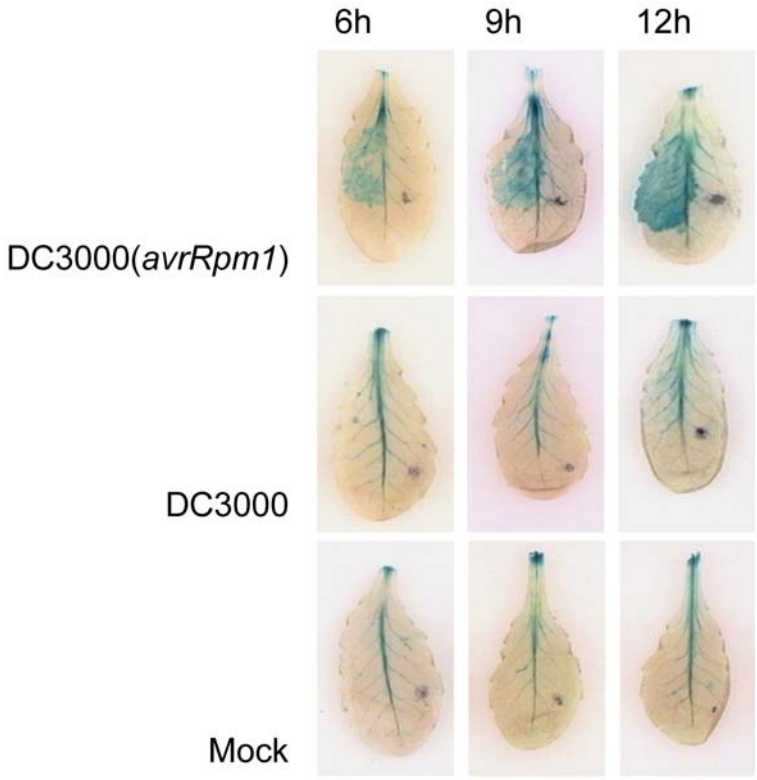
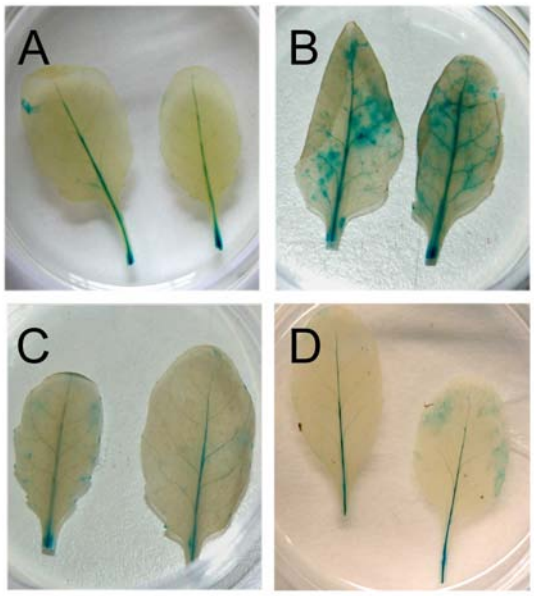
Supplementary material

Supplementary Table 1. Primers used in this study

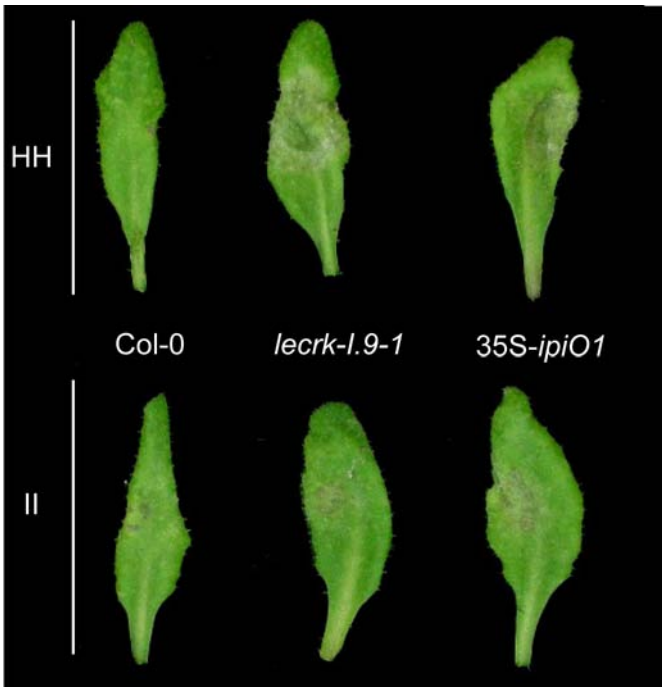
Primer	Primer sequence (5'-3')
mlecat5g60300-s	TCCATGCAACAGTTGCGTTGTCT
mlecat5g60300-as	AGCCAACACCAAAATAGCCAGA
LBb1	GCGTGGACCGCTTGCTGCAACT
Npt-II-as	ATCGGGAGCGGCGATACCGTA
At5g60300-s	TGGAGTTTGTCAGGTCCATCG
Atg60300-as	CTGAGGATCTTCTGCAGGCAA
pro60300-1.5s	TAGTCGACCCTGGTGGTAAAGACACGT
pro60300-as	TAGGATCCACGAGCCATTGCAGATGATGAATC
pK60300-s	CACCATGGCTCGTTGGTTGCTTCAG
pK60300-as	TTACCTCTGACTGCTGATGC
IPIOXHO-F	GCAGTCTCGAGCAATCTCAACACCGCCG
MAL-R	TAACGCCAGGGTTTTCCCAGTC
PstPR1a	TACTATCTGCAGTACCATGGGATTTG
PR1	AATCCAGAAGATGGACAAGTC



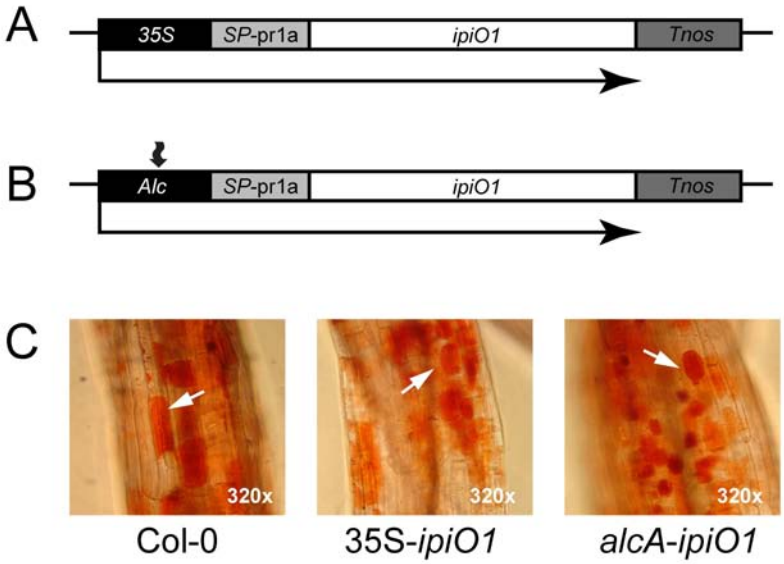
Supplementary Figure 1. Expression of *LecRK-I.9* in Arabidopsis is induced in an incompatible interaction with *Pseudomonas syringae* DC3000(*avrRpm1*), but not in a compatible interaction with strain DC3000. GUS activity in leaves of a transgenic Arabidopsis line carrying promoter-GUS construct $P_{LecRK\text{-}I.9}$-GUS, at 6, 9 and 12 hours after infiltration.



Supplementary Figure 2. *LecRK-I.9* expression in Arabidopsis is induced upon treatment with methyl jasmonate (MeJA). GUS activity after 20 hours in mock-treated $P_{LecRK\text{-}I.9}$-GUS transgenic Arabidopsis (A), and 20 hours after treatment with 0.3 M MeJA (B), 0.3 M salicylic acid (SA) (C), and 0.2 M NaCl (D).



Supplementary Figure 3. Arabidopsis *lecrk-I.9* and 35S-*ipiO1* lines show gain of susceptibility to *Phytophthora brassicae* II. Leaves inoculated with *P. brassicae* isolate HH and II 3 days post-inoculation.



Supplementary Figure 4. Plasmolyzed etiolated Arabidopsis hypocotyls show concave forms of plasmolysis in Col-0, but convex forms in both the 35S-*ipiO1* and *alcA-ipiO1* lines. (A,B) Schematic representation of the constitutive and alcohol-inducible *ipiO1* gene expression constructs, respectively. (C) Etiolated Arabidopsis hypocotyls after plasmolysis using 0.4 M $CaCl_2$.

3.3 Characterization of Arabidopsis and potato lines with constitutive *LecRK-I.9* expression

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In collaboration with WUR Plant Breeding#

Abstract

Plant receptor kinases play an important role in sensing a plethora of self and non-self signals, and hence regulate both developmental and defense-related processes. Previously, it was shown that the lectin receptor kinase LecRK-I.9 is crucial for maintaining the interface between the cell wall and plasma membrane. Moreover, characterization of knock-out lines indicated that *LecRK-I.9* also plays an important role in Arabidopsis disease resistance. In this study we investigated the effects of constitutive expression of *LecRK-I.9* on plant growth and development, and on tolerance to *Phytophthora* pathogens. Transgenic Arabidopsis plants constitutively expressing *LecRK-I.9* were smaller in size, and showed an increase in anthocyanin and lignin. Moreover, these lines displayed an enhanced resistance to *Phytophthora brassicae*. Also, potatoes with constitutive expression of *LecRK-I.9* were found to be smaller in size. Increase in anthocyanin was less obvious, but the leaves displayed an altered morphology and the plants seemed to be less susceptible to *Phytophthora infestans*. However, the increased resistance was not sufficient to block more aggressive *P. infestans* isolates. Although these results support the hypothesis that LecRK-I.9 is involved in defense against *Phytophthora* pathogens, additional experiments are required to confirm and strengthen the data, in particular in potato.

Introduction

Throughout their life cycle, plants have to monitor a dynamic variety of signals to achieve proper regulation of cellular processes such as growth, development and defense activation. These signals can be both of self and non-self origin, such as phytohormones or pathogen effectors. Little is known about the molecular basis underlying the perception of these signals, yet it is generally accepted that membrane-associated proteins with extracellular 'sensing' domains play important roles in signal surveillance and response activation (Humphrey *et al*., 2007). Notable are the receptor-like proteins (RLPs), some of which function in plant development (e.g. CLV2 and TMM), while others play a role in defense, such as the *Cf* genes that confer resistance to *Cladosporium fulvum* (Kruijt *et al*., 2005). Signal sensing of ligand molecules is also governed by receptor-like kinases (RLKs), which in contrast to RLPs have cytoplasmic kinase domains to initiate intracellular signal transduction.

Plant RLKs can be classified in various subfamilies based on their extracellular domain organization, and this high diversity reflects the need to respond to diverse signals (Shiu *et al*., 2004). Several RLKs are known to mediate various aspects of plant growth and development. Examples are SRF4, which appears to work as a regulator of leaf size (Eyüboglu *et al*., 2007), FEI1 and FEI2 that play roles in regulating cell wall architecture (Xu *et al*., 2008), and three RLKs of the CrRLK family which are required for optimal cell elongation (Guo *et al*., 2009). Others RLKs with an assigned function are for instance, the proline-rich extension-like receptor kinase PERK4, which is suggested to play a role at the early stage of ABA signaling to modulate root growth (Bai *et al*., 2009), and various *S*-locus receptor kinases (also named GNA-related lectin receptor kinases, **Chapter 3.1**) that are involved in the self-incompatibility response of various flowering plants (Ivanov and Gaude, 2009). RLKs are also assumed to be crucial in sensing non-self signals. For several leucine-rich repeat RLKs, such as FLS2, EFR and CERK1, there is ample evidence that they are essential for disease resistance; they function as pattern recognition receptors (PRRs) of microbe-associated molecular patterns (MAMPs) (Chinchilla *et al*., 2006; Zipfel *et al*., 2006; Miya *et al*., 2007; Wan *et al*., 2008). Other RLKs have been reported to be involved in both plant development and defense. Well-studied is BAK1, that not only acts as co-receptor of BRI1 to enhance brassinosteroid signaling (Li *et al*., 2002), but also in complexes with the PRRs FLS2 and EFR to initiate MAMP-responses (Chinchilla *et al*., 2007; Heese *et al*., 2007). Additional examples are ERECTA and WAK1, which are involved in development as well as in cell-wall mediated defense to plant pathogens (Sánchez-Rodríguez et *al*., 2009; He *et al*., 1998).

RLKs not only act as signal sensors, but also as physical linkers between the plant cell wall (CW) and plasma membrane (PM) to establish a continuum that

functions as an interface for signal exchange (Baluška *et al*., 2003; Wyatt and Carpita, 1999). Studies by Gouget *et al*. (2006) showed that LecRK-I.9, an Arabidopsis legume-like lectin receptor kinase, participates in protein-protein interactions to mediate this continuum (Gouget *et al*., 2006). LecRK-I.9 interacts via its lectin motif with the RGD tripeptide motif of IPI-O, an RXLR-dEER effector protein from the potato late blight pathogen *Phytophthora infestans*. Interestingly, IPI-O disrupts these CW-PM adhesions through the RGD motif, and hence destabilizes the continuum (Senchou *et al*., 2004; Gouget *et al*., 2006). Disturbance of these linkages can be readily seen upon plasmolysis. Under normal conditions, plasmolysis causes the PM to separate from the CW, but local CW-PM adhesion sites are still maintained. This concave form of plasmolysis changes in the presence of IPI-O and becomes convex.

Previously, we used promoter-GUS fusions to analyze expression of *LecRK-I.9* in Arabidopsis (**Chapter 3.2**; Bouwmeester *et al*., 2008). These analyses revealed that *LecRK-I.9* has a basal level of expression in the main veins of rosette leaves and cotyledons, in hypocotyls and in roots. Treatments with various endogenous stresses did not change the basal expression pattern. In contrast, induction of *LecRK-I.9* expression was observed in a non-host interaction with *P. infestans* and upon inoculation with the necrotrophic fungus *Botrytis cinerea* and avirulent strains – DC3000(*avrRpm1*) and HH, respectively – but not virulent strains, of the biotrophic pathogens *Pseudomonas syringae* and *Phytophthora brassicae*. This suggests that LecRK-I.9 has a role in pathways leading to programmed cell death or HR. Furthermore, we examined Arabidopsis lines with T-DNA insertions within the *LecRK-I.9* gene, and showed that these knock-out lines (*lecrk-I.9*) display an increased disease susceptibility to infection with the oomycete pathogen *P. brassicae*, and show less callose deposition upon MAMP treatment or inoculation with the *Pseudomonas syringae* mutant hrcC, which lacks a functional Type III secretion system (**Chapter 3.2**). Moreover, *lecrk-I.9* mutant lines are altered in their CW-PM continuum, and exhibit a convex type of plasmolysis comparable to the one elicited by IPI-O (Senchou *et al*., 2004; **Chapter 3.2**).

In the present study, we constitutively expressed *LecRK-I.9* in Arabidopsis and potato (*Solanum tuberosum*), and investigated the effect on plant growth and development, and on disease resistance to *Phytophthora* pathogens.

Results and Discussion

Overexpression of *LecRK-I.9* in Arabidopsis disturbs development

In order to obtain Arabidopsis plants with *LecRK-I.9* expression in all tissues, we transferred Arabidopsis with a construct containing the full-length coding sequence under the control of the constitutive cauliflower mosaic virus 35S promoter to Arabidopsis via flower-dip transformation. Two independent transgenic Arabidopsis lines were selected (i.e., C-0123 and C-0126), and their phenotype was compared to the untransformed recipient (i.e. wild-type Col-0). Both transgenic lines exhibited more compact rosettes with smaller and slightly wrinkled leaves (Figure 1). They were also shorter in height (data not shown), and displayed a substantially higher accumulation of anthocyanin and lignin in comparison to the recipient line Col-0 (Figure 1). Anthocyanin pigmentation was most intense along the petioles and midribs of young rosette leaves (Figure 1 and 2), and at the base of the inflorescence stem (data not shown). Increased lignification of the transgenic rosette leaves became apparent after staining with phloroglucinol-HCl (Figure 2). The lignin staining pattern reflected the pattern of anthocyanin pigmentation. Both anthocyanin and lignin are secondary metabolites of the phenylpropanoid pathway, and have been found to accumulate in response to various plant hormones and abiotic stresses. Anthocyanin and lignin are also associated with the basal defense response against plant pathogens. For example, transgenic potato tubers increased in anthocyanin content displayed enhanced resistance to *Erwinia carotovora* (Lorenc-Kukuła *et al*., 2005), whereas imbalanced lignin levels in Arabidopsis correlated with increased susceptibility to plant pathogenic bacteria and fungi, and affected the reproduction of the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Quentin *et al*., 2009).

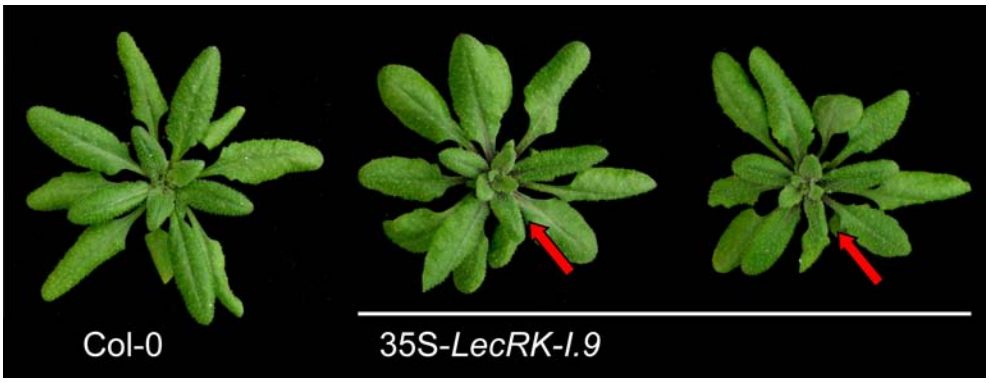

Figure 1. Morphology of Col-0 and transgenic plants overexpressing *LecRK-I.9* (35S-*LecRK-I.9*). Red arrows point to anthocyanin pigmentation.

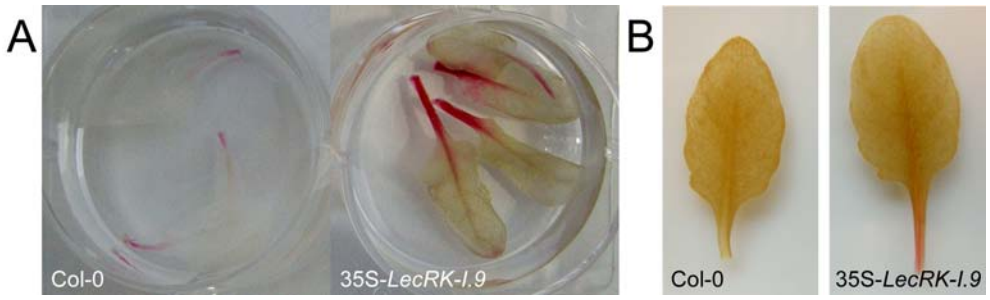

Figure 2. Anthocyanin (A) and lignin (B) staining in rosette leaves of Col-0 and *LecRK-I.9* overexpressing lines.

Overexpression of *LecRK-I.9* in Arabidopsis confers enhanced resistance to *Phytophthora brassicae*

To study the effect of *LecRK-I.9* overexpression in Arabidopsis on disease resistance, we compared the response of the transgenic lines and Col-0 upon inoculation with *P. brassicae*, a *Phytophthora* species capable to infect Arabidopsis. We tested two *P. brassicae* isolates; HH, which is unable to infect Col-0 and CBS686.95 that is virulent on Col-0. Upon inoculation with HH, no changes were observed for both Col-0 and the transgenic Arabidopsis lines that overexpress *LecRK-I.9* (data not shown). In contrast, the transgenic lines exhibited an elevated level of resistance towards CBS686.95, and showed no or only minor disease symptoms, whereas Col-0 was clearly infected (Figure 3; **Chapter 3.2**). The experiment was repeated several times with comparable results.

Figure 3. Col-0 and transgenic Arabidopsis lines with constitutive *LecRK-I.9* expression 4 days post-inoculation with *Phytophthora brassicae* isolate CBS686.95.

Ectopic expression of *LecRK-I.9* in potato results in aberrant leaf development

To generate transgenic potato lines expressing *LecRK-I.9*, the full-length *LecRK-I.9* coding sequence under control of a CaMV 35S promoter was transferred to potato cultivar (cv.) Désirée via *Agrobacterium*-mediated transformation. A total of 41 independent primary transformants was obtained, the majority of which expressed the transgene (data not shown). For seven transgenic potato lines (abbreviated as TPLs) the expression levels determined by Q-RT-PCR using total RNA isolated from leaves as template are shown in Figure 4. As expected *LecRK-I.9* mRNA was not detected in the recipient cv. Désirée (Figure 4), whereas actin mRNA was detected in all cases (data not shown). Four lines with various transgene expression levels were selected for further analysis. TPL38 has the lowest level of *LecRK-I.9* mRNA, TPL31 and TPL42 have intermediate levels and line TPL40 the highest level.

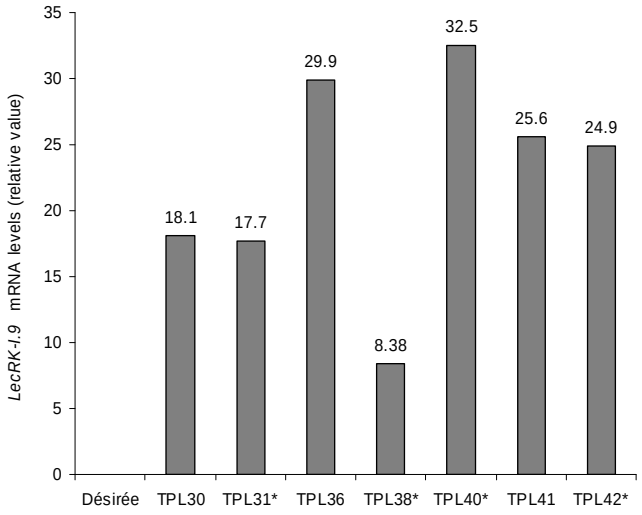

Figure 4. Relative levels of *LecRK-I.9* mRNA in transgenic potato lines.
* Selected for further analysis.

In vitro grown transgenic plantlets were transferred to soil, and growth and development were monitored during six weeks. In comparison to the recipient cv. Désirée, all four transgenic lines were reduced in size and showed early flowering (data not shown). TPL40 was the most severely affected but the stunting was also observed in TPL42, TPL31 and TPL38. Moreover, the mature leaves of the transgenic lines exhibited developmental defects. Like other potato cultivars, Désirée has so-called pinnaltly compound leaves, i.e. leaves with pairs of leaflets

along the midrib ending with a single larger terminal leaflet. In the leaves of the transgenic potato lines we observed deformed leaf laminae which appeared more wrinkled than in Désirée leaves, especially in the three upper leaflets of the compound leaf (Figure 5). The severity of this phenotype differed among the four transgenic lines. TPL38 (Figure 5), TPL42 and TPL31 (data not shown) all have wrinkled leaf laminae. In TPL40, which has the highest expression of *LecRK-I.9*, the wrinkling is more severe, and such that the terminal leaflet is not separated from the two upper secondary leaflets. Accordingly, the leaf veins in TPL40 are radiating outward from a single point at the base, a venation pattern that is more associated with palmately compound leaves (Figure 5, red asterix). The altered leaf morphology found in the transgenic potato lines expressing *LecRK-I.9* resembles more-or-less the disarrangement of digits on animal limbs, which is correlated with a lack of either the RGD-containing ECM-protein laminin or laminin integrin receptors (De Arcangelis *et al*., 1999; Miner *et al*., 1998). The increase in anthocyanin that was observed with the naked eye in the Arabidopsis lines overexpressing *LecRK-I.9* was not visible in the transgenic potato lines. It cannot be excluded, though, that more sensitive detection methods will reveal changes in anthocyanin and lignin levels in the transgenic potatoes.

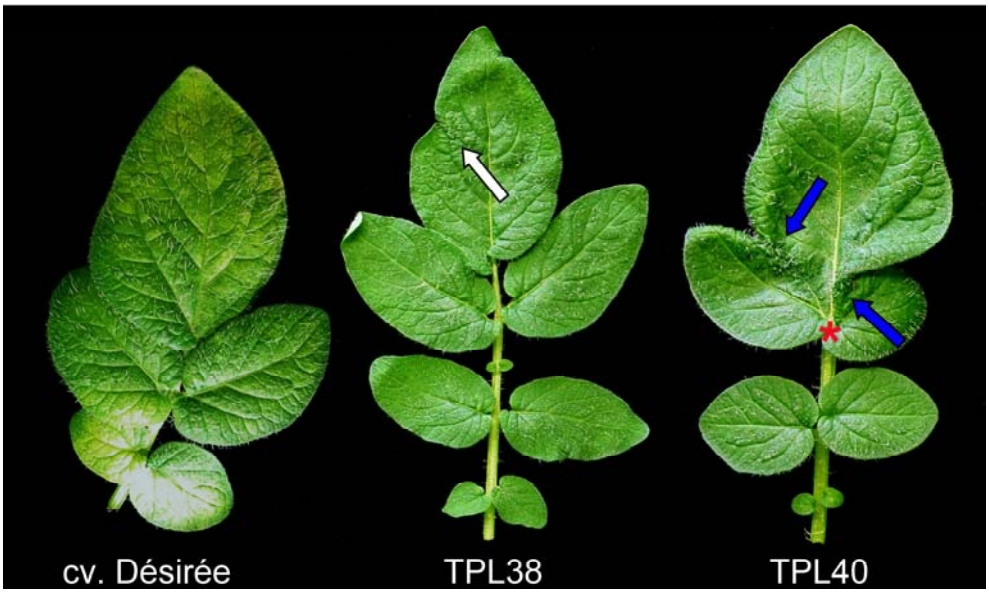

Figure 5. Leaf morphology of cv. Désirée and transgenic potato lines expressing *LecRK-I.9*. Leaf alteration is indicated by arrows. TPL40 displays a more palmate venation pattern (red asterix) and leaf shape, in which the upper three leaflets are not separated (blue arrows).

Expression of *LecRK-I.9* confers enhanced resistance to *P. infestans*

So far, two *LecRK-I.9* expressing transgenic potato lines, i.e. TPL38 and TPL40, were tested in a detached leaf assay for their response to *P. infestans*. We used four isolates of *P. infestans* that differ in their aggressiveness on cv. Désirée and have a different origin (Table 1; Supplementary Table 3 of **Chapter 2.1**). *P. infestans* EC3394 is a strain isolated from tree tomato (*Solanum betaceum*) but unable to infect potato. Equal concentrations of zoospores were used for inoculation. As expected, isolates 90128, PIC97757 and 88069 were capable to reach a 100% infection efficiency (IE) on cv. Désirée, whereas isolate EC3394 did not form any lesion (Figure 6). Strikingly, transgenic line TPL40 – which has the highest level of *LecRK-I.9* mRNA – could not be infected by isolate 88069 (IE = 0%), and on TPL38 the IE of this strain was significantly reduced (12.5%). Also, isolate PIC97757 encountered an increased level of defense in TPL40 (IE = 37.5%). Accordingly, on TPL38 the IE of PIC97757 was only slightly reduced, but much less than 88069 (Figure 6). These data suggest that under moderate disease pressure a higher level of *LecRK-I.9* expression correlates with a higher level of resistance to *P. infestans*. To confirm this correlation more transgenic lines have to be tested with a broader range of *P. infestans* isolates that are moderate aggressive to cv. Desiree.

Table 1. *P. infestans* isolates used in this study

***P. infestans* isolate**	***Solanum* host of origin**	**Aggressiveness on cv. Désirée***	**Year of isolation**	**Country**
90128	*S. tuberosum* (potato)	high	1990	The Netherlands
PIC97757	*S. demissum*	n.d.	1997	Mexico
88069	*S. lycopersicum* (tomato)	moderate	1988	The Netherlands
EC3394	*S. betaceum*	incompatible	2001	Ecuador

n.d= not determined; *= see Supplementary Table 3 of **Chapter 2.1**

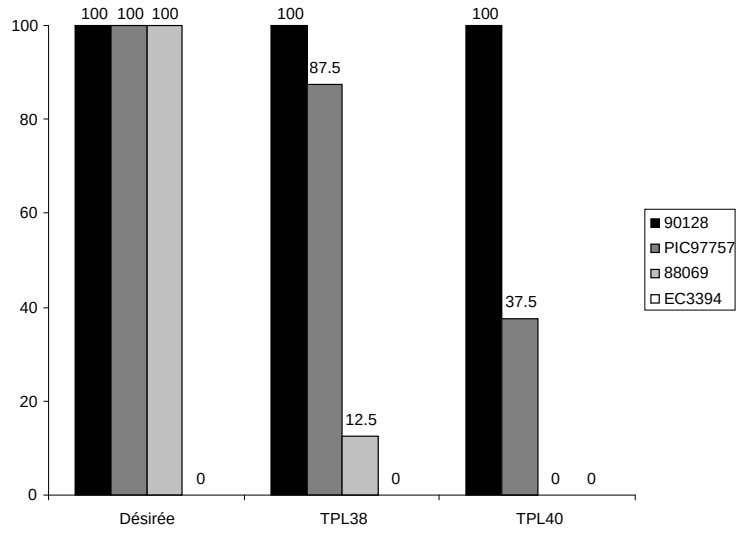

Figure 6. Infection efficiency of four *Phytophthora infestans* isolates on *LecRK-I.9* transgenic and control potato leaves, five days post-inoculation. Data are based on eight inoculation spots per potato line.

Conclusion

The data presented in this study show that *LecRK-I.9* overexpression in Arabidopsis and potato results in quite similar phenotypes, i.e. altered leaf morphology, and altered responses to *Phytophthora* infection.

- Potato plants with ectopic expression of *LecRK-I.9* are reduced in size, are early flowering, and have wrinkled and malformed mature leaves. Similarly, transgenic Arabidopsis plants that overexpress *LecRK-I.9* have reduced rosettes with smaller, slightly wrinkled leaves and, in addition, show accumulation of lignin and anthocyanins.
- The *LecRK-I.9* expressing potato lines are less susceptible to moderate aggressive *P. infestans* strains while overexpression of *LecRK-I.9* in Arabidopsis resulted in resistance to a virulent *P. brassicae* strain. This in contrast to Arabidopsis knock-out lines (*lecrk-I.9*) that showed gain of susceptibility towards avirulent *P. brassicae* strains (**Chapter 3.2**).

The changes in phenotype might be due to the fact that overexpression of *LecRK-I.9* results in cell adhesion strengthening, and hence influences the CW-PM continuum. As a consequence the CW is much stronger attached to PM, thereby disturbing normal plant growth and raising extra barriers for *Phytophthora* to penetrate cells and to grow intercellularly. Alternatively, LecRK-I.9 might function in

the perception of MAMPs or cell wall damage molecules, and as such overexpression could activate an enhanced defense response.

Future research will be focused at (i) repeating and extending the infection assays on potato in order to find a stronger correlation between increased *LecRK-I.9* mRNA levels and *P. infestans* resistance, (ii) testing the transgenic potato and Arabidopsis lines for changes in susceptibility to other pathogens and tolerance to abiotic stresses, and (iii) determining the possible correlation between the increase of anthocyanin and lignin levels and resistance to *Phytophthora*.

Materials and Methods

Plasmid construction

The complete coding sequence of *LecRK-I.9* was PCR amplified from the BAC clone F15L12 with the forward primer pK60300s (5′-CACCATGGCTCGTTGGTTGCTTCAG-3′; containing a CACC overhang to facilitate Gateway cloning) and the reverse primer pK60300as (5′-TTACCTCTGACTGCTGATGC-3′). PCR amplicons were cloned via a BP recombination reaction into pENTR/D-TOPO vector (Invitrogen), and the resultant plasmid was transformed to *E. coli* DH5α. Transformants were checked for the presence of the relevant inserts by colony PCR. The PCR fragment was hereafter subcloned via an LR reaction in the Gateway plant transformation vector pK2GW7 (http://www.psb.ugent.be/gateway; Karimi *et al*., 2002). The resulting construct pK-35S-LecRK-I.9 enables constitutive expression of *LecRK-I.9* under control of the 35S-CaMV promoter and upstream of the 35S-CaMV terminator.

Transformation of Arabidopsis and potato

Binary plasmid pK-35S-LecRK-I.9 was transferred to *Agrobacterium tumefaciens* strain COR308 through electroporation. An overnight culture of a positive clone, selected on LB medium containing spectinomycin at 100 µg ml^{-1}, was used to transform potato tuber discs of cv. Désirée according to Hoekema *et al*. (1989), and to transform Arabidopsis via the flower-dip method (Clough and Bent, 1998). Resultant transgenics were selected for kanamycin resistance on selective medium and via PCR and Southern hybridization. Transgenic potato lines (TPLs) were maintained *in vitro* in climate chambers at 20℃ and a 16 h photoperiod on MS30 medium, and were propagated by transplanting vegetative tissue to fresh medium with regular intervals. To obtain plant material for infection assays plantlets were transferred to soil.

Expression analysis by Q-RT-PCR

Total RNA was isolated from mature green leaves using an RNeasy® Plant Mini Kit (Qiagen) and used as template for Q-RT-PCR with the primer pair 5´-TTTGCCAGATTTCTCACCATACAC-3´and 5´-TCTGTTGACTGCCAAGCGTAAG-3´. Relative expression levels were normalized with respect to actin gene expression and reflect fold-increase compared to the recipient (non-transformed) line cv. Désirée.

Histological staining

Lignification was visualized as described by Mohr and Cahill (2007). In brief, leaves were cleared in ethanol and incubated overnight in a phloroglucinol-ethanol mixture. Leaves were then placed for 5 min. in 20% HCl and subsequently washed with water. To visualize anthocyanin pigmentation, rosette leaves were incubated overnight in the dark at 4°C in 80% methanol containing 1% HCl.

Phytophthora growth and inoculum preparation

The *P. brassicae* isolates HH and CBS686.95 were grown at 18°C on fresh 10% V8-juice (The Campbell Soup Co., Camden, N.J.) agar plates. *P. infestans* isolates 90128, PIC97757, 88069, and EC3394 (Table 1) were routinely grown on rye agar medium supplemented with 20 g l^{-1} sucrose at 18°C (Caten and Jinks, 1968). *P. brassicae* and *P. infestans* zoospores were isolated as described in **Chapter 2.1** and **Section 4**, respectively. For inoculation the concentration of zoospores was adjusted to $1*10^5$ zoospores ml^{-1}.

Plant growth and pathogen inoculations

Arabidopsis plants were grown in a growth chamber at 22°C, with a 16 h photoperiod and at a relative humidity of 75-80%. Inoculation with *P. brassicae* was performed with mycelial plugs (Ø 5mm) or zoospores (10 µl droplets; $1*10^5$ zoospores ml^{-1}) on the abaxial leaf surface of four week old Arabidopsis plants, as described in **Section 4**. Disease symptoms were assessed on a daily basis.

Potato plants were grown in a greenhouse in potting soil. Cultivar Désirée (*R*0) was used as non-transgenic control throughout the experiments. Detached leaf assays as described by Vleeshouwers *et al*. (1999), were used for assessing foliar resistance to *P. infestans*. Leaves were inoculated with 10 µl droplets of inoculum ($1*10^5$ zoospores ml^{-1}) on the abaxial side of the leaf and subsequently incubated at 18°C. The first 24 hours the trays were kept in the dark. Late blight symptoms were evaluated five days after inoculation, and infection efficiencies (IEs) were determined as previously described (Vleeshouwers *et al*., 1999).

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4

A novel method for efficient and abundant production of *Phytophthora brassicae* zoospores on Brussels sprout leaf discs

4 A novel method for efficient and abundant production of *Phytophthora brassicae* zoospores on Brussels sprout leaf discs

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Abstract

Phytophthora species are notorious oomycete pathogens that cause diseases on a wide range of plants. Our understanding how these pathogens are able to infect their host plants will benefit greatly from information obtained from model systems representative for plant–*Phytophthora* interactions. One attractive model system is the interaction between Arabidopsis and *Phytophthora brassicae*. Under laboratory conditions, Arabidopsis can be easily infected with mycelial plugs as inoculum. In the disease cycle, however, sporangia or zoospores are the infectious propagules. Since the current *P*. *brassicae* zoospore isolation methods are generally regarded as inefficient, we aimed at developing an alternative method for obtaining high concentrations of *P*. *brassicae* zoospores. *P*. *brassicae* isolates were tested for pathogenicity on Brussels sprout plants (*Brassica oleracea* var. *gemmifera*). Microscopic examination of leaves, stems and roots infected with a GFP-tagged transformant of *P*. *brassicae* clearly demonstrated the susceptibility of the various tissues. Leaf discs were cut from infected Brussels sprout leaves, transferred to microwell plates and submerged in small amounts of water. In the leaf discs the hyphae proliferated and abundant formation of zoosporangia was observed. Upon maturation the zoosporangia released zoospores in high amounts and zoospore production continued during a period of at least four weeks. The zoospores were shown to be infectious on Brussels sprouts and Arabidopsis. The *in vitro* leaf disc method established from *P*. *brassicae* infected Brussels sprout leaves facilitates convenient and high-throughput production of infectious zoospores and is thus suitable to drive small and large scale inoculation experiments. The system has the advantage that zoospores are produced continuously over a period of at least one month.

Introduction

Plants can be affected by a broad range of plant-pathogenic oomycetes, such as downy mildews and *Phytophthora* species. Comprehensive knowledge of host–pathogen interactions is a prerequisite for designing novel control strategies. Elucidation of these complex interactions will especially benefit from easy and user-friendly model pathosystems. One of the potential model systems is the interaction between *Phytophthora brassicae* and Arabidopsis (Roetschi *et al*., 2001).

P. brassicae was initially classified as *P. porri*, a major pathogen causing white tip disease on *Allium* species (Ogilvie and Mulligan, 1931; Smilde *et al*., 1995), but based on detailed characterization, including isozyme pattern, ITS sequence, morphology and host-pathogenicity, it is now categorized as a new and distinct species (Man in 't Veld *et al*., 2002; De Cock *et al*., 1992). *P. brassicae* has a narrow host range restricted to brassicaceous plants and was shown to be pathogenic on different *Brassica* species, e.g. Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), Brussels sprouts (*Brassica oleracea* var. *gemmifera*) and rutabaga (swedes) (*Brassica napus* var. *napobrassica*) (Semb, 1971; Fagertun and Semb, 1986). *P. brassicae* is mostly associated with post-harvest damage that limits the marketability of cabbage heads and can reach up to 90% losses (Geeson, 1976; Geeson, 1978; Fagertun, 1987). Although less frequently, disease symptoms have been observed on cabbage plants in the field. Colonization often starts in root or stem tissue, and subsequently progresses upwards through the vascular system, eventually colonizing the leaves. Infection and disease spread is more severe under wet weather conditions with moderate temperatures; the optimum lies between 15 and 20°C, although pathogen growth has been observed at lower temperatures down to -3°C (Fagertun, 1987) .

In the last decade, Arabidopsis has become the most attractive model plant for genetic and molecular studies and consequently it is favorable as host plant for studying plant-pathogen interactions. Several oomycete pathogens have been reported to infect Arabidopsis, either naturally or under laboratory conditions. These include *Hyaloperonospora arabidopsidis*, *Albugo candida* and two *Phytophthora* species, *P. cinnamoni* and *P. brassicae* (Roetschi *et al*., 2001; Chou *et al*., 2000; Koch and Slusarenko, 1990). The best studied *Phytophthora* species, i.e. *P. infestans* and *P. sojae*, are incapable to infect Arabidopsis; they trigger defense responses leading to non-host resistance (Huitema *et al*., 2003). Roetschi *et al*. (2001), who first described the *P. brassicae*–Arabidopsis pathosystem, inoculated a variety of *P. brassicae* isolates on multiple Arabidopsis accessions and defense mutants, and showed that certain combinations result in compatible and others in incompatible interactions (Roetschi *et al*., 2001). This pathosystem

has the potential to become a model for studying oomycete–plant interactions, allowing concurrent molecular analysis of the host as well as the pathogen.

A disadvantage of *P. brassicae* is the fact that generating zoospores is troublesome. In nature, *Phytophthora* species produce vegetative spores, the so-called sporangia, that infect the host tissue upon germination. At lower temperatures sporangia often develop into zoosporangia that release zoospores and these then act as the infectious propagules. In the laboratory one can also use mycelium plugs or mycelial suspensions as inoculum but to mimic disease cycle in nature it is more appropriate to use sporangia or zoospores. Various laboratory protocols describe the isolation of zoospores from *in vitro* grown mycelium (Erwin and Ribeiro, 1996) and for several *Phytophthora* species it is relatively easy to obtain sufficient amounts of zoospores for en masse inoculation. For *P. brassicae*, however, efficient production of zoospores is not so straightforward (Mauch *et al*., 2009). To induce sporulation *P. brassicae* has to be cultured on soil medium (http://commonweb.unifr.ch/biol/pub/mauchgroup/zoospores.html) or transferred to Schmitthenner solution (Erwin and Ribeiro, 1996; Mauch *et al*., 2009). The preparation of these media is complicated and laborious and the amount of zoospores generated on these media is low. Moreover, zoospore production is dependent on pH, mycelial age and season (K. Belhaj and F. Mauch, personal communication; Mauch *et al*., 2009). This study aimed at establishing a fast, simple and convenient system for production and isolation of *P. brassicae* zoospores. We first compared the pathogenicity of five *P. brassicae* isolates on Brussels sprouts (*Brassica oleracea* var. *gemmifera*) and monitored infection and colonization using bright field and fluorescence light microscopy. Subsequently, we optimized the zoospore production system. Leaf discs cut from infected Brussels sprout leaves were shown to be an excellent source for large scale production of *P. brassicae* zoospores.

Results and Discussion

Phytophthora brassicae lesion development on Brussels sprouts

Mycelial plugs of *P. brassicae* were inoculated on detached leaves of Brussels sprouts cultivar Cyrus. We tested five *P. brassicae* isolates that were originally isolated from different *Brassica* crop species. Although all five were able to infect Brussels sprout leaves (Table 1), there were differences in disease progression between isolates. For example, foliar lesions caused by *P. brassicae* isolates CBS686.95 and II were predominantly larger than lesions caused by the other isolates. It is noteworthy that these two isolates were originally isolated from Brussels sprouts, possibly explaining their advantage. Foliar lesions on Brussels sprouts had a brownish-grey color and were usually surrounded by a water-soaked

halo (Figure 1A). In later stages of disease development the lesion edges and especially the leaf midribs became darker in color, varying from dark-grey up to black. Another typical symptom often seen at this stage was leaf chlorosis.

The isolates were also tested for their ability to infect stems and roots (Figure 1C, and E). All isolates were infectious on both tissues, but – as on the leaves – there was variation in disease progression between isolates (data not shown). To better visualize the colonization process we used a Green Fluorescent Protein (GFP) tagged *P. brassicae* transformant. Microscopical examination of the infected tissues showed hyphal growth in leaves, stems and roots (Figure 1B, D, and F). In leaves extensive intercellular hyphal growth was found in the intercellular space between mesophyll cells. The few haustoria that were observed were small and – like haustoria of *P. infestans* – digit-like in shape. In late stages of infection, hyphae emerged through the stomata and occasionally protruded the epidermal cell layer but there was no sporulation. Instead, in leaf, stem and root lesions typical protrusions were observed (Figure 1). Supposedly, these protrusions are the sporangiophore initials. Only after being exposed to cold water sporangia were formed, which subsequently developed into zoosporangia.

Table 1. *P. brassicae* isolates used in this study; their origin and foliar lesion sizes on Brussels sprouts cultivar Cyrus

Isolate	Year	Country	Collected from	Lesion size [a]
CBS178.87	1983	Germany	*Brassica rapa* subsp. *pekinensis*	2.9 ± 0.1
CBS212.82	1982	The Netherlands	*Brassica oleracea* var. *alba*	4.9 ± 0.4
CBS686.95	1995	The Netherlands	*Brassica oleracea* var. *gemmifera*	5.9 ± 0.4
HH (CBS782.97)	1994	The Netherlands	*Brassica rapa* subsp. *pekinensis*	3.5 ± 0.2
II	1994	The Netherlands	*Brassica oleracea* var. *gemmifera*	6.0 ± 0.2

[a] the average size in cm^2 of at least 22 lesions at 4 dpi

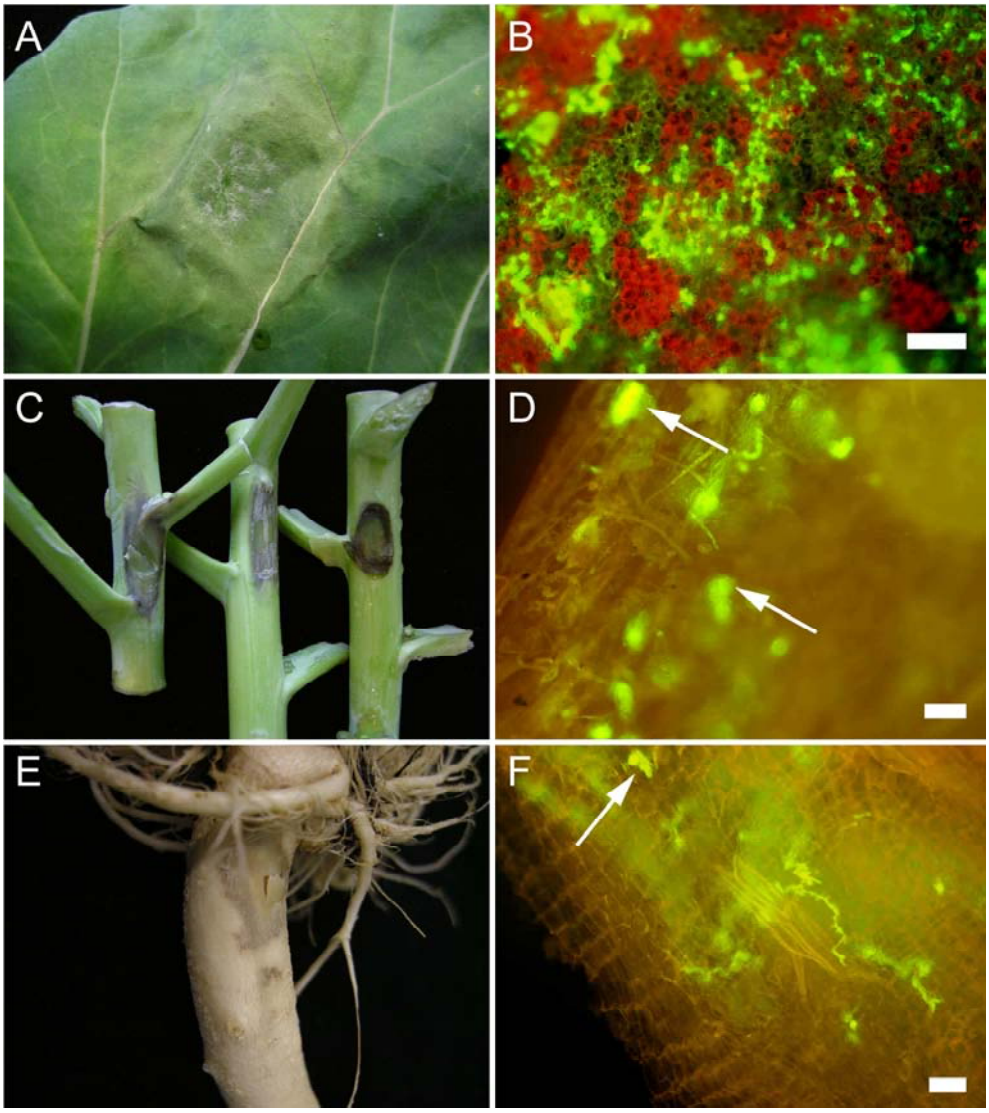

Figure 1. Compatible interaction between the Brussels sprouts cultivar Cyrus and *P. brassicae*. *P. brassicae* infects leaves, stems and roots of Brussels sprouts cultivar Cyrus. Lesion development on the adaxial side of a leaf 4 days post inoculation (dpi) with isolate CBS686.95 (A). Stem lesions 4 dpi with, from left to right, isolates CBS686.95, HH and GFP transformant 155m (C). Root infection 4 dpi with isolate HH (E). Mycelial structures visualized by GFP fluorescence in leaf (B), stem (D) and root (F) tissue, 5 dpi with GFP transformant 155m. Hyphal protrusions are indicated by arrows. Scale bars represent 100 µm (B, D) and 10 µm (F).

Development of a zoospore production method

The susceptibility of Brussels sprout leaves towards *P. brassicae* raised the idea that the lesions could be an excellent source for mass production of zoospores. Figure 2 depicts an overview of the zoospore production procedure. Inoculum was prepared by cutting mycelial plugs from *P. brassicae* colonies grown on V8 agar medium (Figure 2A). The plugs were placed on the Brussels sprout leaves (Figure 2B) with gentle pressure and with the mycelium in direct contact with the leaf surface. Lesions on the Brussels sprout leaves developed quickly and usually 4 days post inoculation (dpi) the lesions were large enough to obtain infected leaf discs with a diameter of 25 mm (Figure 2C). The leaf discs were cut with a cork borer (Ø 25 mm), placed with the abaxial side upwards in 6-well plates with 1-2 ml cold water per well and gently pushed under water (Figure 2D, E). When – after leaf disc cutting – further expansion of the foliar lesions was allowed, the infected leaf could be used to obtain new leaf discs. The first 24 hours the plates were incubated at 4℃ and thereafter at 18℃. Water was refreshed with a two day interval. Infection was checked daily under a stereomicroscope. Newly formed mycelium and sporangia formation were observed after one day. After two days there was a strong increase in the number of sporangia (Figure 2F). Subsequently, the sporangia matured and developed into zoosporangia (Figure 2G). The process from appearance to maturation lasted approximately 3 days. To initiate zoospore release from mature zoosporangia fresh cold water was added and the plates were incubated at 4℃. After one hour the first zoospores were released (Figure 2H), mostly eight from each zoosporangium. The zoospores were able to swim for several hours (5 h average). A time-lapse movie showing discharged zoospores can be seen online (Supplementary File 1: Swimming *P. brassicae* zoospores, http://www.biomedcentral.com/1471-2229/9/111/suppl/S1).

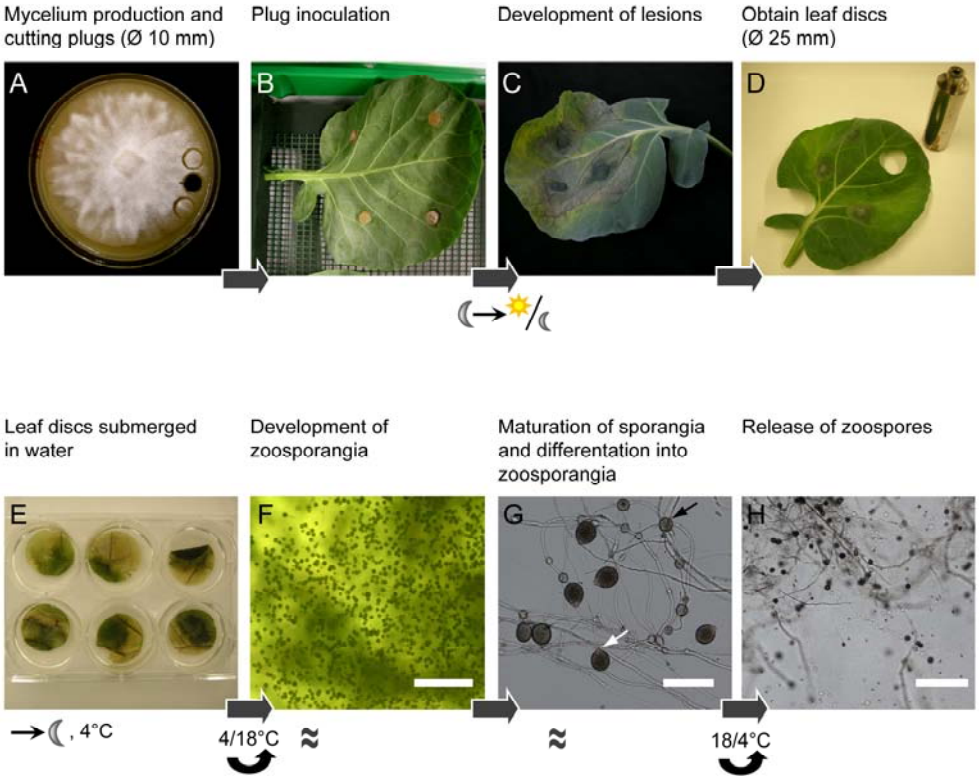

Figure 2. Overview of the *P. brassicae* zoospore production procedure.
From a *P. brassicae* culture grown on V8 agar (A) mycelial plugs (Ø 10 mm) were cut from the actively growing margin and gently pressed on the abaxial side of Brussels sprout leaves (B). From the foliar lesions (C) leaf discs were cut with a cork borer (Ø 25 mm) (D) and transferred to 6-wells plate (E). The infected leaf discs were submerged in water resulting in the formation of sporangia (F) that developed into zoosporangia (G) from which zoospores are released (H) after being exposed to the cold for several hours. Scale bar in (F) and (H) represents 40 µm and in (G) 100 µm. The white arrow in (G) points to a zoosporangium and the black arrow to a hyphal swelling.

All five isolates were tested in this system. In all cases numerous zoospores were produced and we did not observe seasonal influences. The amount of zoospores per leaf disc was semiquantitatively determined with a hemacytometer. Comparable mean numbers of zoospores per leaf disc were found for isolates CBS212.82 and II, whereas isolates HH and CBS686.95 were shown to produce more zoospores, reaching concentrations of $1*10^6$ zoospores per ml (Table 2).

An additional advantage of this system is that the infected leaf discs can be reused after the first harvest. For additional zoospore harvests fresh water was

added to the microwell plates every two days. Subsequently, the microwell plates were placed at 18℃ to allow development and matura tion of fresh zoosporangia. As in the first round, cold water was added and incubation at 4℃ was used to trigger zoospore release. Zoospore yields from successive harvests were lower when compared to initial harvests (Table 2), but the concentrations were still sufficient for infection assays on plants. The leaf discs remained viable and continuously produced zoospores for a period up to one month, albeit that the concentrations became lower as the culture period proceeded.

Furthermore, in accordance with the homothallic nature of *P*. *brassicae*, formation of oospores was observed in the infected leaf discs, although at low frequencies and only in older leaf discs (Supplementary File 2: *In planta* oospore formation).

Table 2. Mean number of zoospores produced by a leaf disc [a]

Isolate	First harvest (zsp. ml^{-1}) [b]	Second harvest (+ 8 days) (zsp. ml^{-1})
CBS212.82	$1*10^5$	n.d. [c]
CBS686.95	$1*10^6$	$0.9*10^5$
HH (CBS782.97)	$0.5*10^6$	$0.3*10^5$
II	$1.5*10^5$	$0.5*10^5$

[a] Ø 25 mm, [b] zsp. ml^{-1}= zoospores per ml, [c] not determined

Zoospores produced on leaf discs can infect Brussels sprouts and Arabidopsis

Infectiousness of zoospores produced on leaf discs in the microwell plates was tested on Brussels sprout leaves and stems, and on Arabidopsis rosette leaves (Figure 3). The inoculations were performed as described in materials and methods. On Brussels sprout leaves, lesion development became clearly evident 2 dpi. At 4 dpi – when the lesions were remarkably larger – a typical discoloration of the tissue was observed (Figure 3A). The zoospores were also shown to infect Brussels sprout stems. Water-soaked, dark brown lesions with dense mycelial growth were observed 4 dpi (Figure 3C). Occasionally, callus formation on stem tissue was observed.

On Arabidopsis, sporulating lesions were observed at 4 dpi. Initially the lesions appeared water-soaked, thereafter the infected tissue wilted and subsequently collapsed (Figure 3D). Dried out lesions turned bleached white in color and papery in appearance. Dense tissue colonization by *P*. *brassicae* was observed microscopically after trypan blue staining in infected Brussels sprout and Arabidopsis tissue (Figure 3B, E, and F).

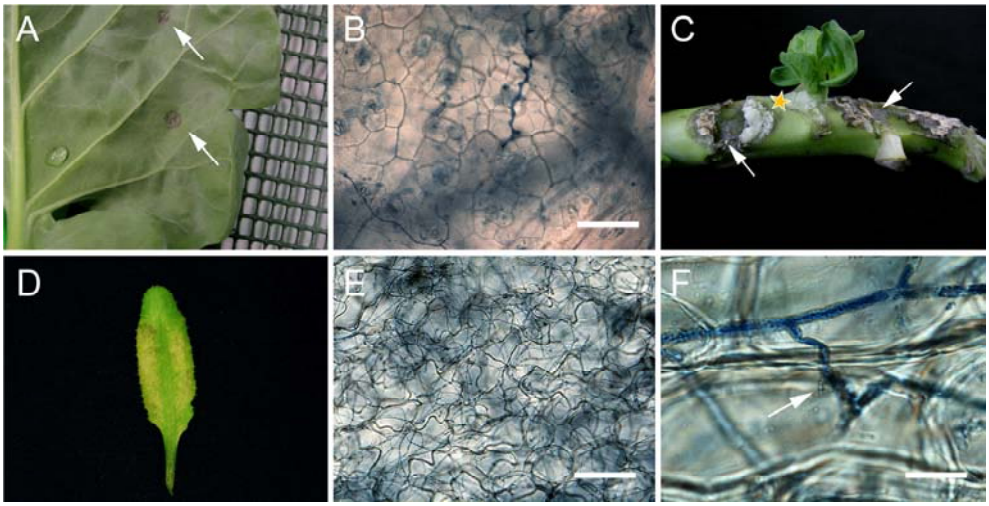

Figure 3. Zoospores produced on leaf discs are infectious.
(A) Foliar lesions (arrows) on Brussels sprouts 4 days post inoculation (dpi) with zoospores of *P. brassicae* isolate CBS686.95. (B) Colonization of Brussels sprout leaf tissue. Scale bar represents 100 µm. (C) Infection on Brussels sprout stem tissue 3 dpi with zoospores of *P. brassicae*. Developing lesions are indicated by arrows and incidental callus formation with a yellow star. (D) An Arabidopsis Col-0 leaf 6 dpi with zoospores of *P. brassicae* isolate CBS686.95. (E). Arabidopsis leaf colonization by intercellularly growing hyphae. Scale bar represents 100 µm. (F). Intercellular hyphal growth in Arabidopsis petiole tissue. A haustorium is indicated by an arrow. Scale bar represents 20 µm. (B, E, F) Intercellular hyphae and haustoria were visualized by trypan blue staining.

Conclusion

In this report we demonstrate that *P. brassicae* easily infects Brussels sprout leaves, stems and roots. An *in vitro* leaf disc method for the isolation of *P. brassicae* zoospores was successfully established and zoospores isolated via this procedure were shown to be infectious. This method opens the opportunity to execute – on small to large scale – zoospore infections on brassicaceous plants, including Arabidopsis. The major advantages are its easy handling, the possibility of inoculating large numbers of plants and the continuous production of zoospores over a period of at least one month, at any season.

Methods

P. brassicae isolates and culture conditions

P. brassicae isolates used in this study were obtained from our in-house collection (i.e., II, HH) and from the Fungal Biodiversity Centre CBS, Utrecht, The Netherlands. *P. brassicae* GFP-transformant 155m (Si-Ammour *et al*., 2003) – which has HH as recipient background – was kindly provided by Dr. F. Mauch, University of Fribourg, Switzerland. *P. brassicae* isolates were cultured at 18℃ on fresh 10% V8-juice (The Campbell Soup Co., Camden, N.J.) agar plates (Erwin and Ribeiro, 1996).

Plant growth conditions

Brussels sprout plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) were grown from seed in a greenhouse in square (11 x 11 cm) plastic pots at 20-25℃, 50/70% relative humidity (RH) and a 16 h photoperiod. Experiments were conducted with 6 week old Brussels sprout plants. Arabidopsis plants were grown in special potting soil (7 parts peat : 6 parts sand : 1 part clay) in a conditioned growth chamber at 18℃ with a 16 h photoperiod and at 75% RH. For inoculation 4 week old Arabidopsis plants were used.

Infection using mycelial plugs as inoculum

Medium sized and large leaves from 6 week old Brussels sprout plants (i.e. the 6^{th} to 14^{th} leaf layer) were detached and washed with water to remove the waxy leaf surface coating. Hereafter, the leaves were placed with their petioles in water-saturated floral foam (Oasis®) in a tray, in such a way that the abaxial sides were facing upwards (Figure 2B). The leaves were sprayed with water and subsequently mycelial plugs (Ø 10 mm), which were taken from the margin of growing colonies, were placed firmly on the abaxial side of the leaf. The trays were closed with transparent lids, wrapped with tape to obtain high humidity, and placed in a growth chamber with a 16 h photoperiod at 18℃ and a RH of 75%. The first day the trays were kept in the dark. Mycelial plugs were removed after 2-3 days to stop nutrition facilitation from the agar. Stem sections were artificially wounded with a razor blade and mycelial plugs (Ø 5 mm) were placed on the wound. The inoculated stems were incubated in the same way as the detached leaves.

Infection using zoospores as inoculum

Leaves of Brussels sprouts (cv. Cyrus) and Arabidopsis (accession Col-0) were drop-inoculated with 10 µl droplets containing $1*10^5$ zoospores ml^{-1}. Inoculations on Arabidopsis Col-0 were conducted with the compatible *P. brassicae* isolate CBS686.95. Plants were kept at 18℃ in the dark at high humidity (100% RH) for

the first 24 hours after inoculation. Subsequently, plants were placed at 18℃ at a relative humidity of 75-80% and a 16 h photoperiod.

Microscopy

Fluorescence microscopy was performed with a Nikon 90i epifluorescence microscope equipped with a digital imaging system (Nikon DS-5Mc camera, Nikon NIS-AR software). GFP fluorescence was examined by using a GFP filter cube (GFP-LP, EX 460-500, DM 505, BA 510). Inoculated plant material was stained with trypan blue (Keogh *et al*., 1980) to visualize hyphal structures and death plant cells.

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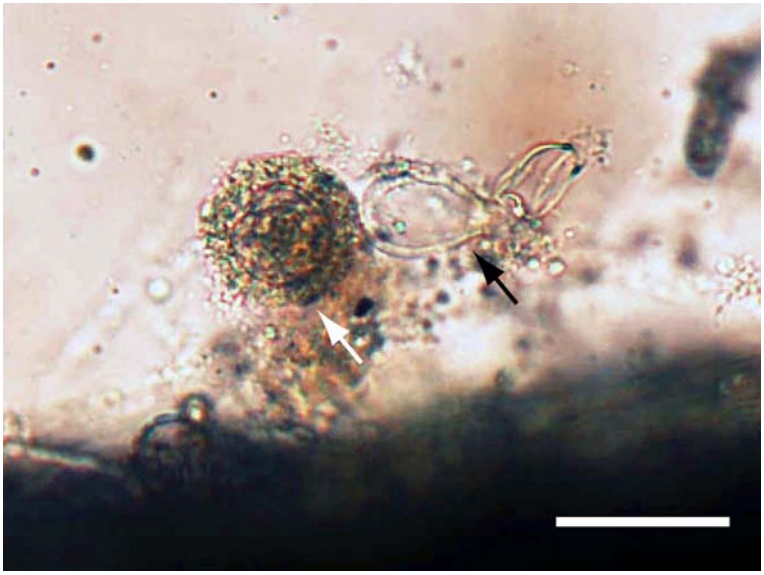
Supplementary material

Supplementary File 1. Swimming *P*. *brassicae* zoospores.
A time-lapse movie corresponding to Figure 2H. The movie shows swimming *P*. *brassicae* zoospores of isolate HH. The movie lasts 3 seconds and is approximately real time. Magnification: 40 x. See: http://www.biomedcentral.com/1471-2229/9/111/suppl/S1



Supplementary File 2. *In planta* oospore formation.
An oospore of *P*. *brassicae* isolate II with a typical thick wall (white arrow). A black arrow points to the antheridium. The scale bar represents 50 µm.

5

General discussion

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Despite the fact that plants possess an effective immune system, pathogens have developed mechanisms to overcome the multi-layered system of plant immunity and are able to invade and cause disease. Plant diseases can be devastating, particularly in crop monocultures that have a narrow genetic base. Many micro-organisms are successful plant pathogens because they have the ability to manipulate the host and suppress plant defenses by secretion of pathogen-specific factors – commonly named effectors – which negatively affect the well-being of the plant. Ergo, the pathogen can colonize plant cells with less hindrance. In the 'arms race' plants acquired specific resistance (R) proteins that directly or indirectly recognize effectors. This recognition leads to effector-triggered immunity (ETI) and is often associated with a hypersensitive response (HR) that blocks further growth of the pathogen (Jones and Dangl, 2006). Since resistance against oomycete species, such as *Phytophthora infestans*, *Phytophthora sojae*, and *Hyaloperonospora arabidopsidis*, is governed by intracellular nucleotide-binding site leucine-rich-repeat (NBS-LRR) R proteins (Table 3 of **Chapter 1.3**), it was anticipated that the cognate effectors should also be targeted into the cytoplasm of host cells. Indeed it has been shown that several oomycete effectors are translocated to the cytoplasm and are recognized intercellularly; they become avirulence proteins when recognized by their cognate R proteins.

This thesis deals with the *Phytophthora infestans* RXLR-dEER effector IPI-O and the interaction with (i) its cognate potato resistance protein Rpi-blb1, and (ii) its target, the Arabidopsis legume-like receptor kinase LecRK-I.9. Here, we will discuss the current concepts on uptake of plant pathogen effectors by host plant cells, and compare them with other protein translocation mechanisms, including those postulated for effector proteins of malaria parasites. Subsequently, diversity of RXLR effectors and the role of the cell adhesion motif RGD present in IPI-O are discussed. This chapter ends with a perspective on the use of Arabidopsis as a tool to study plant–*Phytophthora* interactions.

Effector trafficking: RXLR-dEER as extra gear for delivery into plant cells

When driving a car with automatic transmission one hardly notices that extra gears give more power to the car. But in a car with manual transmission, one is constantly aware that even one gear shift allows you to reach your goal much more efficiently. For *Phytophthora* pathogens, a domain characterized by the amino acid motifs RXLR and dEER seems to function as a special gear. These conserved motifs were discovered in 2004 during a *Phytophthora* genome annotation jamboree, and are located in the N-terminus adjacent to the signal peptide in a domain christened RXLR-dEER (Govers and Gijzen, 2006; Figure 1). At that time, the first few oomycete avirulence (*Avr*) genes were isolated by positional cloning and a genome mining and alignment expedition resulted in the discovery of these conserved motifs in all oomycete Avr proteins and Avr homologs. No conserved

motifs were yet identified in Avr proteins from fungal biotrophic pathogens (Cantanzariti *et al*., 2007) and the presence of a conserved motif in oomycete Avr proteins came as a true surprise. As it happened, shortly after the 2004 jamboree it was reported that many effector proteins of the malaria parasite *Plasmodium falciparum* share the conserved PEXEL motif RXLX$^{E}/_{Q}$. This motif, with a striking resemblance to RXLR, has a role in translocating effectors across membranes that surround vacuoles harboring malaria parasites in red blood cells. Delivery into the cytoplasm of host cells enables these *P. falciparum* PEXEL effectors to carry out virulence and host remodeling functions (Templeton and Deitsch, 2005). This led to the hypothesis that oomycete RXLR effectors are also targeted into host cells, and stimulated many research groups to start addressing the question how oomycete effectors are delivered into plant cells.

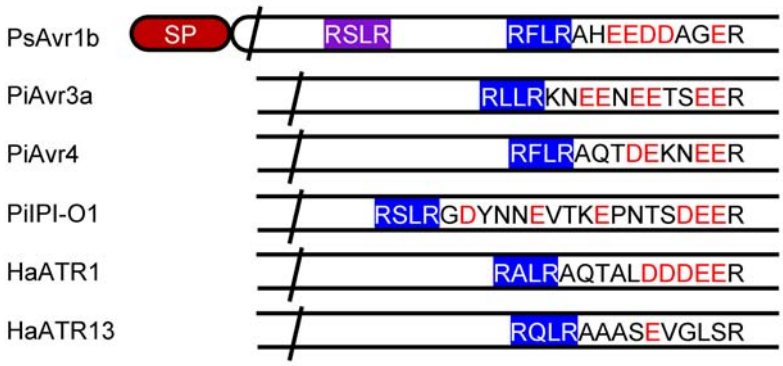

Figure 1. Oomycete effectors share the N-terminal RXLR-dEER domain. The N-terminal part of RXLR effectors commonly contain a signal peptide (SP) and the RXLR-dEER domain. As shown by Dou *et al*. (2008b), Avr1b contains two adjacent RXLR motifs (RXLR-1 in purple and RXLR-2 in blue) and a dEER motif. RXLR-1 is not essential for the avirulence function of Avr1b. Alignment of RXLR-dEER domains of avirulence proteins from *Phytophthora sojae* (Ps), *Phytophthora infestans* (Pi) and the downy mildew *Hyaloperonospora arabidopsidis* (Ha) shows that the dEER motif is less conserved than the RXLR motif but is characterized by a stretch of mainly acidic amino acids (red).

Whisson *et al*. (2007) were the first to show that the RXLR-dEER domain is required for effector translocation into host plant cells. *P. infestans* transformants expressing *Avr3a* in which either the RXLR or dEER motifs were mutated, were shown to be no longer detected by the resistance protein R3a, and as a result did not trigger an HR. Co-agroinfiltration assays with *Avr3a* variants lacking these motifs showed, however, that R3a-mediated cell death was not affected, and

therefore it was concluded that the RXLR-dEER domain is needed for effector translocation to the host cytoplasm (Whisson *et al*., 2007; Bos *et al*., 2006). Translocation of Avr3a was furthermore examined by use of *P*. *infestans* transformants constitutively expressing *Avr3a* fused to a sequence encoding a monomeric red fluorescent protein (mRFP). During infection of potato leaves by these transformants fluorescence was only observed in the haustoria and in the host apoplast, whereas no fluorescence from Avr3a-mRFP could be observed in the cytoplasm of infected plant cells (Whisson *et al*., 2007). The requirement of the RXLR-dEER domain for translocation was supported by experiments with transformants expressing chimeric constructs of *Avr3a* and the ß-glucoronidase (GUS) reporter gene. GUS enzyme activity was observed in infected plant cells when the RXLR-dEER motif was intact, but in plants inoculated with *P*. *infestans* transformants carrying a chimeric construct with a mutated RXLR-dEER motif no GUS activity could be detected in the cytoplasm of infected plant cells. In the latter case, GUS apparently remains in the apoplast where it is quickly degraded.

Another example deals with *P*. *sojae Avr1b*-1, which is the counterpart of the soybean *R* gene *Rps*1b in a 'gene-for-gene' interaction and the first oomycete *Avr* gene that was cloned (Shan *et al*., 2004). To prove Avr function, Shan *et al*. (2004) infiltrated Avr1b protein into the apoplast and observed *Rps*1b-mediated defense responses. This suggests extracellular recognition of the Avr1b protein, an report that was controversial in light of the intracellular nature of R proteins. Subsequently, Dou *et al*. (2008b) showed that the RXLR and dEER motifs are essential for translocation of Avr1b, but not for its recognition by *Rps*1b. They transformed a *P*. *sojae* strain that is virulent on *Rps*1b plants with *Avr1b*-1, with and without mutations in RXLR and dEER. Transgenic strains complemented with wild type *Avr1b*-1 could no longer infect *Rps*1b plants and were avirulent. In contrast, the phenotype of strains complemented with *Avr1b*-1 constructs having mutated motifs did not change, thus demonstrating that dEER and RXLR are pivotal. Avr1b has two adjacent RXLR motifs, 1 and 2, seven amino acid residues apart (Figure 1). Only mutations in RXLR-2, but not RXLR-1, abolished avirulence. Since dEER and RXLR-2 are not required for triggering cell death when *Avr1b*-1 is bombarded into *Rps*1b-leaves, a function of these motifs in effector delivery seems apparent.

To obtain experimental evidence that the RXLR motif in IPI-O is also required for host cell targeting we followed the approach described by Whisson *et al*. (2007) for Avr3a. As described in **Chapter 2.2** we generated *P*. *infestans* transformants expressing an *ipiO-mRFP* chimera and investigated in subcellular localization in various developmental stages. Fluorescence was observed in pre-infectious stages, i.e. in germinating cysts and appressoria. Studying the mRFP localization *in planta* was hampered by the strong autofluorescence of chlorophyll present in plants. This problem was circumvented by using dark-grown etiolated potato

plantlets. Despite the lack of chlorophyll these plantlets are efficiently invaded by *P*. *infestans* and the differential gene-for-gene responses triggered by Avr proteins and mediated by cognate R proteins are retained. By using these etiolated potato plantlets, we could localize fluorescence in haustoria, especially at the haustorial base and neck, during infection by *P*. *infestans* transformants expressing *ipiO-mRFP*. For *P*. *infestans* Avr4, which was also included in this study, comparable patterns of localization were observed. However, as also reported by Whisson *et al*. (2007), we could not detect mRFP fluorescence in the cytoplasm of the host cell during infection. More advanced detection methods (e.g. immunoblotting or immunogold electron microscopy) and mutational studies on the RXLR-dEER motif need to be used to elucidate the precise subcellular localization of IPI-O and Avr4 in infected plant tissues.

Uptake mechanisms: *Phytophthora* RXLR-dEER versus *Plasmodium* PEXEL

Despite the importance of the RXLR-dEER domain, the mechanism underlying effector uptake by host cells remains unclear. Dou *et al* (2008b) took a first step by investigating what happens when Avr1b, produced *in planta* upon bombardment with an *Avr1b*-1 construct, has a secretory leader. Whereas secreted Avr1b triggered *Rps*1b-mediated cell death with the same efficiency as Avr1b lacking the secretory leader, secreted Avr1b with mutated RXLR-2 and dEER motifs triggered no response. This suggested that the motifs mediate re-entry of Avr1b into the cell and, more strikingly, that the entry does not require the presence of the pathogen (Dou *et al*., 2008b). To assess stability of Avr1b and mutated Avr1b, with and without signal peptide, GFP fusions were made and bombarded into onion bulb epidermal cells. Accumulation in the cytoplasm or the apoplast could be distinguished and especially after plasmolysis there was a clear difference between secreted and non-secreted Avr1b, and between secreted wild type and mutated Avr1b. These experiments confirmed a role for RXLR-2 and dEER in re-entry of Avr1b. Autonomous uptake in plant cells was also demonstrated with Avr1b-GFP fusion proteins synthesized in *Escherichia coli*. Soaking root tips of soybean seedlings for 12 hours in a solution containing partly purified fusion protein resulted in uptake of the Avr1b-GFP protein into the root tip cells up to 10 cell layers. Also in this assay mutations in the RXLR or dEER motifs abolished uptake. In retrospect, these results confirm the findings by Shan *et al*. (2004) who reported that infiltrations of Avr1b into the apoplast of soybean elicited *Rps*1b-specific cell death, which had been controversial for some time.

Mutations in RXLR-1 do not compromise the avirulence function of Avr1b and this is in line with the low HMM score of RXLR-1 as compared to RXLR-2. This HMM was created using RXLR flanking regions (10 amino acid residues to the left and right) and could clearly separate sequences with a random RXLR motif (HMM

< 5) from sequences in a curated set of high quality candidate RXLR-effectors (Jiang *et al*., 2008) and in known Avr proteins (*P*. *infestans* Avr3a and *Hyaloperonospora arabidopsidis* ATR1 and ATR13). Altogether these results strongly suggest that sequences flanking RXLR are important for the activity of an RXLR motif. Moreover, it has been shown that RXLR-dEER domains in proteins other than Avr1b can functionally replace the RXLR-dEER domain in Avr1b. Fusion of two RXLR-dEER proteins (HMM > 5) with the C-terminal domain of Avr1b resulted in hybrids that rendered transgenic *P*. *sojae* strains avirulent on *Rps*1b plants. Similarly, by replacing the RXLR-dEER domain in Avr1b with host cell targeting domains of three *Plasmodium falciparum* proteins, the cell death inducing activity of secreted Avr1b in biolistic assays was retained. Mutant analysis revealed that the $RXLX^{E}/_{Q}$ motif by itself is not sufficient to translocate proteins across membranes into red blood cells but requires flanking regions enriched in acidic and hydrophilic amino acid residues (Bhattacharjee *et al*., 2006). Since Dou *et al*. (2008b) did not test an RXLR-dEER domain with an HMM score of 0 or perform mutant analysis of the flanking domains, there is as yet no experimental evidence that flanking regions are required for autonomous uptake. Interestingly, various protein transduction domains have been described to translocate autonomously through the cell plasma membrane (Ziegeler and Seelig, 2007; Sebbage, 2009). Several of these so-called 'cell-penetrating peptides' were found to be rich in arginine and lysine residues. Dou *et al*. (2008b) investigated whether a polyarginine (Arg_9) and the protein transduction domain of an HIV-1 protein, both of which are capable of autonomously carrying proteins across membranes in animal systems, can functionally replace RXLR-2. Biolistics showed they can but, as yet, it is not clear how to interpret these findings in relation to the mechanisms of effector delivery into plant cells.

Based on the results described above, the RXLR-dEER domain seems to embody the complete machinery that the pathogen needs to deliver effectors into host cells. This is in contrast to the bacterial Type III secretion system (T3SS), which requires a multitude of membrane- and periplasm-localized proteins to accomplish this task (Figure 2A and B). These findings are, however, still under discussion and the question remains whether only host-specific molecules are needed, or whether uptake is partially or completely supported by proteins of the pathogen. Macropinocytosis, a specialized form of endocytosis, has been proposed as an option although various research groups favor the hypothesis of receptor-mediated uptake. This would require a targeted machinery of host origin that is exploited by pathogens. Recently, there seems to be new hypothesis, which implies that both the RXLR-dEER and PEXEL motifs bind to phosphatidyl-inositol-phosphates (B. Tyler, personal communication; Kale *et al*., 2009). Moreover, it has been stated that host cell entry could be blocked by an endocytosis inhibitor,

suggesting that entry probably occurs via receptor-mediated endocytosis. Another precedent for this is the membrane spanning lectin receptor kinase LecRK-I.9 of Arabidopsis that binds the RXLR-dEER effector IPI-O from *P. infestans*, and hence it has been hypothesized that LecRK-I.9 could assist effector translocation to host cells (Gouget *et al*., 2006; **Chapters 3.2**, **3.3**).

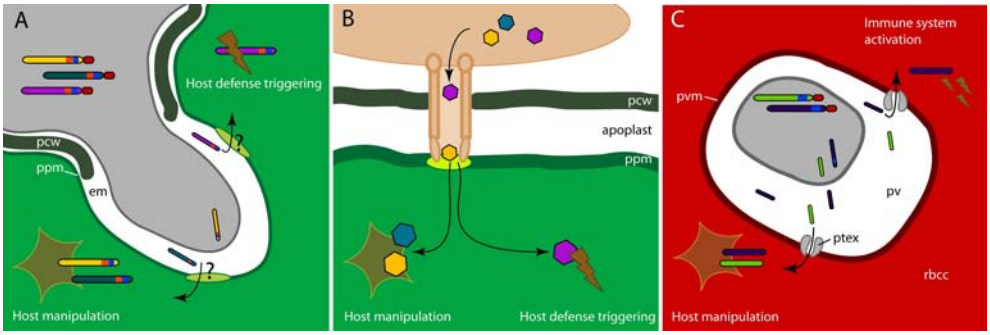

Figure 2. Pathogens deliver effector proteins into the cytoplasm to suppress host defenses and to manipulate the host. When recognized defense responses are triggered. (A) Effector proteins from *Phytophthora* species are secreted from haustoria into the extrahaustorial matrix (em) and cross the plant plasma membrane (ppm). This trafficking is mediated by the RXLR-dEER domain located in the N-terminus of effector proteins. In the model proposed by Dou *et al*. (2008b), RXLR-dEER proteins exploit a targeted host machinery for uptake and do not require other proteins from the pathogen for translocation. (B) By contrast, the type III secretion system (T3SS) of bacteria requires a multitude of proteins from the pathogen to form a molecular syringe that penetrates the plant cell wall (pcw) and plasma membrane, through which effectors are injected into host cells. (C) Malaria parasites (*Plasmodium* spp.) infect red blood cells, and secrete effector proteins into the parasitophorous vacuole (pv). Effectors are translocated to the red blood cell cytoplasm (rbcc) via a pathogen-derived translocon system (ptex), which resides in the parasitophorous vacuole membrane (pvm).

Oomycetes and *Plasmodium* spp. belong to the same eukaryotic supergroup, the Chromalveolates (Figure 1 of **Chapter 1.3**). The similarity between the RXLR motif in oomycetes and $RXLX^{E}/_{Q}$ in *P. falciparum* could point to a common evolutionary origin of the domains carrying these motifs. But what about the host uptake machinery? How widely spread is this machinery and how ancient is it? Considering the fact that the host cell targeting domains in *P. falciparum* and *Phytophthora* spp. are functionally interchangeable, one could wonder whether the uptake machinery in plants is common to that of mammals (Bhattacharjee *et al*.,

2006; Grouffaud *et al*., 2008). Recently, a *Plasmodium* translocon complex (PTEX) has been described, which is proposed to be associated with the parasitophorous vacuolar membrane and to work as an active export machine to deliver PEXEL effectors into the cytosol of the erythrocyte host cell (Figure 2C). Components comprising the PTEX complex are an AAA^{+}-ATPase, the exported protein EXP2, thioredoxin 2, and two novel *Plasmodium* proteins, PTEX88 and PTEX150. In the model proposed by De Koning-Ward *et al*. (2009) it is assumed that also host-derived proteins assist this translocon complex. So far, BLAST searches did not reveal obvious homologs of PTEX components in *Phytophthora* genomes (Bouwmeester and Meijer, unpublished results). This makes it less likely that *Plasmodium* and *Phytophthora* share conserved translocon machineries, although the existence of an analogous translocon complex is still a conceivable hypothesis. Intriguingly it was reported that the PEXEL motif is not involved in a direct interaction with the PTEX, but is functioning as a recognition site for cleavage (Figure 3; Chang *et al*., 2008; Boddey *et al*., 2009). Cleavage of the PEXEL motif was shown to occur after the third residue (RXL↓), and when the conserved L residue was mutated export was prevented (Boddey *et al*., 2009). Recently, it was shown that plasmepsin V, an endoplasmic reticulum (ER) aspartic protease, is responsible for PEXEL cleavage (Boddey *et al*., 2010; Russo *et al*., 2010). Interestingly, homologous sequences can be found in the genome of *P*. *infestans* (Russo *et al*., 2010; H. Meijer, personal communication). An additional step in PEXEL processing is N-acetylation of the new N-terminus, which could well be a crucial step to facilitate translocation to the red blood cell (Chang *et al*., 2008; Boddey *et al*., 2009). Both processes are supposed to be executed early in the trafficking pathway within the ER of *Plasmodium*, but not in the parasitophorous vacuole or during export to the erythrocyte, as was concluded based on inhibition of ER-to-Golgi protein transport with brefeldin A. As yet, the N-acetyltransferase remains to be identified. There is thus far no evidence that RXLR-dEER effectors of oomycetes are processed in a similar way, nor that targeted mutations in the RXLR motif affect protein translocation.

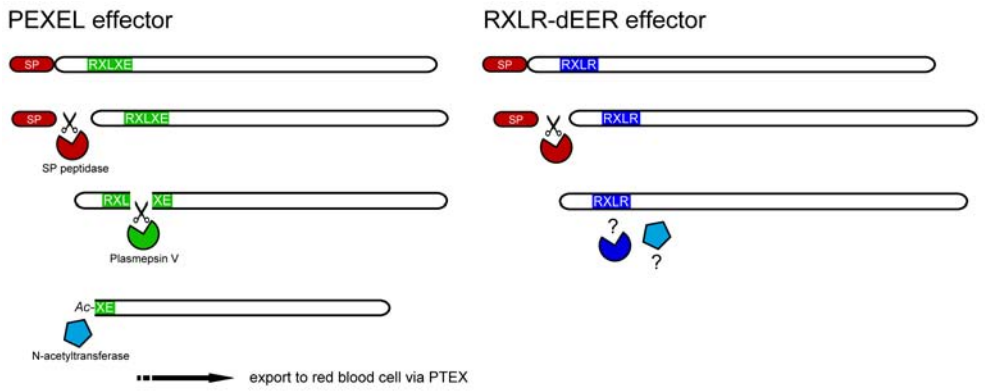

Figure 3. PEXEL effectors of *Plasmodium* spp. are proteolytically processed by a signal peptidase and the ER- localized aspartic protease plasmepsin V that cleaves the PEXEL motif after the leucine (L). This results in a new N-terminus which is then N-acetylated. *Phytophthora* spp. share related transport motifs, but it is unknown whether they use similar processing steps to facilitate correct export to host cells.

Diversity in RXLR-dEER effectors

The size of the RXLR-dEER gene family in each of the sequenced *Phytophthora* genomes is astonishing. Jiang *et al*. (2008) reported 385 RXLR-dEER genes in *P*. *sojae* and 370 in *P*. *ramorum*. With the same mining strategy of reiterated BLAST searches and HMMs, around 560 family members were found in the genome of *P*. *infestans* (Haas *et al*., 2009). Unlike their conserved N-terminal domain, RXLR effectors have a high-turnover and contain extensive sequence diversity in their C-terminal domains. This is likely reminiscent of rapid birth and death evolution, where the pathogen needs to adapt its effector arsenal to circumvent R protein recognition. Mechanisms underlying this molecular adaptation include gene duplication, relaxation of selection, diversifying selection and gene loss (Haas *et al*., 2009). Due to the high sequence divergence in the RXLR-dEER gene family it seems at first sight that these effectors are not related to each other. Recursive similarity searches showed, however, that the majority of them has a common evolutionary origin (Jiang *et al*., 2008). It can be assumed that there is functional redundancy within the large repertoire of RXLR-dEER effectors, and no necessity to maintain them all in the population. Loss or adaptation of RXLR-dEER effectors that function as Avr factors is therefore a tactical move to evade R-mediated recognition, which is in accordance with the rapid adaptation to late blight resistance observed in the field. So far, the genetic diversity of only few *P*. *infestans* RXLR-dEER effectors has been studied in great detail (Table 3 of **Chapter 1.3**). *Avr3a* has only a few alleles and the encoded proteins, one of which

is recognized by *R3a*, are highly similar. The low number of allelic variants suggests that *Avr3a* has been subjected to purifying selection (Armstrong *et al*., 2005). For *Avr4*, four out of 16 alleles were shown to encode a full-length Avr4 protein that elicits an HR in potato plants carrying the *R4* gene. All *P*. *infestans* isolates that could infect R4 plants have *Avr4* alleles with frame-shift mutations that lead to truncated proteins (Van Poppel *et al*., 2008). Complementation of such a virulent race 4 isolate with a full length *Avr4* resulted in gain of avirulence. Hence, it seems that abolishment of Avr4 production leads to loss of recognition, but moreover shows its redundancy. A limited number of alleles was also described for the recently identified *Avr1* gene (Guo, 2008; Govers *et al*., unpublished results). In this case, it seems that *Avr1* is retained in avirulent isolates, whereas all virulent isolates lack the *Avr1* allele completely. In contrast, the genetic diversity of *Avr-blb2*, which is recognized in a gene-for-gene manner by the *S*. *bulbocastanum R* gene *Rpi-blb2*, was shown to be high and under diversifying selection (Oh *et al*., 2009). Till now, 19 Avr-blb2 variants could be identified. Interestingly, none of them contained a premature stop codon, but instead showed an increased polymorphism localized in the C-terminal domain. Another RXLR-dEER effector gene with a high level of genetic variation is *ipiO*, which was identified as the counterpart of *Rpi-blb1* (Vleeshouwers *et al*., 2008). We found 16 IPI-O variants in *P*. *infestans* and in the closely related species *P*. *andina*, *P*. *mirabilis*, *P*. *ipomoeae* and *P*. *phaseoli* (**Chapter 2.1**). It is likely that more variants can be found in clade 1 *Phytophthora* species; a prelude to this is the recent identification of additional IPI-O variants in *P*. *infestans* (R. Weide and P. van 't Hof, personal communication). No *ipiO* variants were identified by PCR amplification in *Phytophthora* species that reside in other phylogenetic clades, and the existence of analogous proteins in other oomycetes is still hypothetical. The identified IPI-O variants could be divided in three phylogenetic classes, the largest of which is class I that contains 11 variants. IPI-O3 and IPI-O13 belong to class II. They are highly similar, with the exception of a C-terminal extension of 10 amino acids in IPI-O13 that is lacking in IPI-O3. The most divergent variant, with 16 amino acid differences compared to IPI-O2, is IPI-O4, the only protein variant that belongs to class III. Thus far, all isolates were found to contain an *ipiO* allele, hence IPI-O is – in contrast to Avr4 and Avr1 – likely important for pathogen fitness. By means of co-agroinfiltration assays we determined that only class I and II variants trigger *Rpi-blb1*-mediated cell death. *P*. *infestans* isolates that are fully virulent on *Rpi-blbl1*-containing plants were shown to lack variants of class I, and it was therefore concluded that the co-occurrence of class I variants determines whether the pathogen is recognized or not. The class II variant *ipiO3* was, however, also found to be expressed in a virulent strain. This was unexpected since the presence of IPI-O3 and Rpi-blb1 should trigger an HR. This unexpected result could be

explained by protein activity or instability, effector synergism, virulence target competition, or by the possibility that IPI-O3 has specific features allowing it to escape recognition (**Chapter 2.1)**.

As shown by Jiang and co-workers (2008) the C-termini of the majority of RXLR-dEER effectors have a modular and repetitive architecture comprised of W, Y, and L motifs, which were named after their conserved amino acid residue at a fixed position (Figure 3 of **Chapter 1.3**). In **Chapter 2.1** we show that IPI-O contains a single W motif that harbors various amino acids under positive selection. Co-agroinfiltration assays with several deletion mutants of *ipiO2* showed that the C-terminus comprising the W motif is sufficient to trigger an *Rpi-blb1*-specific cell death. Also the C-termini of the RXLR-dEER proteins Avr1b, Avr3a and Avr4 were shown to harbor regions that are responsible for recognition by their cognate R proteins (Dou *et al*., 2008; Bos *et al*., 2006; Van Poppel *et al*., 2009). The C-terminal domain of Avr1b contains one W, one Y, and an additional K motif, which contains various conserved lysine residues. Mutational analysis showed that the conserved residues within the W and Y motifs are essential for recognition of Avr1b by Rps1b (Dou *et al*., 2008a). In the same set of experiments the conserved residues in the W motif of Avr1b were shown to be required for suppression of BAX-induced programmed cell death. Avr3a is homologous to Avr1b, and has a comparable motif architecture. The avirulent and virulent variant of Avr3a differ only in two amino acid residues in the C-terminus; one of which is located in a K motif, and the other in a W motif. The C-terminal region of Avr3a, including the K and W motifs, was defined to be required for recognition by *R3a*, but also for suppression of HR induced by the elicitin INF1 (Bos *et al*., 2006). Strangely, only the avirulent form of Avr3a is capable to suppress INF1-triggered cell death. Avrblb2 was shown to be under positive selection, and to contain a 34 amino acid C-terminal region which is sufficient to trigger Rpi-blb2 hypersensitivity (Oh *et al*., 2009). This region comprises a single positively selected amino acid site, i.e. at position 69, which was found to be critical for recognition by Rpi-blb2. Up till now, isolates of *P*. *infestans* that have overcome *Rpi-blb2*-mediated resistance are yet to be detected, and it is thus unclear how Avrblb2 functions as virulence factor. Van Poppel and co-workers (2009) identified three W motifs – i.e. W1, W2 and W3 – and one Y motif in Avr4. Motif W2 was found to be essential for recognition by R4, but not sufficient and flanking regions are needed for HR elicitation. It is as yet unknown whether the C-terminus of Avr4 is capable of suppressing HR, and which amino acid residues are responsible for recognition by R4. The same holds for IPI-O, although we have shown that this effector is able to suppress callose deposition, and to promote disease susceptibility to *P*. *brassicae* (**Chapter 3.2)**. Moreover, preliminary results indicate that IPI-O can also suppress elicitor-induced HR.

RGD-containing effectors of animal and plant pathogens

The *P. infestans ipiO* gene was one of the first oomycete genes that was isolated and has been studied in detail (Pieterse *et al*., 1993). Assignment of a putative function in pathogenesis was solely based on expression studies (*in planta* induced), and attempts to determine a function by mutant analysis such as gene silencing or by overexpression failed (Van West, 2000). In the pre-genomic era comparative analysis, that could have led to the discovery of the RXLR-dEER motif in IPI-O was not yet feasible and the only potentially interesting feature that could be distinguished was the presence of a cell attachment motif (Pieterse *et al*., 1994). This motif is a tripeptide sequence consisting of Arg-Gly-Asp (RGD), which is found in a large number of extracellular proteins, such as the mammalian glycoproteins fibronectin and vitronectin that reside in the extracellular matrix (ECM). A wide variety of these proteins binds to integrins, a family of transmembrane receptors that play a role in many signaling pathways. The fact that membrane-to-matrix interactions can be easily disrupted by adding synthetic RGD peptides shows that these interactions are important for the ECM-plasma membrane continuum (Canut *et al*., 1998; Gronowicz and Derome, 1994). Integrins are also chosen by pathogens as a target for binding and their role in animal innate immune responses has been emphasized in several reports (Isberg and Van Nhieu, 1994; Pribila *et al*., 2004). Integrins, for instance, interact with the RGD-containing penton base proteins of adenoviruses. Following this interaction integrins initiate clathrin-mediated endocytosis and promote virus internalization. It was demonstrated, however, that the RGD motif is not necessary for initial virus attachment *per se*, as was shown by adenoviruses with deleted RGD motifs. However, these mutants were impaired in endosomal internalization and in escape to the host cytoplasm (Shayakhmetov *et al*., 2005). Another example can be found in *Helicobacter pylori*, a bacterium causal to stomach inflammation, that exploits integrins for effector translocation into the host cell (Kwok *et al*., 2007). This process is dependent on CagL, a RGD-containing protein that is situated on the surface of the pilus and interacts in an RGD-dependent manner with the integrin α5ß1. Upon interaction, various downstream components are activated that were shown to be essential for effector translocation. Other potent effectors of integrin function are the disintegrins from snake venoms of various viper species (Calvete *et al*., 2007; Matsui *et al*., 2010). Disintegrins are usually short cysteine-rich peptides that contain a RGD motif on an exposed hairpin loop. Disintegrins selectively bind and activate integrin receptors, and hence compete with their natural RGD-containing ligands, such as fibrinogen and Von Willebrand factor, subsequently disturbing platelet aggregation and cell-ECM adhesion. Several RGD-disintegrins – such as contortrostatin and jarastatin – have been shown to activate the focal adhesion kinase FAK that

regulates actin dynamics, and to trigger phosphoinositide 3-kinase and MAPK pathways upon integrin-binding (Coelho *et al*., 2001, Oliva *et al*., 2007).

Nowadays the hypothesis is adopted that plant pathogens also produce proteins with RGD motifs to promote uptake and to manipulate the host cell. *Pyrenophora tritici-repentis* and *Stagonospora nodorum*, two fungal pathogens causing leaf spot disease on wheat, require the host-selective toxin ToxA to induce necrosis in host cells (Sarma *et al*., 2005; Friesen *et al*., 2008). The *ToxA* gene encodes a pre-pro-protein that contains a signal peptide (pre-region) and an N terminal pro-region. Both are cleaved by the fungus prior to secretion of the mature 13.2 kDa protein. ToxA contains a RGD motif which is found to be required for internalization into mesophyll cells (Meinhardt *et al*., 2002; Manning *et al*., 2008). It is proposed that internalization occurs by means of receptor-meditated endocytosis. ToxA induces various transcriptomic changes when applied to wheat (Pandelova *et al*., 2009). Interestingly, this resulted in a significant fold increase in the expression of a number of genes encoding endocytosis-related proteins and lectin receptor kinases. Moreover, exogenously applied RGD-peptides have a strong disrupting effect on plant cells. When added, adhesions between the cell wall (CW) and plasma membrane (PM) are easily disrupted, and can result in reduced levels of phytoalexins and phenolic compounds (Schindler *et al*., 1989; Canut *et al*., 1998; Kiba *et al*.,1998; Gao *et al*., 2007). Mellersh and Heath (2001) showed that RGD-treated cowpea plants are attenuated in their defense response, which correlated with enhanced fungal penetration efficiency. The *P*. *infestans* effector IPI-O has the capacity to disrupt CW-PM adhesions through its RGD motif (Senchou *et al*., 2004; **Chapter 3.1**). Disruption of these adhesions is, however, lost when IPI-O is mutated in the RGD motif (Senchou *et al*., 2004; **Chapter 3.1**). Moreover, it was shown that IPI-O can suppress MAMP- and pathogen-induced callose deposition in Arabidopsis (**Chapter 3.1**). Arabidopsis plants expressing *ipiO* also displayed gain of susceptibility to *P*. *brassicae*, suggesting that IPI-O functions as a suppressor of defense, thus promoting disease susceptibility.

The RGD motif in IPI-O overlaps with the second R of the RXLR motif (Figure 1 of **Chapter 2.1**). There are, however, very few RXLR-dEER effectors that contain an overlap between RXLR and RGD (RXLRGD). *P*. *infestans* has only five, and both *P*. *sojae* and *P*. *ramorum* have two of these effectors. In total, the *P*. *infestans* genome contains 28 genes encoding RXLR-dEER effectors with an RGD motif (including Avrblb2), whereas 12 can be found in *P*. *sojae* and five in *P*. *ramorum*. The larger number found in *P*. *infestans* could reflect a recent family expansion of RGD-containing *P*. *infestans* effectors (Bouwmeester and Jiang, unpublished results).

Legume-like lectin receptor kinases and their role in disease resistance

Extensive studies did not reveal the occurrence of integrin homologs in plants (The Arabidopsis Genome Initiative, 2000). Canut *et al*. (1998) showed, however, that Arabidopsis contains high affinity RGD-binding sites in the plasma membrane pointing towards functional homologs of integrins in plants. Surprisingly, one of these binding sites showed a very high affinity for IPI-O. Since the affinity for IPI-O was lost when mutated into RGA or RGE, the RGD motif seems to be crucial for binding (Senchou *et al*., 2004). To identify proteins that potentially interact with the RGD motif in IPI-O, a phage display screen was performed. This resulted in two heptamers that could inhibit plasma membrane RGD-binding activity in Arabidopsis and disrupt CW-PM membrane adhesions. Blasting the Arabidopsis proteome with these peptide sequences led to the identification of an Arabidopsis protein that functions in CW-PM adhesions (Gouget *et al*., 2006). It is a legume-like lectin receptor kinase, named LecRK-I.9, that binds IPI-O via its RGD motif (Gouget *et al*., 2006; Senchou *et al*., 2004). Although its natural ligand has not yet been discovered, results thus far suggest that LecRK-I.9 is involved in RGD-specific interactions and likely plays a structural and signaling role in Arabidopsis.

The extracellular lectin motifs structurally resemble soluble lectins of leguminous plants, which are described to have carbohydrate-binding capacities and function in multiple biological processes including cell-cell and host–pathogen interactions. The sugar-binding residues within the lectin motifs of LecRKs are poorly conserved and probably do not bind monosaccharides. However, they do contain a rather conserved hydrophobic binding site which is regarded to be involved in the recognition of small hydrophobic ligands, such as oligosaccharides, plant hormones or microbe-associated molecular patterns (MAMPs) (**Chapter 3.1**; Barre *et al*., 2002). It remains to be determined how LecRKs function: how do they mediate the cell wall-plasma membrane continuum, and which signals are sensed? In **Chapter 3.2** we show that LecRK-I.9 plays a role in oomycete disease resistance, as suggested by the observation that *LecRK-I.9* expression in Arabidopsis is induced upon inoculation with pathogen strains that cannot infect Arabidopsis (avirulent strains) (Bouwmeester *et al*., 2008). For functional analysis we obtained Arabidopsis knock-out lines (*lecrk-I.9*) and lines expressing *ipiO*. Both lines gained susceptibility to *P*. *brassicae* and preliminary assays showed significantly more haustoria produced when inoculated with the downy mildew *H*. *arabidopsidis* (unpublished results). The *ipiO*-expressing lines and *lecrk-I.9* lines are also affected in their response to compounds classified as MAMPs; there is no callose deposition upon MAMP treatment. Interestingly, lines overexpressing *LecRK-I.9* gained resistance to *P*. *brassicae*, but remained susceptible to *Botrytis* (**Chapter 3.2**). These findings undoubtedly suggest that LecRKs are involved in response to plant pathogens, including oomycete species.

Arabidopsis, a model to study plant–*Phytophthora* interactions

Our understanding of how oomycete pathogens are able to infect their host plants could benefit greatly from information obtained from model systems of representative interactions (**Chapter 1**). Up to now, Arabidopsis has only been reported to function as a host plant for two *Phytophthora* species; i.e. *P. cinnamomi* and *P. brassicae* (Robinson and Cahill, 2003; Roetschi *et al*., 2001). Several Arabidopsis accessions were tested with *P. brassicae*, and distinct incompatible and compatible interactions were detected among various accession-isolate combinations (Roetschi *et al*., 2001; Mauch *et al*., 2009). Research on this pathosystem is hampered by the difficulty of obtaining enough *P. brassicae* zoospores, and an extensive screening based on zoospore inoculation has not yet been performed (Mauch *et al*., 2009). As described in **Chapter 3.2**, we used the *P. brassicae*–Arabidopsis pathosystem as a model to unravel the interaction between IPI-O and LecRK-I.9. We also established a novel method for abundant production of *P. brassicae* zoospores, which opens the opportunity to execute large scale inoculation experiments (**Section 4**). Recently, we tested various combinations between Arabidopsis accessions and isolates of *P. capsici*, and revealed differential interactions (Bouwmeester, unpublished results). This pathosystem will be an additional tool to dissect Arabidopsis–*Phytophthora* interactions. The above-named *Phytophthora* spp. can be cultured *in vitro* and are amenable to DNA transformation, thus allowing concurrent molecular analysis of the host as well as the pathogen.

The use of Arabidopsis as a tool will certainly help us to increase our insight into the mechanisms by which pathogens manipulate their hosts and to address the following questions; how do disease resistance complexes in plants recognize and block pathogens?, how do pathogens manage to circumvent this recognition?, and what are the roles of virulence targets in these processes? Since the introgression of resistance in crop species is time-consuming and laborious, the use of Arabidopsis can increase the speed of functional identification and characterization of putative resistance genes. For example, V*e1* – a tomato gene conferring resistance to *Verticillium* – retains its function in Arabidopsis, and this offers a new research path to study this *R* gene in depth by mutant analysis of putative downstream targets (E. Fradin, personal communication). In a similar way, *MbR7* – a NBS-LRR resistance gene isolated from a wild apple species – was shown to confer enhanced resistance in Arabidopsis to *P. syringae*, and was therefore proposed as a novel target gene for the development of disease resistant apple varieties (Lee *et al*. 2007). It should, however, be noted that *R* genes from distantly related plant species are not always functional in Arabidopsis, for example *Cf* genes (Van der Hoorn *et al*., 2001). This is likely due to their inability to guard virulence targets or the absence of specific downstream signaling components.

The same phenomenon can occur *vice versa*, not every Arabidopsis *R* gene will be functional on other plant species. For various Arabidopsis genes though it was shown that they function ectopically in other plant species, and some of which were reported to confer enhanced tolerance to abiotic and biotic stresses (Kim *et al*., 2009; Lacombe *et al*., 2010). As described in this thesis, overexpression of *LecRK-I.9* in Arabidopsis and constitutive expression of *LecRK-I.9* in potato resulted in increase of resistance to *P. brassicae* and *P. infestans*, respectively (**Chapter 3.2**, **3.3**). LecRKs are widely spread in higher plants, with 45 genes in Arabidopsis and 103 in rice (**Chapter 3.1**; Shiu *et al*., 2004), and hence it is worth to investigate whether these potential novel resistance components can be exploited for disease control in crop plants.

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Summary

Late blight is a devastating plant disease caused by the oomycete *Phytophthora infestans*, and one of the most important production-limiting factors for cultivation of potato. During plant infection *P. infestans* secretes a plethora of effectors, toxins and cell-wall degrading enzymes, several of which are known to be involved in host manipulation and defense suppression. **Chapter 1.3** is a review focused on oomycete genome biology. It covers the typical features of oomycetes and describes various plant pathogenic oomycetes in relation to their hosts. Also the currently available oomycete genomic resources are listed and the various strategies that have been used to accelerate gene discovery in oomycetes are summarized.

The central subject of **this thesis** is *ipiO*, one of the first cloned *Phytophthora* genes with a putative function in pathogenicity as was anticipated based on its *in planta induced* (*ipi*) expression, in particular during early stages of host infection. IPI-O contains two striking motifs: RXLR-dEER and RGD. RGD is a cell adhesion motif and was shown to be involved in binding to the extracellular lectin domain of LecRK-I.9, a lectin receptor kinase of Arabidopsis. The RXLR-dEER motif plays a role in effector trafficking into host cells and is shared by several secreted oomycete effector proteins which are known to function as race-specific avirulence (Avr) factors. The fact that IPI-O is a potential Avr factor was revealed in an effector genomics approach aimed at identifying novel pairs of *P. infestans Avr* genes and host plant resistance (*R*) genes. In this high-throughput screen *ipiO* was identified as *Avr-blb1*, the counterpart of the late blight *R* gene *Rpi-blb1* which originates from the nightshade *Solanum bulbocastanum*. Often *R* genes exploited in late blight resistance breeding become rapidly ineffective as a result of adaptation of *P. infestans*. However, unlike most late blight *R* genes that interact in a gene-for-gene manner with *Avr* genes, *Rpi-blb1* seemed to have the potential to remain its effectiveness.

Section 2 focuses on the role of IPI-O as both an avirulence and virulence factor during infection of potato. In **Chapter 2.1** we monitored the genetic variation and distribution of the *ipiO* family in an extensive isolate collection of *P. infestans* and closely related species. This resulted in the identification of 16 IPI-O variants that could be sub-divided in three distinct classes. Variants from class I and class II were shown to induce cell death when co-infiltrated with *Rpi-blb1* in *Nicotiana benthamiana*. Class III consists solely of the highly divergent variant IPI-O4, that is not able to trigger Rpi-blb1-mediated cell death. Class I is highly diverse and represented in all *P. infestans* isolates analyzed so far, except in two Mexican *P. infestans* isolates. The latter two are capable to infect *Rpi-blb1* plants, suggesting that the lack of class I variants in the genome of these strains allows

them to escape recognition by *Rpi-blb1* plants. Furthermore, we show that the C-terminal region of IPI-O is essential to elicit Rpi-blb1-mediated cell death. This region comprises a single W motif that is under positive selection. For other RXLR effectors it was shown that amino acid residues in the W motif are required for R protein-mediated recognition and suppression of cell death. We propose that profiling of the *ipiO* variants within *P. infestans* populations can predict the effectiveness of Rpi-blb1-mediated resistance in potato and, as such, can facilitate integrated disease management.

In **Chapter 2.2**, we investigated the subcellular localization of IPI-O during pre-infection and infection stages of potato. *P. infestans* was transformed with a chimeric construct of *ipiO* C-terminally-linked to *mRFP* encoding a red fluorescent protein tag. In transformants mRFP fluorescence was observed in germinating cysts, appressoria and tips of germ tubes. During infection of potato mRFP fluorescence was detected in haustoria, but not in the cytoplasm of the host cell. In the same study similar localization patterns were found for Avr4, another *P. infestans* RXLR-dEER effector that functions as an avirulence factor. In our inoculation assays we used etiolated potato plantlets, which do not show autofluorescence and this facilitated quick and easy monitoring of the infection by *P. infestans*.

Section 3 of this thesis deals with legume-like lectin receptor kinases (LecRKs), membrane-spanning proteins with potential roles in adaptive responses and cell wall integrity. In **Chapter 3.1**, we present an inventory and a phylogenetic analysis of the Arabidopsis *LecRK* gene family, which consists of 45 members. The rationale behind this study was to gain better insight into the diversity of LecRKs and their potential roles in plant development and plant defense. A comprehensive expression analysis based on exploration of existing databases revealed that several *LecRK* genes are differentially expressed during plant growth and development, whereas others are induced upon treatment with elicitors or during pathogen infection. In the literature, *LecRK* nomenclature is not uniform; different codes are used for the same genes leading to confusion and miscommunication. Based on the phylogenetic analysis we have reclassified the *LecRK* genes into nine clades and proposed a new nomenclature.

LecRK-I.9, one of the clade I Arabidopsis LecRKs which binds the RGD cell adhesion motif of IPI-O, was shown to mediate adhesion between the cell wall (CW) and plasma membrane (PM). In contrast, IPI-O disrupts these adhesions by virtue of its RGD motif. In **Chapter 3.2**, we analyzed Arabidopsis *LecRK-I.9* knock-out lines (*lecrk-I.9*) for their response to pathogen infection, in particular to *Phytophthora brassicae*. We also analyzed transgenic Arabidopsis lines expressing *ipiO*, and observed that both the *lecrk-I.9* lines and *ipiO*-expressing lines are impaired in their resistance to oomycete pathogens. To unravel the mechanisms

underlying this phenomenon we analysed callose deposition upon MAMP (i.e. flg22) treatment and investigated the strength of CW-PM adhesions under plasmolysis-inducing conditions. The results indicated that LecRK-I.9 is not only important for the maintenance of the CW-PM continuum, but also in MAMP-triggered immunity. Also here, both the *lecrk-I.9* knock-outs and the *ipiO*-expressing lines displayed a destabilized CW-PM continuum and impaired callose deposition, and hence, they can be regarded as phenocopies. Arabidopsis plants that constitutively express *LecRK-I.9* were smaller in size, and displayed increased levels of anthocyanin and lignin. Additionally, these lines were shown to exhibit enhanced resistance to *P. brassicae*. In **Chapter 3.3**, we studied the effects of LecRK-I.9 on growth and development of potato, and on tolerance to *P. infestans*. Constitutive expression of Arabidopsis *LecRK-I.9* in transgenic potatoes resulted in decreased plant size and altered leaf morphology. An increase in anthocyanin, such as observed in the *LecRK-I.9*-overexpressing Arabidopsis lines was, however, less obvious. In comparison to the parental control potato line the transgenic lines were less susceptible to mild and moderately aggressive *P. infestans* isolates, but the increased tolerance was not sufficient to provide resistance to aggressive isolates. These results strongly suggest that LecRK-I.9 is a novel resistance component that plays a role in defense against *Phytophthora*.
Several pathogens, including some oomycete species, have been reported to infect Arabidopsis, either naturally or under laboratory conditions. Most *Phytophthora* species, however, trigger specific defense cascades in Arabidopsis, leading to a non-host resistance response. *P. brassicae* is capable of infecting Arabidopsis, and the *P. brassicae*–Arabidopsis pathosystem is becoming a model to study various aspects of *Phytophthora*–plant interactions. One disadvantage of using *P. brassicae* is the laborious, time-consuming and inefficient procedure to generate zoospores from this pathogen. In **Section 4** we describe a novel method for propagating *P. brassicae* zoospores on an intermediate host plant. This resulted in the production of high numbers of zoospores thereby facilitating highly reproducible small and large scale inoculation experiments.

This thesis is completed with a general discussion (**Section 5)** addressing the current understanding of effector uptake by host cells, the subsequent recognition by cognate R proteins mediating effector-triggered immunity, and RXLR-dEER effector diversity. We also discuss the role of the RGD motif in effectors of both animal and plant pathogens, and the potential functions of LecRKs. Finally, we high-light the advantages of *Phytophthora*–Arabidopsis pathosystems as research object.

Samenvatting

De aardappelziekte, die veroorzaakt wordt door de oömyceet *Phytophthora infestans*, is één van de meest beperkende factoren in de aardappelteelt. Om aardappel te infecteren scheidt *P*. *infestans* een breed spectrum aan effectoren, toxines en celwandafbrekende eiwitten uit. Voor enkele daarvan is aangetoond dat ze betrokken zijn bij manipulatie en onderdrukking van afweerreacties in de plant. **Hoofdstuk 1.3** geeft een overzicht van de genoombiologie van plantenpathogene oömyceten, de typische eigenschappen van oömyceten en de relatie tot hun gastheren. Daarnaast wordt de vooruitgang in de genomica van oömyceten beschreven, en worden de diverse strategieën die gebruikt zijn om identificatie van genen in oömyceten te versnellen toegelicht.

Het centrale onderwerp in **dit proefschrift** is *ipiO*, één van de eerste gekloonde *Phytophthora* genen die, op basis van zijn *in planta* geïnduceerde (*ipi*) expressie vroeg tijdens infectie van de gastheer, als pathogeniciteitsgen gekenmerkt werd. IPI-O bevat twee opvallende aminozuurmotieven: RXLR-dEER en RGD. RGD is een celadhesie motief, en betrokken bij binding aan het extracellulaire lectine domein van LecRK-I.9, een lectine receptor kinase van Arabidopsis. Het RXLR-dEER motief speelt een rol in effector-transport naar gastheercellen en komt voor in verscheidene extracellulaire oomycete effector-eiwitten waarvan bekend is dat ze als cultivarspecifieke avirulentie (Avr) factor fungeren. De aanwijzing dat IPI-O een potentiële Avr factor is kwam uit een studie waarin via een effector-genomica benadering getracht werd nieuwe paren van *P*. *infestans Avr*-genen en resistentiegenen (*R*) te identificeren. In deze 'high-throughput screen' werd *ipiO* geïdentificeerd als *Avr-blb1*, de tegenhanger van *Rpi-blb1*, een *R*-gen afkomstig uit de nachtschade *Solanum bulbocastanum*. *R*-genen die de basis vormen van *P. infestans* resistentie ingekruisd in aardappel worden vaak spoedig doorbroken door snelle adaptatie van *P*. *infestans*. In tegenstelling echter, tot de meeste *R*-genen die op een 'gen-om-gen' manier met *Avr*-genen interacteren, bleek *Rpi-blb1* het potentieel te hebben zijn effectiviteit te behouden. **Sectie 2** concentreert zich op de rol van *ipiO* als zowel een avirulentie- als een virulentiefactor tijdens de interactie met aardappel. In **Hoofdstuk 2.1** analyseerden we de genetische variatie en de verspreiding van de *ipiO* genfamilie in een uitgebreide isolatenverzameling van *P*. *infestans* en nauw verwante soorten. Dit resulteerde in de identificatie van 16 IPI-O varianten die in drie verschillende klassen konden worden onderverdeeld. Varianten uit klasse I en II bleken celdood te induceren wanneer ze tezamen met *Rpi-blb1* in *Nicotiana benthamiana* werden geïnfiltreerd. Klasse III bestaat alleen uit de afwijkende variant IPI-O4 die geen celdood induceert in een *Rpi-blb1* bevattende aardappellijn. Klasse I is zeer divers en aanwezig in alle tot nu toe geteste *P*. *infestans* isolaten, met uitzondering van

twee Mexicaanse isolaten. Deze laatsten zijn in staat *Rpi-blb1*-planten te infecteren en dit suggereert dat het ontbreken van klasse I *ipiO* varianten in het genoom van deze isolaten het mogelijk maakt herkenning door *Rpi-blb1*-planten te omzeilen. Daarnaast laten we zien dat het C-terminale deel van IPI-O essentieel is voor het induceren van *Rpi-blb1*-afhankelijke celdood. Dit deel van het eiwit bevat een W-motief dat onder positieve selectiedruk staat. Voor andere RXLR-effectoren is aangetoond dat aminozuurresiduen in het W-motief cruciaal zijn voor herkenning door een R-eiwit en onderdrukking van celdood. We postuleren dat analyse van *ipiO* variatie in *P. infestans* populaties de effectiviteit van *Rpi-blb1*-afhankelijke resistentie in aardappel kan voorspellen, en op deze manier kan bijdragen aan een geïntegreerde bestrijding van *P. infestans* in het veld.

In **Hoofdstuk 2.2** onderzochten we de subcellulaire lokalisatie van IPI-O tijdens pre-infectie- en infectiestadia. *P. infestans* werd hiervoor getransformeerd met een hybride construct dat codeert voor IPI-O met een, aan de C-terminus gekoppeld, rood fluorescerend eiwit (mRFP). In de transformanten werd mRFP fluorescentie waargenomen in kiemende cysten, appressoria, en in de toppen van kiembuizen. Tijdens infectie was mRFP fluorescentie zichtbaar in de haustoria van *P. infestans*, maar niet in het cytoplasma van gastheercellen. Een soortgelijk experiment liet eenzelfde lokalisatie zien voor Avr4, een andere *P. infestans* RXLR-dEER effector die fungeert als Avr-factor. Voor deze infectieproeven werden geëtioleerde aardappelplantjes gebruikt. Deze geven geen autofluorescentie, met als voordeel dat het infectieproces beter te volgen was.

Sectie 3 van dit proefschrift gaat over een subklasse van lectine receptor kinases; de 'legume-like' lectine receptor kinases (LecRKs). Dit zijn membraaneiwitten met een potentiële rol in adaptieve afweer en celwandintegriteit. In **Hoofdstuk 3.1** presenteren we een inventarisatie en fylogenetische analyse van de Arabidopsis *LecRK* familie die 45 genen omvat. Dit had tot doel een beter inzicht te krijgen in de diversiteit van LecRKs en in hun potentiële rol in zowel plantontwikkeling als tijdens afweerreacties. Een uitvoerige expressieanalyse op basis van bestaande databestanden, toonde aan dat sommige *LecRK* genen differentieel tot expressie komen tijdens de groei en ontwikkeling van de plant, terwijl expressie van andere *LecRK* genen wordt geïnduceerd na behandeling met elicitors of tijdens infectie door pathogenen. In de literatuur is de *LecRK* nomenclatuur niet uniform; voor eenzelfde gen worden verschillende coderingen gebruikt hetgeen leidt tot verwarring en miscommunicatie. Op basis van de fylogenetische analyse hebben we de *LecRK* genen opnieuw geclassificeerd in negen clades en een nieuwe nomenclatuur voorgesteld.

LecRK-I.9 is een clade I Arabidopsis LecRK die een rol speelt in de adhesie tussen de celwand (CW) en het plasmamembraan (PM), en bindt aan het RGD celadhesie motief in IPI-O. IPI-O kan daarentegen deze adhesies verstoren door

middel van zijn RGD motief. In **Hoofdstuk 3.2** analyseerden wij de reactie van Arabidopsis LecRK-I.9 knock-out lijnen (*lecrk-I.9*) op infectie met pathogenen, in het bijzonder *Phytophthora brassicae*. We analyseerden ook transgene Arabidopsis planten waarin *ipiO* tot expressie komt. Zowel de *lecrk-I.9* lijnen als de *ipiO*-expressie lijnen bleken verminderd resistent te zijn tegen plantenpathogene oömyceten. Om de mechanismen te ontrafelen die aan dit fenomeen ten grondslag liggen analyseerden we callose depositie na behandeling met flg22, een 'microbe-associated molecular pattern" (MAMP) die basale afweer oproept, en onderzochten we de sterkte van de CW-PM adhesie na inductie van plasmolyse. De resultaten suggereren dat LecRK-I.9 niet alleen belangrijk is voor het behoud van het CW-PM continuüm, maar ook voor MAMP-geïnduceerde immuniteit. Omdat ook hier zowel de *lecrk-I.9* knock-outs als de *ipiO*-expressie lijnen een verstoorde CW-PM integriteit en verminderde callose depositie vertoonden kunnen beide lijnen als elkanders fenokopieën worden beschouwd. Arabidopsis planten die constitutief *LecRK-I.9* tot expressie brengen waren kleiner en toonden meer anthocyaan en lignine. Bovendien bleken deze lijnen resistenter te zijn tegen *P. brassicae*. In **Hoofdstuk 3.3** bestudeerden wij het effect van LecRK-I.9 op de groei en ontwikkeling van aardappel, en tolerantie tegen *P. infestans*. Constitutieve expressie van Arabidopsis *LecRK-I.9* in transgene aardappels resulteerde in kleinere planten en in een veranderde bladvorm maar een verhoging in anthocyaan, zoals waargenomen in de Arabidopsis *LecRK-I.9*-overexpressie lijnen , was niet evident. In vergelijking met de ouderlijn waren de transgene aardappellijnen minder vatbaar voor milde en matig agressieve *P. infestans* isolaten, maar deze verhoogde tolerantie resulteerde niet in resistentie tegen agressieve isolaten. Deze resultaten tonen aan dat *LecRK-I.9* een nieuwe component is in resistentie tegen *Phytophthora*.

Voor verscheidene plantenpathogenen met inbegrip van oömyceten, is het aangetoond dat ze Arabidopsis kunnen infecteren, hetzij onder laboratoriumomstandigheden dan wel in een natuurlijke omgeving. De meeste *Phytophthora* soorten echter initiëren specifieke afweerreacties in Arabidopsis waardoor infectie wordt gestopt. *P. brassicae* kan Arabidopsis daarentegen wel infecteren en het *P. brassicae*–Arabidopsis pathosysteem kan dus als model gebruikt worden om de diverse aspecten van *Phytophthora*-waardplant interacties te bestuderen. Een nadeel van *P. brassicae* is de tijdrovende en inefficiënte procedure om zoösporen van dit pathogeen te verkrijgen. In **Sectie 4** beschrijven we een nieuwe methode voor het genereren en isoleren van zoösporen van *P. brassicae* waarbij gebruikt wordt gemaakt van een tussengastheer. Dit vergemakkelijkt de productie van grote hoeveelheden zoösporen en het uitvoeren van reproduceerbare infectieproeven – op zowel kleine als grote schaal.

Het proefschrift wordt afgesloten met een algemene discussie (**Sectie 5**) over het huidige inzicht in effector-opname door de plantencellen, de daaropvolgende effector-specifieke herkenning door R-eiwitten, en de diversiteit van RXLR-dEER effectoren. We bespreken de rol van het RGD motief in effectoreiwitten van ziekteverwekkers van zowel plant als dier en de mogelijke functies van LecRKs in planten, en eindigen met eenbeschouwing over de voordelen van het gebruik van *Phytophthora*–Arabidopsis pathosystemen.

Dankwoord

En nu, na al die pagina's aan wetenschappelijke schrijfsels wil ik maar zo vrij zijn om het dankwoord kort en bondig te houden. Of me dat lukt is nog de vraag want vele mensen hebben mij geholpen bij de totstandkoming van al dit werk.

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Beste familie, een verblijf in het Noorden was altijd weer plezierig. Bedankt voor alle fijne momenten. Lieve oma, ik weet dat je heel trots op me bent, en ik ook op jou. Lieve zus, ik vind het heel tof vind dat je altijd tijd neemt voor je broer met z'n rare Wageningse fratsen. Lieve papa en mama, ik wil jullie graag bedanken voor de vaste overtuiging dat alles uiteindelijk goed komt. Ik heb altijd mijn eigen

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Wetenschap is een mooi tijdverdrijf, ik kan er veel in kwijt. Maar soms leek het ook wel of er weinig anders was dan dat. Nu deze grote klus geklaard is moet ik denken aan het Groninger spreekwoord '*ain mens is gain eerdappel*', dat wil zeggen geen inspanning zonder ontspanning. Dus op naar buiten en op naar 't lab.

Curriculum vitae

Klaas Bouwmeester werd geboren op 30 juni 1978 te Zevenaar. Na het behalen van het mavo en havo diploma aan het Ommelander college te Appingedam begon hij in 1996 aan de HLO opleiding Biologie en Medisch Laboratoriumonderzoek met de specialisatierichting biotechnologie aan de Hanzehogeschool te Groningen. Tijdens zijn HBO afstudeeronderzoek bij Plant Research International te Wageningen heeft hij onder begeleiding van Dr. ing. Martijn Fiers en Dr. Chun-Ming Liu gewerkt aan de karakterisering van CLE19, een signaalmolecuul functionerend in de stamcelregulatie van Arabidopsis en *Brassica napus*. Na het succesvol afronden van zijn HBO opleiding in 2000, begon hij in datzelfde jaar met de M.Sc. opleiding Biotechnology aan de Wageningen Universiteit. Zijn afstudeervak werd uitgevoerd bij de Leerstoelgroep voor Erfelijkheidsleer van de Wageningen Universiteit onder begeleiding van Dr. ing. Leónie Bentsink en Prof. dr. ir. Maarten Koorneef aan de genetische regulatie van kiemrust in Arabidopsis. Na het afstuderen in 2002 werkte hij vervolgens als onderzoeker aan het voormalige onderzoeksinstituut IMAG, en als projectadviseur Life Sciences bij het agentschap SenterNovem van het Ministerie van Economische Zaken. In november 2003 begon hij met zijn promotieonderzoek bij het Laboratorium voor Fytopathologie aan de Wageningen Universiteit onder begeleiding van Prof. dr. ir. Francine Govers en Prof. dr. ir. Pierre de Wit. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Sinds maart 2010 is hij bij hetzelfde laboratorium aangesteld als postdoctoraal onderzoeker op een project gericht op de karakterisering van lectine receptor kinasen en hun rol in resistentie tegen plantenpathogenen, in het bijzonder tegen oömyceten.

Publications

Bouwmeester K, Govers F. 2009. Arabidopsis L-type lectin receptor kinases: phylogeny, classification, and expression profiles. *Journal of Experimental Botany* **60**, 4383-96.

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[*] shared first authorship

Champouret N[*], Bouwmeester K[*], Rietman H[*], van der Lee T, Maliepaard CA, Heupink A, van de Vondervoort PJI, Jacobsen E, Visser RGF, van der Vossen EAG, Govers F[*], Vleeshouwers VGAA[*]. 2009. *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on Rpi-blb1 potato. *Molecular Plant-Microbe Interactions* **22**, 1535-45
[*] shared first and last authorships

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Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Klaas Bouwmeester
Date: 31 May 2010
Group: Laboratory of Phytopathology, Wageningen University

1) Start-up phase	date
▶ **First presentation of your project**	
Identification of virulence targets in the *Phytophthora infestans* - potato interaction	Dec 01, 2003
▶ **Writing a review or book chapter**	
Genome biology cracks enigmas of oomycete plant pathogens. Annual Reviews 34	2009
▶ **MSc courses**	
Plant-microbe Interactions, PHP-30306	Mar-Apr 2004
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	date
▶ **EPS PhD Student Days**	
Vrije Universiteit Amsterdam	Jun 03, 2004
Radboud University Nijmegen	Jun 02, 2005
Wageningen University	Sep 19, 2006
Wageningen University	Sep 13, 2007
▶ **EPS Theme Symposia**	
EPS theme 2, Interactions between Plants and Biotic Agents, Wageningen	Dec 12, 2003
EPS theme 1, Developmental Biology of Plants, Wageningen	Feb 17, 2004
EPS theme 2, Interactions between Plants and Biotic Agents, Utrecht	Sep 17, 2004
EPS theme 2, Interactions between Plants and Biotic Agents, Leiden	Jun 23, 2005
EPS theme 2 symposium in combination with Willy Commelin Scholten day, Amsterdam	Feb 02, 2007
EPS theme 2 symposium in combination with Willy Commelin Scholten day, Utrecht	Jan 22, 2009
▶ **NWO Lunteren days and other National Platforms**	
NBC-10, Netherlands Biotechnology Congress, Royal Netherlands Chemical Society (KNCV), Ede	Mar 11-12, 2004
ALW/ EPW Annual Meeting, Lunteren	Apr 05-06, 2004
ALW/ EPW Annual Meeting, Lunteren	Apr 04-05, 2005
ALW/ EPW Annual Meeting, Lunteren	Apr 03-04, 2006
ALW/ EPW Annual Meeting, Lunteren	Apr 02-03 2007
ALW/ EPW Annual Meeting, Lunteren	Apr 07-08, 2008
ALW/ EPW Annual Meeting, Lunteren	Apr 19-20, 2010
Willie Commelin Scholten day, Royal Botan. Society Netherlands (KNBV), section Phytopathology, Utrecht	Jan 22, 2004
Willie Commelin Scholten day, Royal Botan. Society Netherlands (KNBV), section Phytopathology, Utrecht	Jan 19, 2006
Summit CBSG2012	Mar 16-17, 2010
▶ **Seminars (series), workshops and symposia**	
Seminars (Barbara Baker, Martin Parniske, Jean Ristaino, Matteo Garbelotto, Mohammed Babadoost, Sophien Kamoun, Khaoula Belhaj, Jim Beynon, Philip Zimmerman, Andre Drenth, James Bradeen, Julio Diez, Mike Coffey, Richard Oliver, Pieter van West, Cyril Zipfel, Rays Jiang, Thorsten Nürriberger, Ricardo Oliva, Laurent Zimmerli)	2004 -2010
CBSG Disease Resistance Workshop, Wageningen	Jun 10, 2004
Flying seminars (Steven Clark, Philip Benfey	2004-2005
Dutch Phytophthora Umbrella meeting	Oct 06, 2005
▶ **Seminar plus**	
▶ **International symposia and congresses**	
Eucablight Potato Late Blight Network for Europe,*P. infestans* population biology meeting, Wageningen	Dec 07-09, 2005
XII International Congress on Molecular Plant-Microbe Interactions, Merida (Mexico)	Dec 14-19, 2005
Annual meeting Oomycete Molecular Genetics Network, Wageningen	May 04-07, 2006
XIII International Congress on Molecular Plant-Microbe Interactions, Sorrento (Italy)	Jul 21-27, 2007
Annual meeting Oomycete Molecular Genetics Network, Birnam (Scotland)	May 06-08, 2008
XIV International Congress on Molecular Plant-Microbe Interactions, Quebec (Canada)	Jul 19-23, 2009
▶ **Presentations**	
Oral presentation, ALW/ EPW Annual Meeting, Lunteren	Apr 03-04, 2006
Oral presentation, Annual meeting Oomycete Molecular Genetics Network, Wageningen	May, 04-07, 2006
Oral presentation, BioExploit meeting SP1+3, Wageningen	Mar 28, 2007
Oral presentation, EPS theme 2 symposium in combination with Willy Commelin Scholten day, Utrecht	Jan 22, 2009
Poster presentation, XII International Congress on Molecular Plant-Microbe Interactions, Merida (Mexico)	Dec 14-19, 2005
Poster presentation, 8th European Conference on Fungal Genetics, Vienna (Austria)	Apr 08-11, 2006
Poster presentation, XIII International Congress on Molecular Plant-Microbe Interactions, Sorrento (Italy)	Jul 21-27, 2007
Poster presentation, Annual meeting Oomycete Molecular Genetics Network, Asilomar (USA), (2x)	Mar 15-17, 2009
Poster presentation, XIV International Congress on Molecular Plant-Microbe Interactions, Quebec (Canada)	Jul 19-23, 2009
Poster presentation, Summit CBSG2012	Mar 16-17, 2010
Poster presentation, ALW/ EPW Annual Meeting, Lunteren	Apr 19-20, 2010
▶ **IAB interview**	
Interview with Prof.dr. Michel Dron	Sep 07, 2006
▶ **Excursions**	
Field visit, field trial for screening late blight resistance of wild *Solanum* species, Wageningen	Jul 14, 2005
Subtotal Scientific Exposure	*28.1 credits**

3) In-Depth Studies	date
▶ **EPS courses or other PhD courses**	
EPS Summer school 'Signalling in plant development and defence: towards systems biology'	Jun 19-21, 2006
EPS Summer school 'Evironmental signalling: Arabidopsis as a model'	Aug 27-29, 2007
WIAS course: Introduction for R statistical analysis	Apr 21-22, 2008
Summer School 'On the evolution of plant pathogen interactions: from principles to practice'	Jun 18-20, 2008
▶ **Journal club**	
Literature study group Phytopathology	2003-2010
▶ **Individual research training**	
Subtotal In-Depth Studies	*6.3 credits**

4) Personal development	date
▶ **Skill training courses**	
Endnote advanced	Apr 12, 2007
Guide to Digital Scientific Artwork	Apr 10-11, 2007
▶ **Organisation of PhD students day, course or conference**	
Organisation of the annual outing of the Laboratory of Phytopathology	Jun 11, 2004
Organisation of the 5th annual meeting Oomycete Molecular Genetics Network, Wageningen	May 04, 2006
Organisation Summer School 'On the evolution of plant pathogen interactions: from principles to practice'	Jun 18-20, 2008
▶ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**51.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises a study load of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study*

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