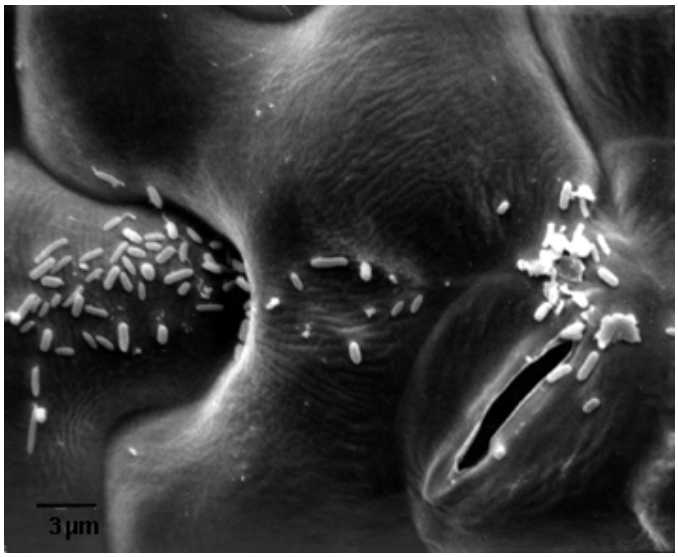
Role of Arabidopsis resistance proteins and *Pseudomonas syringae* effector proteins in host-microbe interactions



About the cover

Pseudomonas syringae on the leaf surface of *Phaseolus vulgaris.* The structure in the right bottom is the stomate, which is a natural opening to secrete extensive water. *P. syringae* mainly lives on the surface of the leaf, but once the bacterium has entered the plant it can cause disease.

Adapted from: www.micro.iastate.edu

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Abstract

The plant Arabidopsis can be infected by several pathogens, such as bacteria, fungi, and viruses. The plant reacts on the infections by pathogens by inducing a pathogen-associated molecular patterns induced immune response (PTI). This response can be evaded by bacteria that secrete specific effector protein via a type III secretion system. Bacteria that can evade the PTI are known as the true pathogens. Effector proteins alter several cellular processes in the plant. Plants have evolved resistance proteins as a reaction on the effector proteins. Once the effector protein is recognized by the resistance protein the effector triggered immunity is activated. Furthermore, plants can prime cells on a distance to be prepared for the bacterium. The exact working mechanism of the resistance proteins is not yet fully understood. In this thesis the plant immune system will be briefly introduced, followed by the working mechanism of the effector proteins of the bacterium *Pseudomonas syringae* and the resistance proteins.

List of abbreviations

Avr protein	Avirulence protein
AtMKK4/AtMKK5	MAP kinase proteins
AtMPK3/AtMPK6	MAP kinase proteins
CC	Coiled Coil
CDR1	Constitutive disease resistance1
ETI	Effector triggered immunity
EFR	EF-Tu receptor
EF-Tu	Elongation factor Tu
FLS2	Flagellin sensitive2
LPS	lipopolysaccharides
LRR	Leucine rich repeats
MeSA	Methyl salicylate
NBS	Nucleotide binding site
NPR1	Non-Expressor of Pathogenesis Ralated1
PAMP	Pathogen-associated molecular patterns
PRR	Pattern recognition receptors
PTI	PAMP-triggered immunity
R protein	Resistance protein
SA	Salicylic acid
SAP2	SA-Binding Protein2
SAR	Systemic acquired response
siRNA	Small interference RNA
TIR	Toll-receptor from Drosophila or IL-1

Table of Contents

Introduction

Many causative agents of infectious diseases, such as bacteria, viruses, parasites and fungi are known. Although infectious diseases are not the only cause of death in the world, it is one of the main causes. In 2002 over 25% of all deaths were caused by infectious diseases. Of the deaths caused by infectious diseases bacterial infections are the major player (Prithiviraj et al 2005).

First the pathogenesis of the different causative agents needs to be understood to cure or prevent the infectious disease. Many model organisms are used for the search of cures and prevention of infectious diseases. In this thesis the focus will be on the interaction of the plant Arabidopsis with bacterium *Pseudomonas syringae.*

Bacteria in general

Bacteria can be distinguished into gram-positive and gram-negative bacteria by their differences in the composition of their cell wall. Gram-negative bacteria possess two cell walls; an inner cell wall, which surrounds the cytoplasm, and the outer cell wall. The space between the cell walls is the periplasm wherein a peptidoglycan layer is situated. The most striking feature of the outer membrane of the gram-negative bacteria is the LPS that is situated there (Willey 2009). Via secretion systems bacteria secrete virulence factors that assist them in colonizing the host. Eventually, the virulence factors can lead to invasive and toxinogenic infections (Prithiviraj et al 2005).

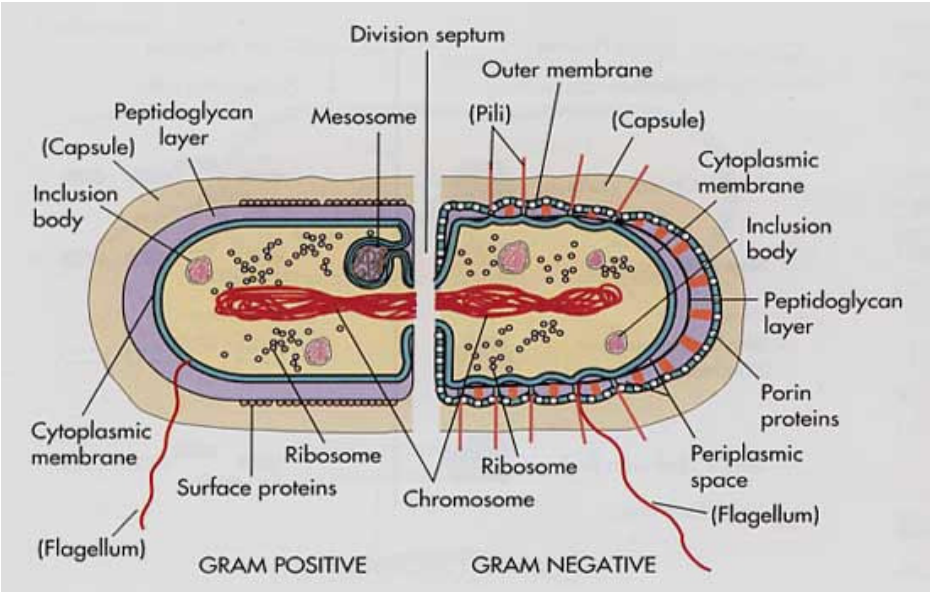

Figure 1: gram positive and gram negative bacterium.
A schematic overview of the gram positive bacterium is shown on the left side of this figure. In this figure the most distinct differences between gram positive and gram negative bacteria can be observed. Gram-negative bacteria have an inner and outer membrane while gram positive bacteria only possess a plasma membrane. Adapted from http://micro.digitalproteus.com.

P. syringae is a rod shaped gram-negative bacterium that infects the plant Arabidopsis, which is a member of the family of the *Pseudomonadaceae* and the genus *Pseudomonadales*. All bacteria in this genus are motile by flagella and contain straight or slightly curved rods. *Pseudomonadales* bacteria obtain their energy by O2 dependent oxidation, although sometimes also nitrate is used as the electron receptor. The *Pseudomonas* family is again divided in subgroups. Because *P. aeruginosa, P. fluorescens, and P. syringae* all produce a yellow-green pigment that is fluorescent if UV-radiation is used, those are placed in the same group. The *Pseudomonas* family has also a great impact. First of all, several organisms can be infected by *Pseudomonas* bacteria. *P. aeruginosa* is an opportunistic bacterium that infects immune compromised humans, but is also able to infect plant under certain conditions. *P. fluorescens* infects plant, but has bio-control function in the infected plant. It is suggested that the infected plant is protected from other microbe infection by the bacterial infected because of priming of the plant. *P. syringae* is an opportunistic bacterium that also infects plants. Within the *P. Syringae* group, several subtypes are known, nomenclature depending on the plant it is infecting. In this thesis the focus will lie on the *P. syringae* and Arabidopsis interaction (Willey 2009).

Plants in general

The plant is build of three main organs; roots, stems, and leafs. Furthermore, the plant can be divided in two systems, the root system, which consists of the roots, and the shoot system, which consists of stems and leafs. The entire root system anchors the plant, but in most plants uptake of water and minerals only occurs near the root tips. This area of the root is increased by many tiny root hairs. Roots can also function as storage place for nutrients in plants that flower or produce fruits. That also why many vegetables, such as potatoes, are harvested before the plant flowers. The leave is the organ of the plant that is responsible for photosynthesis (Campbell 2005).

In several ways plants are as well similar as different from mammals. First of all mammals can move; plants are not able to run or hide neither are they able to move in search for nutrients. Another striking difference between plants and mammals is how they generate energy. Plants can generate energy from light by their chloroplasts, which are energy producing organelles in plant cells. This process is dependent on CO_2 and O_2 is produced in this process, whereas humans use O_2 and generate in CO_2 in the process of energy production. Another difference between plant cells and mammalian cells is the cell wall that plant cells posses. This cell wall is not present in mammalian cells (see figure 2). Because of the cell wall, the cells of plants cannot freely move through the plant and therefore the plant is dependent on specific and tightly regulated cell division. Also for accessibility of the nutrients the plant is dependent on cell division because the plant cannot move towards the nutrients but has to grow towards it (Taiz 2006).

Grow of Arabidopsis is dependent on auxin. The main functions of auxin are stimulation of stem elongation, root growth, and cell differentiation. Another important function of auxin is stimulation of cell elongation in new shoots. Auxin also induces cell division. How these processes are regulated and the mechanism

of auxin signal transduction is not yet fully understood and more research is needed on this field (Campbell 2005).

Arabidopsis is the main model used in unravelling the processes in the plant. Arabidopsis is easily genetic manipulated, has many descendents and can be grown in a test tube. Another advantage of performing experiments with Arabidopsis is that the ethical questions are non-existing (Campbell 2005). In addition, the whole genome of Arabidopsis has been sequenced which make it very easy to analyse outcomes of experiments on genetic level (Kaul 2000)

Bacterial infections in plants

Plants can get infected by viruses, fungi, and bacteria just like the mammals can. The mechanism of defence of plants differs in several ways from mammals, but also similarities can be observed. As previously mentioned *P. syringae* infects Arabidopsis and is the causative agent of foliar spots and blights on this plant. The infection starts with infected seeds and if the disease progresses the bacterium moves to the leaf surfaces and grows there. It is critical for the survival of *P. syringae* that it is able to grow on the leaves. Entering of the bacterium into the plant is achieved by, and entirely dependent on, wounds or natural openings, like the stomata. Stomata are small openings in the leaf where through excessive water normally leaves the plant. Thereafter it will grow in the intracellular space, the apoplast. From this moment on two things can happen. The bacteria can be eliminated by the host or if the host has not the correct defence, the bacterium causes necrotic lesions in the plant that are often surrounded by chlorotic halos, which are brown spots on the surface of the plant (Kim et al 2008).

When *P. syringae* has reached the apoplastic area, this is the area that is covered by the two cell walls of neighbouring cells (see figure 2), it will become in direct contact with the cells of Arabidopsis. The cell of Arabidopsis is surrounded with a cell wall that is over 100.000 times larger than the whole bacterium. In the apoplast nutrients are scarce, but *P. syringae* needs the nutrients to flourish in the apoplast. To fulfil the need in nutrients, *P. syringae* secretes virulence factors that make the host cell secrete nutrients, such as photosynthates, and water over the cell wall. The virulence factors also impair the defence of Arabidopsis against *P. syringae* (Kim et al 2008; Melotto et al 2006).

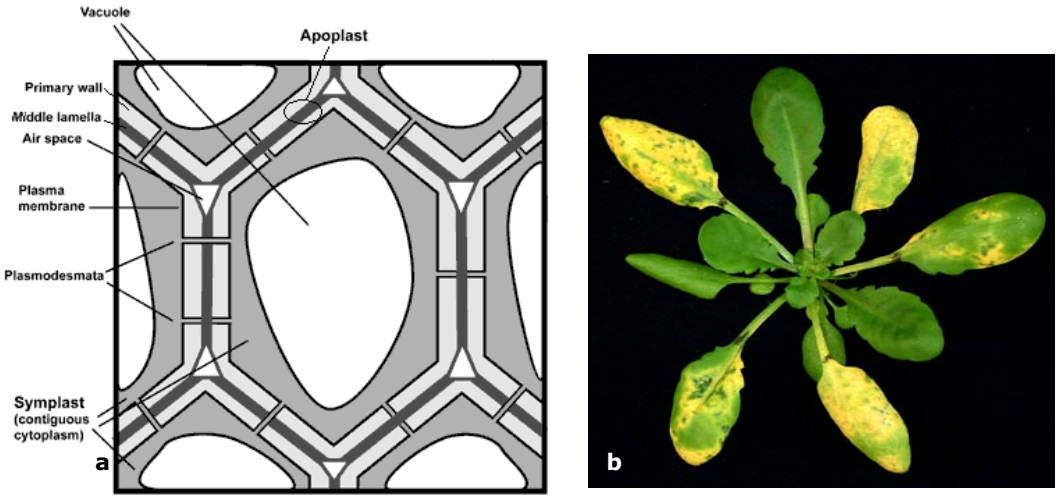

Figure 2: Schematic overview and a *P. syringae* infection in Arabidopsis. a) The schematic overview of a plant cell with some important structures. For the *P. syringae* the apoplast is important because this is the place where the bacterium move to once the plant is infected (adapted from http://www.ccrc.uga.edu/~mao/intro/ouline.htm. b) An infected Arabidopsis. Adapted from pseudomonas-syringae.org.

Aim of the Thesis

This thesis will be focussed on the interaction between *P. syringae* and Arabidopsis. In the first chapter the immune system of the plant in general will be discussed. This is necessary to understand the interaction between the plant and the bacterium. In the second chapter the immune evasion by *P. syringae* will be discussed with regard to the secretion systems of the bacterium and how the secreted proteins, the 'effector' proteins, alter the host cell. How Arabidopsis deals with the secreted proteins by so-called resistance proteins will be discussed in the third chapter. In addition in the third chapter also the co-evolution and the ongoing competition between pathogen and host will be addressed. The thesis will end with a discussion section. In this section the difficulties in identifying resistance proteins and their working mechanism and the link that can be made towards mammalian cells will be discussed.

Chapter 1 – Plant immune system

Unlike mammals, plants do not possess a specific immune system. Therefore they rely entirely on their innate immune system. Each single cell has an innate immune reaction. In addition the plant as a whole has a systemic innate immune system that reacts on excreted substances from the pathogen. Plants have several natural barriers against pathogens and insects. They have rigid cell walls, pre-existing toxic compounds and leaf hairs. If a pathogen is able to pass these barriers, the pathogen specific innate immune system comes into action. Plants recognize the pathogens in three different manners; via microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) (primary immune response), via Resistance-proteins (effector triggered immunity), and via systemic immune response.

Primary immune response

The primary immune response relies on the recognition of PAPMs by pattern recognition receptors (PRRs). PAMPs of gram-negative bacteria recognized by the PRRs are for example flaggelin, lipopolysaccharides (LPS), and elongation factor Tu (EF-Tu). Recognition of the PAMPs can have different cellular reactions. The reaction includes cell wall alterations, deposition of callose to thicken the cell wall and the accumulation of defense-related proteins such as chitinases, glucanases and proteases. All these alterations can negatively influence the pathogen. However several PAMPs are known and several PRRs are identified; only a few downstream pathways are known. This suggests that PRRs make use of only a limited amount of downstream pathways to up-regulate genes that stimulate cellular alterations (de Wit 2007; Galletti et al 2009).

Bacterial Flg22, which is a part of flagellin, is recognized by Flagellin sensitive2 (FLS2), a Leucine-rich Receptor. Furthermore, EF-Tu is recognized by EFR. These two different PRRs activate the same MAP kinase pathway (Zipfel et al 2006). Furthermore, the MAPK-pathway is not only activated by PAMPs, but can also be activated by abiotic of biotic stress factors (Galletti et al 2009). These results taken together underlie the possibility that different PAMPs activate the same pathway. This pathway results in resistance to bacteria as well as fungi and viruses, implying that the pathway initiated results in a converged MAPK pathway. Because the primary response is not affected by protein synthesis inhibitors it is suggested that the molecules needed are already available in the cell (Zipfel et al 2006). But upon binding of Flg22 to FLS2 not only the MAPK is activated, but also an early oxidative burst occurs and other cellular proteins are phosphorylated. Due to the activation of the kinases, a broad gene expression alteration occurs (Asai et al 2002).

Genes important for the innate immunity of the plant are transcribed as a reaction of the MAPK signalling. It is thought that other leucine rich receptors, which are not identified yet, also activate the similar MAPK pathway. In this pathway the intercellular kinase is activated by the LRR (see figure 3)

Upon activation, the kinase activates AtMEKK1, followed by activation of AtMKK4/AtMMK5 and AtMPK3/AtMPK6. This results in phosphorylation and thereby inhibition of the repressor (R) of WRKY22/29. In this manner WRKY22/29

can conduct their function as transcription factor and the innate immune reaction gene transcription is initiated (Asai et al 2002).

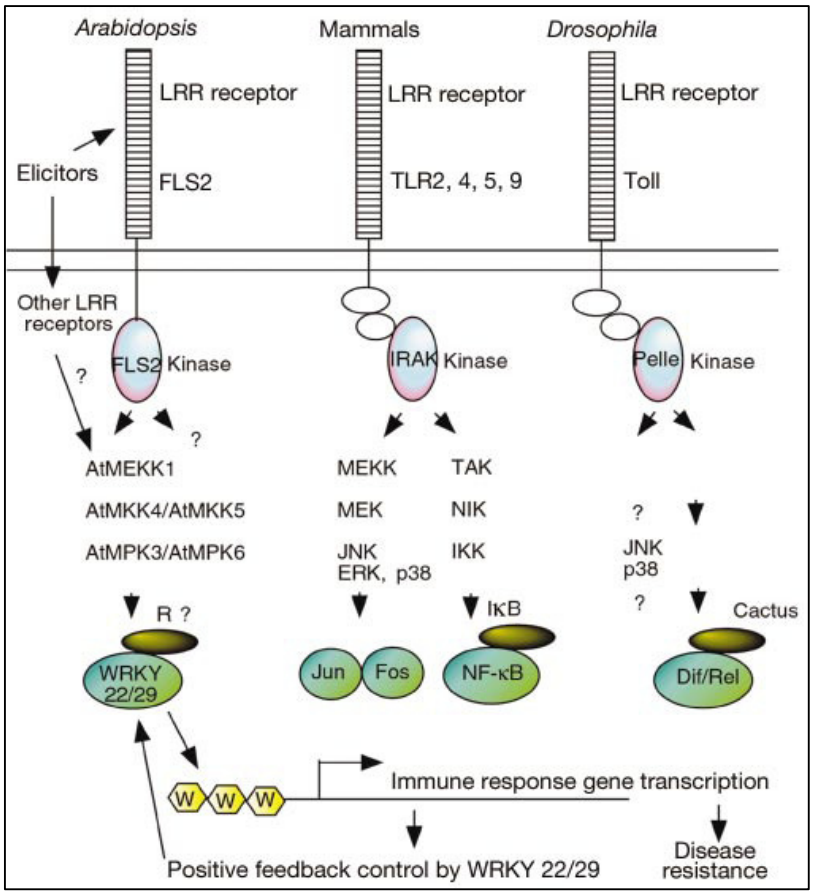

Figure 3: Schematic overview of activation of the MAPK pathway by recognition of the elicitors.
Once the elicitor, in this overview Flg22, is recognized by its receptor, which for Flg22 is FLS2, the MAPK pathway is activated. By deactivation of the repressor, the WRKY transcription factors can bind to its promoters whereby the immune response gene transcription is activated. In mammals and Drosophila a similar pattern can be observed. Upon recognition of the PAMP-recognition receptors the transcription of gene that are responsible for the immune reaction is initiated via a similar pathway. Adapted from (Asai et al., 2002)

Another outcome of Flg22 perception by the cell is the production of small interfering RNA (siRNAs). siRNA are believed to be important in regulation of protein expression by guiding mRNA to destruction. It has been showed that some pathogens can trigger the production of siRNAs to interfere with the pathogen eventually resulting into cell death (Hou et al 2009). The main function of siRNA is defence against viral infection (Jones & Dangl 2006). This lies beyond the scope of this thesis and will therefore not further be discussed.

Secondary immune response

Phytopathogens have evolved a way to circumvent the primary immune response; the true pathogens. True pathogens secrete effector proteins that disrupt the primary immune reaction. Those proteins are called avirulence (avr) proteins and several have been identified. In the next chapter the manner of secretion of the effector proteins by bacteria and the effector proteins itself will be discussed more thoroughly. Plants have evolved proteins, the resistance (R) proteins, which counteracts the avr-proteins. In this paragraph the functional way of counteraction will be discussed.

Most R proteins contain a nucleotide binding site (NBS) and a series of leucine-rich repeats (LRRs), together called NBS-LRRs. The recognition of bacterial avr-proteins by R proteins results in activation of NBS-LRR. NBS-LRR on their turn activates the MAP kinase pathway that will lead to activation of defence genes. The recognition of bacterial effector genes by R proteins usually results in a the cell death of the infected cells, the hypersensitive response (Chisholm et al 2006; de Wit 2007; Gohre & Robatzek 2008).

The NBS-LRRs can be distinguished in two different groups depending on their N-terminus. On the N-terminus is either a coiled-coil (CC) motif or a motif that is related to the Toll-receptor from Drosophila or IL-1 in mammalian cells (TIR) (Dangl & Jones 2001). The different N-termini determine which cellular complexes are needed to fully achieve the effector-triggered immune response. Cellular NDR1 is needed by some CC-NBS-LRR proteins to achieve the full defence response. However, not all CC-NBS-LRR require NDR1. The mechanism behind this is only poorly understood (Axtell & Staskawicz 2003).

The R proteins in plants are important for plants. The R protein is activated upon recognition of its specific pathogenic avr-partner. This interaction or recognition will trigger a pathway that will lead to programmed cell death, production of reactive oxidative stress, and production of antimicrobial compounds and eventually activation of the *R*-genes will result in resistance of the plant to the bacterium. If either the pathogenic *avr*-gene or the *R*-gene in the plant is absence, disease will be the outcome (Dangl & Jones 2001). Although the gene-for-gene hypothesis is strongly supported by identification of several avr-R protein couples, a direct interaction between effector proteins and R proteins is rarely found, suggesting another method of recognition of effector proteins by the R protein (Dangl & Jones 2001).

Another conceptual framework is that the R protein 'guards' several host-proteins that are targeted by the bacterial effector proteins. The Guard Model also explains how one R protein can protect several targets of effector proteins by suggesting that one R protein can guard several host proteins. The protection of the host proteins can be achieved in a two different manners (see figure 4). First, the R-protein is associated with the protein complex and upon binding of the effector protein to the host-protein-R-protein-complex the NBS-LRR is activated. Second, the host protein-R-protein complex conformation changes once the bacterial effector protein binds to the complex. NBS-LRR is recruited to the protein complex by the conformational change and activated there. Activation of NSB-LRR eventually leads to transcription of the gene that regulates the defence against that particular bacterium (Dangl & Jones 2001; Hou et al 2009).

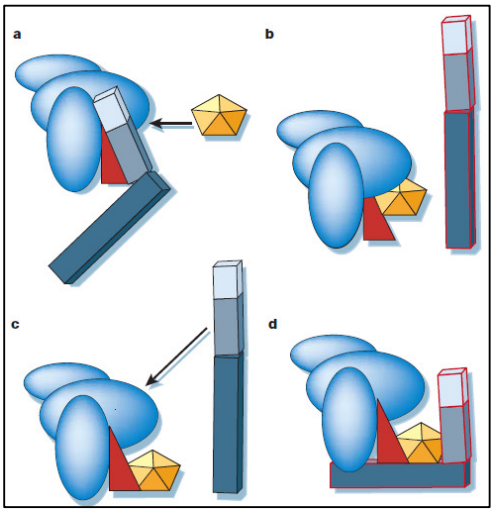

Figure 4: Model for the indirect activation of the secondary immune response.
A and B: The R protein (red) is bound to the host protein complex (light blue). The NBS-LRR is bound to the R protein. Once the effector protein (yellow) binds to the R protein NBS-LRR is released and activates the effector triggered immune defence.
C and D: A second hypothesis is that once the effector proteins bind to the R protein, the structure of the R protein changes in such a way that NBS-LRR can bind to it and thereby gets activated and in turn activates the defence genes. Adapted from (Dangl & Jones 2001)

However, the Guard Model is not in line with the natural selection of plants and bacteria. The target of the R protein is unstable if the R protein is present, but also has problems with the bacterial effector if the R protein is not present. This conflict in natural selection results, according to the Decoy Model, into proteins that mimic the target protein with the R protein to perceive the effector, but has no longer another function in the host (Hou et al 2009; van der Hoorn & Kamoun 2008).

In the Guard model, the R protein physically interacts with the targets of the effector proteins. If the effector protein binds to the protein complex, the R protein perceives this and is activated resulting in the host defence. In another mechanistic scenario the effector protein binds to its host target resulting its disassociating of the R protein and activation of the R protein. Another possibility is that due to binding of the effector protein to its target protein complex the conformation of the complex changes resulting in recruitment and activation of the R protein. Each model implies that the R protein perceives the effector protein if the target of the effector protein is altered (Dangl & Jones 2001; Hou et al 2009). In figure 5 an overview is shown of how the PAMP-triggered immune response and the effector triggered immune response are integrated.

Systemic resistance

Upon activation of the primary and secondary immune response also a systemic immune response is triggered. The systemic acquired resistance (SAR) protects the cells of the plant that are further away from the primary infection site and is not specific. SAR is dependent on salicylic acid (SA), and a subset of pathogenesis resistance (Prithiviraj et al 2005) genes. The plant does not only defence itself from pathogens such as bacteria and fungi, but also defence itself against insect with SAR. A second systemic resistance process is the induced systemic resistance, which is more specific for a pathogen, and relies on jasmonic acid (Vernooij et al 1994) and ethylene (de Wit 2007; Grant & Lamb 2006).

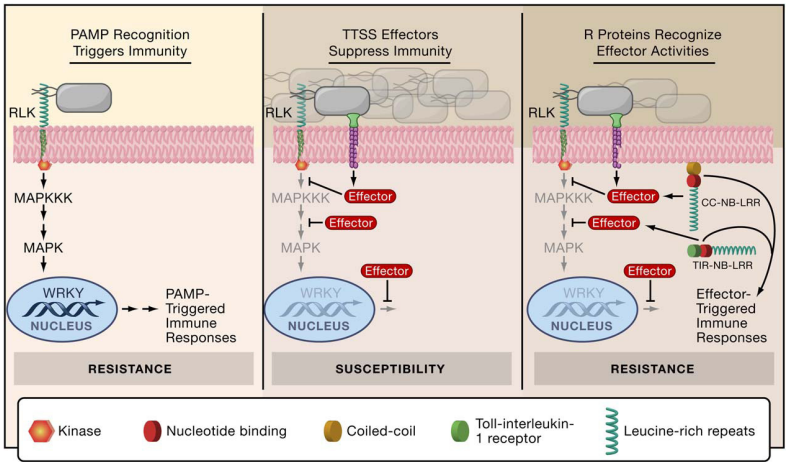

Figure 5: Overview of primary and secondary immune response.
(a). The plant recognizes through its PRR the PAMP/MAMP of the pathogen and the primary defense is activated. (b). The pathogen secretes effector proteins that target the proteins that are transcribed in the primary defense response. (c). The plant protects the proteins that are targeted by bacterial effector proteins by Resistance (R) proteins. Through the activation of the R proteins a effector triggered immune response is activated to impair the bacterial infection. (Adapted from (Chisholm et al 2006)

It was thought for a long time that SA was the systemic signal for SAR, several experiments excluded this possibility (Rasmussen et al 1991; Vernooij et al 1994). Nowadays it is thought that lipid-derived signalling proteins are responsible for transmission of SAR through the plant (Grant & Lamb 2006). One lipid derived molecule is DIR1, although it is not known how this molecule is activated (Maldonado et al 2002).

In addition to the lipid derived signal molecules, SA-derivative methyl salicylate (MeSA) is implied to be a signal molecule for SAR in distant cells. Once MeSA is present in the distant cells, hydrolysis of MeSA into SA is triggered by SA-Binding Protein2 (SAP2), resulting in accumulation of SA and thereby SAR (Park et al 2007). MeSA is only essential to trigger SAR in the distant cells, but not in the infected cell. Upon silencing the *SAP2-gene* SAR was no longer induced (Dong 2004; Pieterse & Van Loon 2004). Also peptides may be involved in SAR-signalling. Peptides generated by Constitutive disease resistance1 (CDR1) may be involved in SAR signalling. However, the exact mechanism of this process still needs to be excluded (Vlot et al 2008).

Once the SAR signal reaches the distant non-infected cells or upon infection of cells, SA accumulates. Accumulation of SA is achieved by *de novo* synthesizing through chorismate via the enzyme isochorismate synthase (Wildermuth et al 2001). Through this accumulation of SA the redox of the cells is altered resulting in active monomeric forms of non-Expressor of Pathogenesis Related1 (NPR1), which is the crucial factor in SAR (Mou et al 2003). The monomeric forms of NPR1 relocate to the nucleus and interact with TGA-transcription factors initiating the transcriptional reprogramming and thereby inducing the transcription of

pathogenesis-related (Prithiviraj et al 2005) genes, which means that non-infected cells are primed from a distance (Mou et al 2003; Pieterse & Van Loon 2004). It is implied that NPR1 is the downstream player in *Resistance*-gene mediated defence (Dong 2004).

NPR1 is an important regulator for SAR but also for induced systemic resistance and can function in both pathways simultaneously (van Wees et al 2000). NPR1 is located downstream of jasmonic acid and may regulate this responses according to the signals given by upstream proteins. However, induced systemic resistance is induced in infections where the pathogen survival relies on the degradation of the host while SAR is induced in infections where the pathogen survival relies in the survival of the host (Fan et al 2009; Truman et al 2007). Because *P. syringae* is dependent on the survival of the host, induced systemic resistance lies beyond the scope of the thesis and will therefore not be discussed.

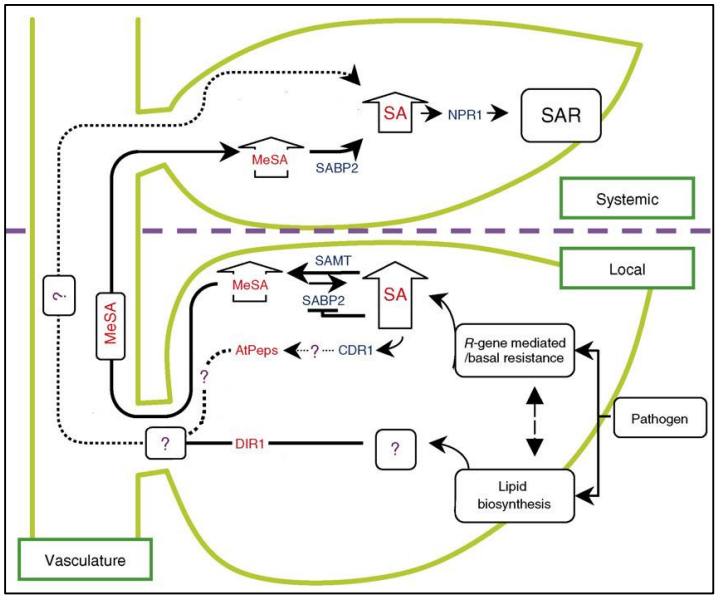

Figure 6: Inducing systemic acquired resistance
Three different manners of transmitting SAR through the plant are predicted in this figure. The first hormone that is implied to communicate SAR to systemic cells is the phytohormone MeSA. In addition, lipid derived (DIR1) and peptide derived (CDR1) signal proteins are suggested to function as signal molecules. Furthermore, their working mechanism in signal transmission is shown. However, much needs to be investigated yet. Adapted from (Vlot et al 2008)

Chapter 2 – Secretion of effector proteins of *Pseudomonas syringae*

In the previous chapter the immune system of plant is described. Bacteria have evolved several ways to evade this immune system. In this chapter the question how bacteria secrete the effector proteins into the target cells will be addressed. Furthermore the host cell targets of the effector proteins of *P. syringae* to evade the immune system will be discussed.

Secretion systems of gram-negative bacteria

Five different secretion pathways have been identified so far in gram-negative bacteria, type I, II, III, IV, and V. Also another pathway that transports proteins from the cytosol to the periplasm is identified; the Sec dependent and the Tat dependent pathway. All pathways are schematic shown in figure 7

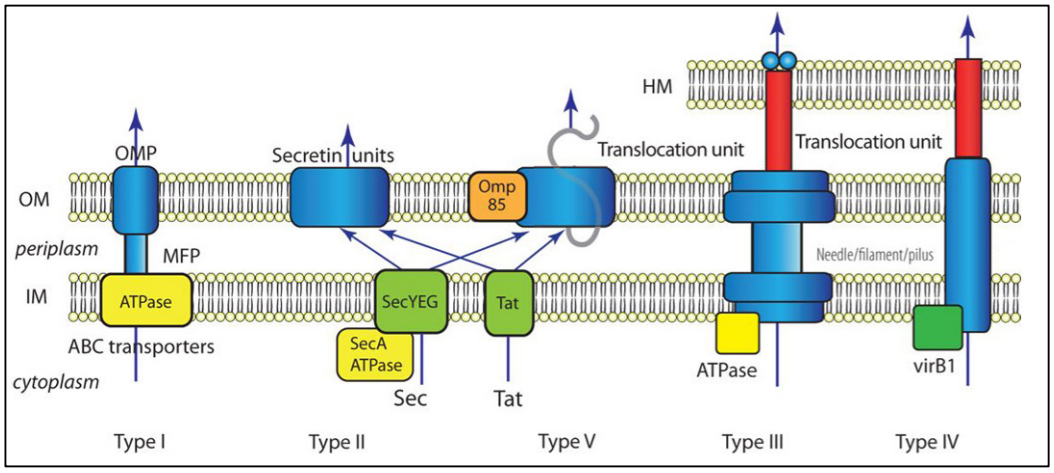

Figure 7: schematic overview over the different secretion systems.
In this figure the composition of 5 types of secretions systems are shown. Mainly type III secretion system is used for the secretion of virulence factors. However, all the other secretion systems are also essential for the survival of the bacterium. Adapted from (Cornelis 2006).

The Sec dependent pathway translocates unfolded protein from the cytosol to the periplasm or integrates the protein into the plasma membrane. This pathway is highly conserved in prokaryotes. All proteins that are translocted by this mechanism carry a signal peptide that is recognized by this machinery. When the signal peptide is synthesised, several chaperon proteins bind to the signal peptide. Folding of the protein that needs to be translocated is delayed through the binding of the chaperone proteins (SecB). Several Sec proteins, SecY, SecE, and SecG are implied to form a pore where through the protein is transported. The process of transport is as follows: Sec A binds to the SecB-protein complex and acts as a motor to transport the protein through the pore. Once the protein is in the periplasm or plasma membrane it folds into its final conformation (Tseng et al 2009; Willey 2009).

Another pathway that transports proteins over the plasma membrane is the type I secretion system (T1SS), also known as the ABC-protein-secretion

pathway. T1SS transports proteins from the cytosol directly into the exterior of the bacterium. Proteins that are transported by the T1SS usually carry a C-terminus signal sequence to direct them to the T1SS (Tseng et al 2009; Willey 2009).

The TAT dependent pathway transports proteins that are already folded across the plasma membrane and delivers them directly to the type II secretion system (T2SS). Only proteins that carry two arignines in their signal sequence are transported by the Tat machinery. Although T2SS spans the plasma membrane, periplasm and the outer membrane, it seems that it only transports proteins from the periplasm over the outer membrane to the exterior environment of the bacterium. Furthermore, proteins that are transported by the Sec dependent pathway can be transported to the exterior environment by the T2SS (Willey 2009).

Type V secretion systems are also dependent on the Sec-pathway. Once proteins are in the periplasm it seems as if they form a pore themselves and transport themselves over the outer membrane. Other proteins are supported by a separate helper protein (Tseng et al 2009; Willey 2009). Type IV secretion systems transport not only proteins but also DNA to other bacteria. The secretion of DNA occurs in a process called conjuration (for more information on conjuration see Willey et al.).

The final and most important secretion system for the scope of this thesis is the type III secretion system (T3SS) since this secretion system injects virulence factors, also known as effector proteins, into host cells. It covers the plasma membrane, the periplasm, the outer membrane and the plasma membrane of the host cells. Thereby T3SS transports the effector proteins directly into the host-cell or sometimes also in the exterior environment of the bacterium. T3SS bears resembles to a needle, the injectisome (see figure 8).

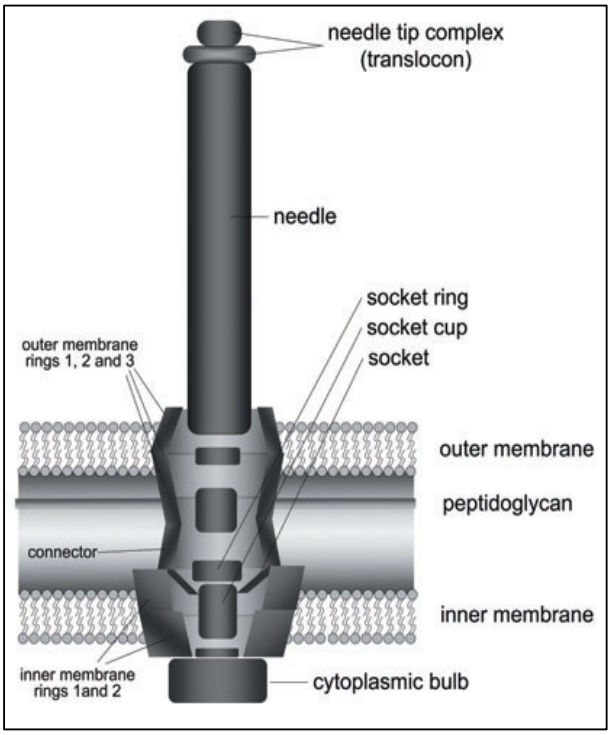

Figure 8: The schematic composition of the injectisomes of the *P. syringae.*
The assembly of the injectisome is tightly regulated in a conserved process. One of the essential proteins in the injectisome is de ATPase, which is located in the cystoplasmic bulb. Adapted from (Enninga & Rosenshine 2009)

The injectisome needle can be visualized as a straight hollow tube. This hollow tube is constructed from 100-150 molecules of the YscF family. The injectisome is approximately 25A in diameter and 60 nm in length, although the length of the injectisome may vary. A translocation pore, the translocon, is situated in the exterior part of the active injectisome. The translocon is inserted in the plasma membrane of the host cell (Cornelis 2006). Direct contact with the host cell triggers the activation of the injectisome (Cornelis 2006; Enninga & Rosenshine 2009). The assembly of the injectisome is tightly regulated and over 30 genes are involved. Many of the genes involved are co-activated. The assembly of the injectisome itself is depending on the different components that are brought to the assembly site in a tight hierarchal temporal process. It remains unclear how the bacterium senses when sufficient amounts of components are delivered at the assembly site and how the bacterium knows when the assembly is completed (Enninga & Rosenshine 2009).

One of the essential proteins of the injectisome is an ATPase from the YscN family. This protein is highly conserved among the injectisomes in different genera of bacteria. The ATPase delivers energy for the translocation of the proteins. In *P. syringae*, the ATPase forms hexamers and docamers and are associated with the cytoplasmic site of the injectisome. This ATPase is activated by oligomerization. Furthermore, it is implied that the ATPase is involved in the detachment of the chaperon proteins from the proteins that needs to be transported (Cornelis 2006).

As mentioned in the previous paragraph, many effector proteins have a cytoplasmic chaperone protein to transport the protein to the T3SS. The exact function of the chaperones remains to be elucidated, but it is suggested that they are 3D targeting factors, or that they determine which protein is transported first with the T3SS injectisome. Other results imply that the chaperone proteins are necessary to store the effector proteins prior to transport. Chaperone binding does not prevent the folding of the effector protein to its mature conformation (Alfano & Collmer 2004; Birtalan et al 2002; Boyd et al 2000; Cornelis 2006).

Targets of effector proteins

In the previous paragraph is discussed how bacteria secrete their effector proteins in host cells. In this paragraph will be discussed how the effector proteins are beneficial for the bacterium and are malicious for the plant. Secreted effector proteins can suppress the primary as well as the secondary immunity. Furthermore, they can influence many essential cellular processes.

The most important function of effector proteins is influencing the immune system so that it does not affect the bacterium anymore. Effector proteins can target several levels of the immune system of the plant. Experiments suggest that one third of the effectors secreted by *P. syringae* targets the immune system (Alfano & Collmer 2004; Espinosa et al 2003).

Experiments have predicted many effector triggered immunity suppressors. Nevertheless, the exact function and working mechanism of many of them remains to be resolved. Effector proteins can function either as elictors or as suppressors of the plant immune system. Effector proteins can also act on more than one pathway. An example of a effector protein that acts on two immune pathways simultaneously by targeting proteins that act within two pathways is

HopAO1 (Espinosa et al 2003; Gohre & Robatzek 2008). It targets host proteins downstream of MAPK. MAPK is a kinase in a pathway where many signals converge to. Thus by inhibiting one kinase, more than one pathway is impaired. The main function of HopAO1 is inhibiting the PAMP-mediated immune response (see figure 9) (Alfano & Collmer 2004; Espinosa et al 2003). This impairment of the MAPK-pathway is conducted by (de)phosphorylation of the kinases. HopAI1 and HopAO1 dephosphorylate their target and thereby irreversible inactivate the MAPK. HopAI1 binds to more than one kinase *in vitro,* thus *in vivo* even more targets may exist (Block et al 2008). Another effector that targets the early PAMP-induced pathway is AvrPto. This protein recognizes FLS2 and inhibit the kinase function of this receptor (Angot et al 2007; Zhou & Chai 2008).

An example of an effector protein that is able to interfere with the effector triggered immunity is *avrRpt2.* avrRpt2 suppresses the fast immune reaction that was triggered by avrRpm1 (Alfano & Collmer 2004). But effector proteins that target the immune system of the plant do not alone target the effector triggered immune response, but also the PR response and the oxidative burst.

Effector proteins are also capable of influencing the hormone signalling that is necessary to establish a systemic immune response. Two different ways of how the hormone signalling is influenced are identified in Arabidopsis. For a proper immune response the signal hormone auxin, which is a growth hormone, needs to be suppressed by miRNA that is transcribed upon recognition of a PAMP. This process can be altered in two ways by effector proteins. First, effector proteins can influence the PAMP-induced pathway so that the signal for miRNA production does not reach the nucleus. Second, effectors can manipulate the host cell in such manner that they produce more auxin (da Cunha et al 2007). If the amount of auxin increases, the immune response is impaired. A second hormone of which the expression is modulated, is the hormone jasmonic acid (Shang et al 2006; Vernooij et al). Bacteria that affect the amount of jasmonic acid conduct this by inducing genes that express jasmonic acid. As mentioned in the previous chapter, jasmonic acid is the antagonist of salicylic acid and by increasing the amount of jasmonic acid the systemic resistance of the plant will be demolished (da Cunha et al 2007).

Trafficking of proteins from the endoplasmic reticulum via the Golgi to the plasma membrane is very important for the host immune system. This trafficking can be a target for the effector proteins of the bacterium. The effector protein HopM1 suppresses callose deposition, which is the plant host cell wall associated defence. HopM1 targets AtMIN7, which is guanine exchange factor that activates ARF GTPases. ARF GTPases are important for the intracellular trafficking. If Arabidopsis has a non-functional AtMIN7 then their immune system is impaired, indicating the importance of intracellular trafficking for the plant immune system and thereby the importance of HopM1 for the bacterium (da Cunha et al 2007; Hou et al 2009; Speth et al 2007).

Infections by pathogens results in a change in host gene expression. First of all, this is associated with the defence of the host cell to the pathogen and thereby the transcription of defence related proteins. However, research have revealed that also bacterial effector proteins are directed to the genome of the host cell and thereby changing the gene expression in such manner that it is favourable for the bacterium. Modulation of host gene transcription can occur by

effector proteins that either bind DNA directly or by effector proteins that function as transcription factors that not bind the DNA directly (Block et al 2008; da Cunha et al 2007; Speth et al 2007).

Effector proteins can also target the proteasome-mediated degradation pathway in host cells. Proteins that are targeted for the proteasome are ubiquitinated and thereby directed to the proteasome and degraded there. Pathogens can use this pathway to fine tune the expression of effector proteins. In addition, the proteasome pathway is explored by the bacterium to degrade host proteins and thereby promoting disease. Host proteins can be directed to the proteasome in different manners. HopM1 of *P. syringae* mediates degradation of AtMIN7 via the host cell ubiquitination pathway. However, HopM1 has no ubiquitin ligase features, which implies that HopM1 may work as a mediator that ensures that AtMIN7 is recognized and degraded by the plant ubiquitination program. Furthermore, effector proteins can mimic the function of one of the players in the ubiquitination pathway to target host proteins to the proteasome (Angot et al 2007; da Cunha et al 2007).

A recent study revealed that effector proteins alter the secreted proteins of Arabidopsis. They found proteins without signal sequences or translocation sequences in the medium once Arabidopsis was infected with *P. Syringae*. The question remains what the physiological, or better to say beneficial, function of the secreted proteins are for *P. Syringae* (Kaffarnik et al 2009).

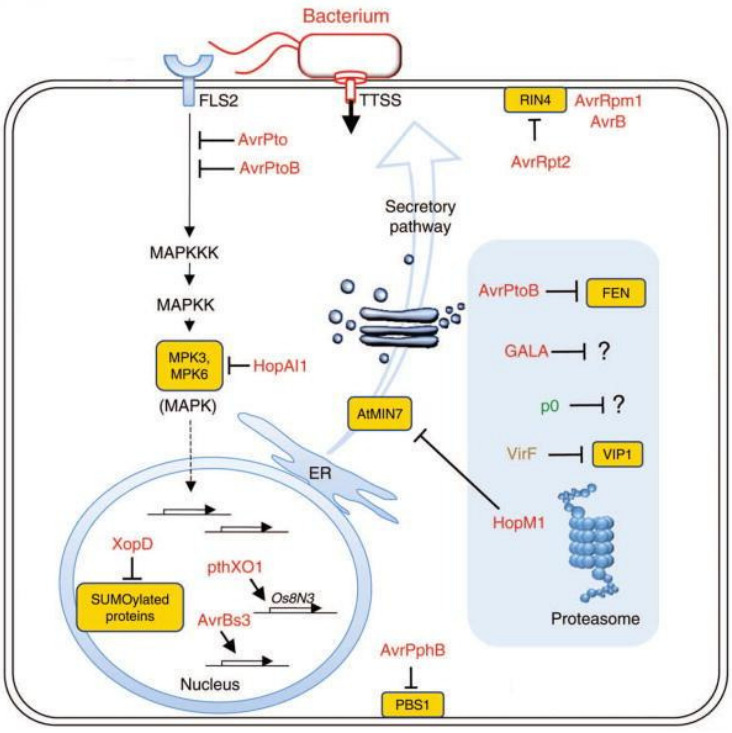

Figure 9: The different targets of host cell modulation by bacteria.
Effector proteins target all different cellular processes in order that the bacterium survives and multiplies. Processes that are modulated are gene expression, protein degradation, protein trafficking and the immune system processes. Adapted from (Speth et al 2007).

Chapter 3 - Plant (Arabidopsis) vs. Bacteria (*P. syringae)*

This chapter will focus on the interaction between the plant and the bacterium. Specific R-proteins will be discussed and how they target different Avr (effector)-proteins. Also the enduring struggle between plant en bacterium will be discussed in this chapter.

R-proteins and their Avr targets

Plants have evolved together with the bacteria to escape from the effector proteins. However many R-proteins are identified, the working mechanism of many of those R-proteins remains to be elucidated (Meyers et al 2003).

In the first chapter the hypothesized working mechanisms of R proteins are discussed; either by direct interaction (the gene for gene hypothesis) of the R-protein with the Avr protein or by indirect recognition (the guard hypothesis), or by a protein that structurally mimics the target protein (decoy hypothesis). AvrPphB, which is an effector of *P. syringae* with yet unknown biological function or target*,* is recognized via indirect recognition by RPS5, which is the R-protein responsible for recognizing AvrPphB. PBS1 is a protein kinase of which the function is not yet known. RPS5 binds with its coiled-coil to PBS1 (Ade et al 2007). PBS1 is cleaved by AvrPphB at a specific site. This cleavage is recognized by RPS5 and the inhibitory effect on RPS5 is released and ADP is exchanged for ATP resulting in an active RPS5. Therefore it seems that the conformation of PBS1 is monitored by RPS5. If the status of PBS1 is specifically changed, RPS5 recognizes this and the signal transduction pathway towards an immune response is started (Ade et al 2007; DeYoung & Innes 2006).

Another example of indirect recognition of the effector proteins is the R-protein Pto and the effector AvrPto. AvrPto binds to FLS2 to abolish the flg22, which is a part of flagellin, mediated immune response. However, AvrPto also binds to Pto in a similar way as it binds to FLG2, suggesting that Pto functions as a decoy for FLS22 (Zong et al 2008). AvrPto interacts with two loops of Pto. If AvrPto is not present in the cell these two loops are required to keep Prf inactive. If mutations occur in the two loops, Prf is activated and the immune response is triggered even in absence of a pathogen. This taken together suggests that AvrPto triggers the activation of Prf by inhibiting the inhibitory effect that Pto has on Prf. Another possibility of activation of Prf is that AvrPto can trigger a conformational change in Pto that is detected by Prf and Prf auto-activates upon recognition of the intermolecular disturbance of Pto (Hou et al 2009; Zhou & Chai 2008). Another working mechanism of Pto that was recently shown is that Pto phosphorylates AvrPto and thereby inhibiting its E3-ligase function and abolishing its function as a inhibitor of FLS2 (Ntoukakis et al 2009).

Three different effector proteins target RIN4 by at least two different mechanisms. AvrB and AvrRpm1 phosphorylates RIN4 and phosphorylzed RIN4 is recognized by RPM1 (Mackey et al 2002) and an effector triggered immune recognition occurs. AvrRpt2 is a cysteine protease and inhibits the recognition of phosphorylized RIN4 by cleaving RIN4. However, cleavage of RIN4 is recognized by RPS2 and also results in a defence response (Kim et al 2005; McHale et al

2006). It is thought that uncleaved RIN4 keeps RPS2 inactive, but once RIN4 is cleaved, RPS2 will be activated and initiate an immune response (Axtell & Staskawicz 2003). AvrRpt does not only cleave RIN4 but also cleaves other host targets that contain the cleavage sites for this cysteine protease (Chisholm et al 2005).

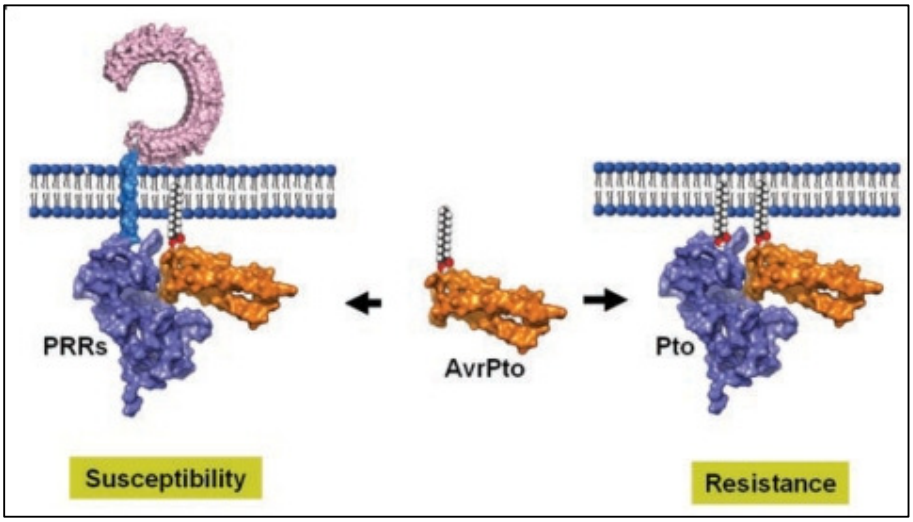

Figure 10: Model for working mechanism of AvrPto
AvrPto binds either to the FLS2 or to Pto. If AvrPto has bound to FLS2, the immune defence is inhibited. When AvrPto binds to Pto, Pto no longer can inactivate Prf and a immune reaction is established. Adapted from (Zong et al 2008)

R-proteins also need to be regulated tightly to balance the side-effects of the defence reaction. This regulation is conducted in several ways. Some *R*-genes are only expressed during the immune response. RPS4 is an example of one of those genes (Zhang and Gassmann, 2007). Another manner of regulation of the R proteins is alternative splicing, this also occurs with RPS4. A third mechanism used to control the R protein is by chaperones. Furthermore, R proteins are kept inactive by interaction of the proteins they guard. However some mechanisms of regulation are known, the whole mechanism of activation and regulation of the R proteins remains to be addressed (Kwon et al 2009).

Evolution of Resistance Genes

It is suggested that R proteins originated from endogenous signalling proteins that are required in normal processes in the plant and in development of the plant. This is suggested because many endogenous signalling proteins in mammalian cells have similarities with the R proteins. NBS-LRRs show a high degree of structural homology with the human major histocompatibility complex class II transcription activator and also with a mouse protein that is involved in B cell proliferation and protection against programmes cell death. This homology with mammalian proteins implies that they have a common evolutionary origin (Hammond-Kosack & Jones 1997). Nevertheless, research has showed that two *R* genes with common target show no similarity in their sequence or orthology, suggesting that two distinct R proteins are developed against the same target (Ashfield et al 2004) .

In the previous paragraph the manner of how R proteins recognize effector proteins is discussed. It is put forward that recognition can occur in two different manners, via direct recognition and via indirect recognition. If an effector protein is recognized via direct recognition negative selection with that specific effector protein will occur, this can be by a mutation in the effector protein that influences the recognition of that protein, but does not abolish the virulence function of the effector protein (Xiao 2008). R-proteins that indirectly recognize effector proteins are believed to have an evolutionary advantage and are more stable in the population. It is more difficult for a bacterium to escape from this kind of recognition because adaptations on the effector protein do not only abolish recognition by the R-protein but also the effector protein lost its virulence (Xiao 2008).

The co-evolution of R proteins and bacterial effector proteins can be seen in 4 steps. First, the PAMPS trigger an immune response in the plants that eliminate the bacterium. In response to the immune response the bacterium injects effector proteins that impair the PAMP-triggered immune response. In reaction on those effector proteins Resistance proteins evolve. Resistance protein can recognize effector protein directly or indirectly. If the resistance proteins recognize the effector protein the effector triggered immunity will be activated. In the following step the bacterium evade this effector triggered immunity. The effector protein that is directly recognized mutates in such a way that is no longer recognized by Resistance proteins. If the recognition occurs indirectly, the effector protein is deleted from the bacterium and also the selection pressure on the Resistance gene decreases. In the end the variation of selection pressure leads to stable polymorphisms in the *R* and *effector loci.*

The driver of evolution of resistance proteins is as follows (see figure 11). In the first phase the plant has an R protein that provides resistance to a specific effector protein. Plants carrying this R protein will have an advantage and so this specific R protein will become more abundant in the population. Then the bacterium carrying this specific effector gene will be eliminated more and the effector gene will become less abundant in the bacterial population. This results in less need of the specific R protein again and then this gene will also become less abundant in the plant population. Some bacteria in the population still carry the gene for the effector protein and when the R protein against this effector protein becomes less abundant the bacteria carrying the effector protein can flourish again. In this manner the circle goes on and on (Jones & Dangl 2006).

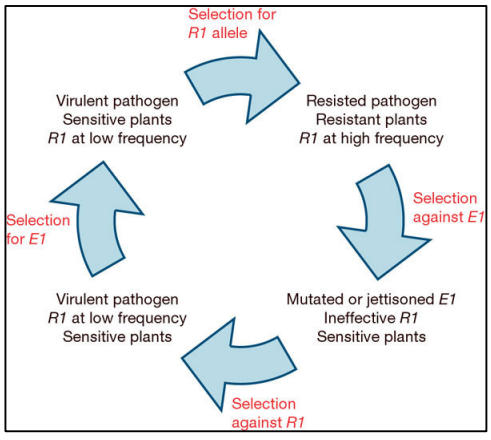

Figure 11: Suggested process in R-protein and Avr-protein selection. The process of selection of R-proteins and effector proteins never stops and is a circle. Once one of the proteins is less abundant the other will become less abundant as well as a reaction on this (Jones & Dangl 2006).

Discussion

In this thesis the proteins involved in the interaction between microbe and plant are discussed. The thesis started with a brief overview of the immune system of the plant. It became clear that the plant, although it does not have an adaptive specific immune system, has a very good defence mechanism against pathogens. The defence cascade starts with the recognition of PAMPs resulting in the up regulation of defence related proteins. The bacterium can evade this defence response by secreting so called effector proteins. Bacteria that can evade the PAMP-triggered immunity are the true pathogens. The plant has evolved a mechanism to perceive the effector proteins. The perception of the effector proteins by resistance proteins results in effector triggered immunity that eventually results in cell death of the infected cell. Effector proteins and resistance proteins are subdued to selection and thereby polymorphisms in the different proteins can be found.

Although many resistance genes are suggested, over 150 in Arabidopsis, only of a few the function and working mechanism is known (Kim et al 2008; Meyers et al 2003). It could be that breakthroughs are rare because of the low abundance or R proteins in the plant cells. Also the studying of the R proteins biochemically is very difficult. Because of their complex structure it is very hard to prove interaction with a yeast-two hybrid and other protein-protein screens and thus the number of identified partners is limited (Holt et al 2002). Another possibility for the difficulty in identifying the working mechanism of R proteins and their partners can be due the working mechanism of the resistance proteins. Most R proteins do not recognize the effector proteins by a direct interaction. Mostly, the status of cellular targets of the effector proteins is monitored. If this status alters, the R protein recognizes that and initiates the effector triggered immunity. This indirect interaction makes it very hard to identify the targets of the R proteins. The indirect interaction between effectors, R proteins, and cellular targets makes it likely that the whole working process occurs in a multi-protein complex and therefore highly sophisticated biochemical methods are needed to conduct research on those complexes (Martin et al 2003).

The structure of resistance genes shows similarities with PAMP recognition proteins in mammalian cells. As discussed previously in this thesis, R proteins possess a leucine rich region (LRR) and a nucleotide-binding site (NBS). Some of the R proteins also have coiled coil or TIR (Drosophila Toll and human IL-1 receptor) domain on the N-terminus of the NBS-LRR. Both the subsets, CC-NBS-LRR and TIR-NBS-LRR, are widely found in the R protein repertoire (Postel & Kemmerling 2009). It is fascinating that structures responsible for PAMP-recognition that are similar to these R proteins can be found in animal cells (Aderem & Ulevitch 2000; Nurnberger et al 2004). In animal cells these NBS-LRR proteins that recognize PAMPS carry caspase activity (NOD1 and NOD2) (Girardin et al 2002; Ting & Davis 2005), whereas NBS-LRR proteins in plant cells are responsible for recognizing their effector protein partners (Nurnberger et al 2004).

In this thesis also several models for the function resistance proteins against Avr-proteins have been discussed. First the Gene for Gene model was introduced. This model implies that there is an R protein for every Avr-protein. However this

model was confirmed by many Avr-R protein couples, a direct interaction between the two proteins was not shown (Dangl & Jones 2001). This taken together suggested that an indirect working mechanism is responsible for the perception of Avr proteins by R proteins. The Guard model was generated. The guard model predicts that R proteins protect the host proteins by monitoring their status in the cell. If the status is altered by the effector protein, by for example cleavage or phosphorylation, this is noticed by the R protein and via a signalling cascade the effector triggered immunity response is activated. This model explains how a relatively small amount of R proteins can protect the host cell against many effector proteins. Several effector proteins can have the same host target that is protected by one R protein (Chisholm et al 2006; Dangl & Jones 2001).

A decoy is a protein that recognizes the effector protein and thereby activating the R protein and thereafter the R protein activates the immune response. The decoy does not carry out its cellular function anymore. It is suggested that a decoy originates from two conflicting evolutionary processes. First of all, if the R protein is absence and the Avr protein is presence the target protein is driven to mutating its binding region of the effector protein to decrease the affinity. This is all to avoid recognition and destruction by the effector. But, when the R protein is present nature is expected to select the target proteins that do recognize the effector protein to promote an immune response. This whole process can result into target proteins, 'decoys', that no longer carry out their cellular function but do recognize the effector protein (van der Hoorn & Kamoun 2008).

In last couple of years several models have been proposed to explain the working mechanism of the R proteins. None of the models seemed to suit all the experimental data and can be seen as an addition on to each other. The gene-for-gene model still is correct if only considered the Avr-R protein couples. The guard model is correct, considered the guardians of host targets by R protein against the effector protein. And also the decoy model is correct if looked from an evolutionary perspective. However, the model that exclusively explains all the different mechanism that can be found thus far in the R protein-Avr detection still needs to be found. Obviously more research is needed to unravel the exact working mechanism of the R proteins and maybe there is not just one working mechanism but are several methods developed by plants to evade from bacteria.

Effectors alter processes in the cell so the bacterium can survive. Bacteria mainly alter cellular processes to suppress the defence system (Alfano & Collmer 2004). Viruses also alter the infected host cells. They have various strategies for it and also various functions of the host cell are being misused. Viruses transcribe proteins that alter for example the cellular protein turnover and those proteins also take care that the virus can use the transcription/translation machinery of the host cell (Flint 2004). The main difference between viral and bacterial effectors is that bacterial effectors mainly function as suppressors of the host immune system, while the viral proteins are used to alter the host cell in such way that the virus can replicate in the cell. This also shows the difference of viruses and bacteria; viruses are totally dependent for their survival on their host cells while bacteria are cell themselves and are otherwise dependent of the host, more via nutrients.

P. aeruginosa carries a type III secretion system that secretes four effector proteins. The effector proteins of *P. aeruginosa* are not necessary for infection but do enhance the severity and burden of disease. Although the exact function and mechanism of the effector proteins in disease is not clear, a model for the function of the effector proteins is suggested. For the model the acute pneumonia is used as a model disease. It is suggested that the effector proteins of *P. aeruginosa* affect the early immune response by delaying or impairing it. This is thought to be conducted by inhibiting the inflammasome related IL-1β and IL-18 production. However, eventually a break out of the immune reaction will occur, leading to the attraction macrophages and neutrophil to the infection site. However, those immune cells are not capable of eradicating the bacteria, but do cause severe damage in the lungs. It is implied that the neutrophils and macrophages are not capable in eradicating the bacterium because they are in some way impaired by the secreted effectors. The damage caused by the immune cells is used by the bacterium to further colonize the, in this case, the lungs (Hauser 2009).

It would be interesting to investigate whether plant resistance proteins can inhibit the infection of *P. aeruginosa* by targeting the effector proteins. However, plant resistance proteins do not target the effector protein directly but conduct their protective work by recognizing the host protein that is modulated by the bacterial effector. Thus, first it needs to be investigated whether the resistance proteins can also recognize cellular proteins. Thereafter research should be conducted to determine if the activation of the resistance protein is sufficient to initiate a protective immune response.

Clearly, the immune response of plants against bacterial effector proteins is very interesting. Nevertheless, more research is needed to fully unravel the effector triggered immunity. Especially on the area of mechanism, much remains to be found. It would also be interesting to determine whether resistance proteins can be used to overcome severe bacterial infections in humans. But first of all the exact working mechanism needs to be determined.

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