Chapter I: Introduction

1 Plant pathogen resistance

1.1 General mechanisms of plant pathogen resistance

Plants are haunted by various diseases caused by phytopathogenic fungi, bacteria, viruses, insects and nematodes. In agriculture, severe damage is especially caused by rust and mildew fungi, *Fusarium* spp., Barley yellow dwarf virus (BYDV) as well as cyst and root knot nematode species. Rice is mainly attacked by *Xanthomonas, Fusarium* spp. and *Magnaporthe* causing bacterial blight, root rot and stem rot disease. The lack of genetic diversity within the genomes of cultivated crop species as well as changes in cultivation techniques such as large-scale cropping of genetically uniform plants, reduced crop rotation and the expansion of crops into less suitable regions, resulted in an increasing susceptibility to different pests. For crops, the total global actual loss due to pests varies between about 26% in soybean and more than 40% in potato production (Table 1).

Table 1: Overall summary of the loss potential and the actual losses due to fungal and bacterial pathogens, viruses, animal pests and weeds in wheat, rice, maize, potatoes, soybean and cotton, in 2001-03. According to Oerke et al. 2006.

	Crop losses due to in %									
	Pathogens		Viruses		Animal pests		Weeds		Total	
	Potential	Actual	Potential	Actual	Potential	Actual	Potential	Actual	Potential	Actual
Wheat	15.6	10.2	2.5	2.4	8.7	7.9	23	7.7	49.8	28.2
Rice	13.5	10.8	1.7	1.4	24.7	15.1	37.1	10.2	77.0	37.4
Maize	9.4	8.5	2.9	2.7	15.9	9.6	40.3	10.5	68.5	31.2
Potatoes	21.2	14.5	8.1	6.6	15.3	10.9	30.2	8.3	74.9	40.3
Soybeans	11	8.9	1.4	1.2	10.7	8.8	37	7.5	60	26.3
Cotton	8.5	7.2	0.8	0.7	36.8	12.3	35	8.6	82	28.8

One of the most important ways of protecting plants against harmful organisms and of improving agricultural production is the use of plant protection products. The use of pesticides has increased dramatically since the early 1960s. Even though pesticides may provide a certain control level, their use may also involve risks and hazards for humans, animals and the environment. Despite crop protection, about 32%, 29% and 40% of attainable

maize, cotton and potatoe production is still lost to pests (Table 1). Therefore, breeding for disease resistance in plants is a promising alternative for controlling plant diseases.

To counter pathogen attacks plants have evolved sophisticated and multi-faceted defense mechanisms. In essence, two branches of the plant immune system do exist. The older, basal MAMP-/PAMP-triggered immunity (PTI) (Figure 1), that is reminiscent of innate immunity in vertebrates, uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs) (Figure 1). The second one, the effector-triggered immunity (ETI) (Figure 1) relying on resistance (R) proteins confers a pathogen-specific resistance that is often associated with a form of programmed cell death around the infection site termed the hypersensitive response (HR). PTI activates a MAP kinase signaling cascade and an extensive transcriptional reprogramming leading to downstream defense responses as production of reactive oxygen species, accumulation of phenolics, production of phytoalexins, papilla formation, induction of PR genes and callose deposition to reinforce the cell wall at sites of infection (Chisholm et al. 2006; Truman et al. 2007; Zipfel et al. 2004).
The best characterized szenario complementing our understanding of the plant response to PAMPs relates to flagellin, the major protein of flagella which is recognized by a receptor like protein kinase (RLK) from *Arabidopsis thaliana*, FLS2, carrying extracellular leucinerich repeats (LRRs), a transmembrane domain (TM) and a cytoplasmic serine/threonine protein kinase domain (Gomez-Gomez and Boller 2002). Other examples of MAMPs include lipopolysaccharides, fungal chitin, oomycete Pep-13 or heptaglucosides. Immune responses induced by the interaction of bacterial flagellin (elicitor) with the plasmamembrane-localized FLS2 receptor restrict the growth of the virulent *Pseudomonas syringae* pv. tomato strain DC3000, whereas *fls2* mutant plants are more susceptible to bacterial infection (Nürnberger et al. 2006). Sheen and colleagues identified a complete MAP kinase cascade and WRKY transcription factors that function downstream of flg22 perception (Kovtun et al. 2000; Tena et al. 2001). Even though this signaling machinery was identified in response to a bacterial PAMP, activation of defenses by WRKY overexpression decreased symptoms caused by both bacteria and fungi, indicating that the resistance mechanisms induced are not specific to bacteria (Asai et al. 2002). Interestingly, PAMP perception in animals is also predominantly mediated by pattern recognition receptors carrying extracellular LRR domains (Toll-like receptors, TLR) (Nürnberger et al. 2004).

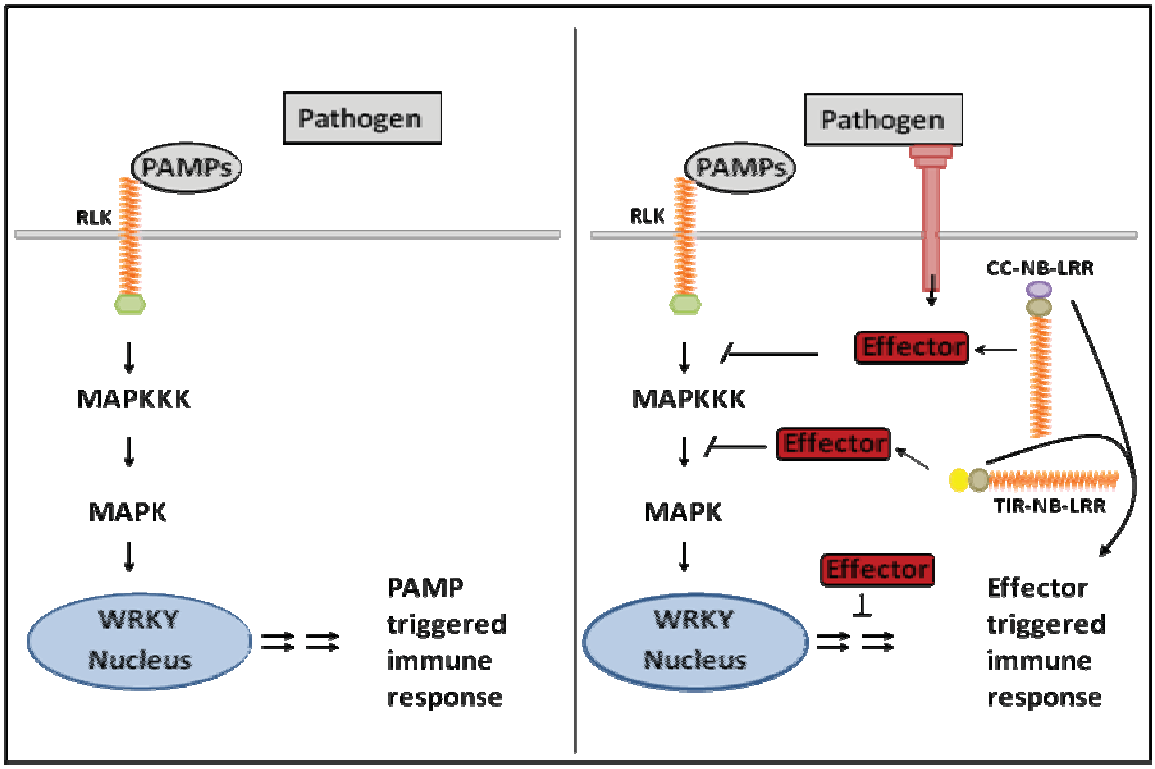

Figure 1: Simplified model for the evolution of resistance in plants according to Chisholm et al. 2006 and Bent 2007. Left to right, recognition of pathogen-associated molecular patterns (PAMPs) by extracellular receptor-like kinases (RLKs) triggers basal immunity, which requires signaling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors. Effector proteins target multiple host proteins and suppress basal immune responses. Plant R- proteins (CC-NB-LRR and TIR-NB-LRR) recognize effector activity and restore resistance through effector-triggered immune responses. kinase, LRR, TIR, NB, CC.

1.2 Plant susceptibility

Pathogens can overcome basal immune systems and colonize the plant successfully by the delivery of effector proteins into the plant cell, which can interfere directly with components of PTI or lead to changes in the transcritption of PTI genes (Li et al. 2005; Thilmony et al. 2006) resulting in effector-triggered susceptibility (ETS) (Murray et al. 2007; Truman et al. 2006) (Figure 1). Effectors, such as toxins and effector proteins, are virulence factors that interact with the host. Thus, the *P. syringae* effectors AvrPto and AvrRpt2 inhibit defense responses elicited by PAMP recognition (Hauck et al. 2003; Kim et al. 2005b). Several effector proteins from *P. syringae* pathovars are known to inhibit the HR localized to infection sites (Nomura et al. 2006). Fungal pathogens deliver their effectors; most of them are small proteins of unknown function containing a signal for secretion, via a specialized infection structure, the haustorium into the plant intercellular space (apoplast). Cyst nematodes secrete their parasitism proteins that often function in syncytium induction and

maintenance, through a stylet into the cytoplasma (Fuller et al. 2008). The secretions of interest originate from three pharyngeal (oesophageal) glands, one located dorsally and two subventrally (Lilley et al. 2005).

1.3 R-gene mediated resistance

Once the pathogen succeeded in suppressing the insufficient basal defenses, plants evolve R-proteins which directly or indirectly interact in a specific manner with microbial effector proteins and thereby trigger plant immune responses. This is referred to as ETI and is synonymous to pathogen race-plant cultivar-specific host resistance or gene-for-gene resistance (Jones et al. 2004, 2006) (Figure 1). The recognized effector is termed an avirulence (Avr) protein. Pathogens evolve further and suppress ETI, which again results in new R-gene specificities so that ETI can be triggered again (Jones et al. 2004, 2006).
To date numerous R-genes have been cloned which confer resistance to several classes of pathogens, including viruses, bacteria, fungi, oomycetes, insects, and even nematodes. R-gene products can be categorized into two main classes based on conserved structural features (Dangl et al. 2001; Chisholm et al. 2006). The largest class of R-proteins possessess, in addition to a leucine-rich repeat (LRR) domain implicated in signal perception, a central nucleotide binding site (NBS) domain shared by plant disease R-proteins, mammalian NLR (NOD-like receptor or CATERPILLER) proteins, and animal apoptotic proteins, such as mammalian Apaf-1 and C. elegans CED-4 (Chisholm et al. 2006; DeYoung and Innes 2006; Jones and Dangl 2006; Ting et al. 2005). There is considerable evidence that plant and animal innate immune systems are conserved as a consequence of convergent evolution suggesting that common signaling events are the basis of defense cascades (Palma et al. 2007; Afza et al. 2008). In mammals, two families of soluble pathogen recognition proteins (PRRs) called NOD1 and NOD2 are intracellular sensors of pathogenicity that recognize molecules derived from pathogens as well as from the host itself (Inohara et al. 2002; Ryan et al. 2007). The NBS-LRR class of R-proteins is further subdivided into coiled-coil (CC) NB-LRR and Toll-interleukin-1 receptor (TIR) NB-LRR according to their amino-terminal domain (Burch-Smith et al. 2007).

LRR domains are located at the carboxy termini of plant NBS-LRR R-proteins and are composed of tandem LRRs, thought to be involved in effector binding and maintenance of regulatory functions (DeYoung et al. 2006). The NBS domain (also called the NB, NB-ARC, Nod or NACHT domain) contains blocks of sequence that are conserved in both plant and

animal proteins. Those include the nucleotide-binding kinase 1a or P-loop and kinase 2 motifs (also called Walker's A and B boxes) and the kinase 3a motif, as well as several blocks of conserved motifs of unknown function (RNBS-A, RNBS-C, GLPL, RNBS-D and MHD) (Traut et al.1994; Aravind et al. 1999). ATP binding coordionnating by the histidine residue of the MHD motif is necessary for signaling in plant NBS-LRR R-proteins because binding of ATP initiates a conformational change in plant NBS-LRR proteins, resulting in their activation. The amino-terminal domain also seems to mediate the physical association between R-proteins and pathogen effector targets, at least for those R-proteins that use an indirect recognition mechanism.

A second major class of R-genes encodes extracellular LRR (eLRR) proteins. Three subclasses of eLRRs have been classified according to their domain structures (Fritz Laylin et al. 2005). These subclasses include RLP (receptor-like proteins; extracellular LRR and TM domain, RLK (extracellular LRR, TM domain, and cytoplasmic kinase) and PGIP (polygalacturonaseinhibiting protein; cell wall LRR). RLPs, for example, are represented by the tomato *Cf* genes, which confer resistance to infection by the biotrophic leaf-mold pathogen *C. fulvum* carrying the elicitors Avr2, Avr4, and Avr9 (Jones et al. 1994). The nematode resistance gene $Hs1^{pro-1}$ from sugar beet (Cai et al. 1997) encodes for a protein that forms a LRR-TM structure as well.

Many R-genes are located in clusters that comprise several copies of homologous R-gene sequences arising from a single gene family (simple clusters) or colocalized R-gene sequences derived from two or more unrelated families (complex clusters). Intergenic unequal crossover has the potential to place R-genes in new structural contexts that may alter expression, whereas intragenic mispairing generates chimeric genes that may encode novel functions. In the absence of pathogen pressure, recombination and transposon activity at R-gene clusters are expected to be inhibited presumably by chromatin modification. This is also described by the Birth and Death Model (Michelmore and Meyers 1998). Although a very limited number of R-proteins are functionally characterized in detail there is now evidence that plants use both direct and indirect mechanisms of pathogen detection (DeYoung et al. 2006).

Although there is evidence that some plant NBS-LRR R-proteins have been under diversifying selection, the direct detection hypothesis for pathogen recognition fails to explain how a relatively limited number of plant R-proteins can specifically recognize the vast diversity of potential pathogens and their effectors. Not only this apparent disparity but also the lack of substantial evidence for direct Avr-R-protein interaction led to the 'guard hypothesis' (Van der Biezen and Jones 1998), which proposes that the Avr-protein induces a

change in a host protein that is normally recruited by the pathogen via its Avr-protein to establish a successful infection, and that this change sensed by the R-protein (guard) leads to the activation of the R-protein and subsequent defense signaling (Dangl and Jones et al. 2001; Bent and Mackey 2007; van der Hoorn 2008). For instance, in rice, Xa-21 protein requires XB3 a ubiquitin ligase that is phosphorylated by Xa-21 for complete *Xa-21*-mediated disease resistance (Wang et al. 2006). In Arabidopsis, the host protein RIN4 is structurally modified by Avr elicitors AvrRpm1 or AvrB from the bacterial pathogen *P. syringae* which in turn leads to the activation of *RPM1*-mediated resistance (Axtell and Staskawicz 2003; Mackey et al. 2002; Kim et al. 2005b; Coaker et al. 2005; Day et al. 2005). This model may provide a good explanation for resistance response networks triggered by other R-genes which for example has been proven for the $Hs1^{pro-1}$ -mediated nematode resistance. Additional support for the guard hypothesis comes from the tomato protein *Prf* involved in the indirect detection of *P. syringae* effectors AvrPto and AvrPtoB (Tang et al. 1999; Xiao et al. 2003).

As it is generally known, a large number of sequences with similarity to R-genes exist in plant genomes, which are referred to as RGAs (resistance gene analogs, Leister et al. 1996). The common motifs within the NBS domain are sufficient for PCR amplification of resistance gene analogs (RGAs) from a wide variety of plant species using degenerated primers, for example from soybean (Kanazin et al. 1996), potato (Leister et al. 1996), lettuce (Meyers et al. 1999), cereals (Pan et al. 2000), sugar beet (Tian et al. 2004), rape (Tanhuanpaa 2004) and cotton (He et al. 2004). The cloned RGAs have been found to cluster in plant genomes and some are located in close genetic distance to known resistance loci thus suggesting their possible role in disease resistance response in plants (Kanazin et al. 1996; Collins et al. 1998; Aarts et al. 1998; Ashfield et al. 2003; Radwan et al. 2005). RGAs not only provide a source for new R-gene species, but also represent candidates for putative interacting partners for functional R-genes according to the guard hypothesis described above. Van Hoorn (2008) describes an improved system of plant-pathogen interactions, the Decoy Model. In the absence of a functional R-gene, natural selection is expected to drive the guardee to decrease its binding affinity to the effector. However, in the presence of a functional R-gene, natural selection is expected to favor guardees with improved interaction with an effector to enhance pathogen perception. These two conflicting selection pressures result in an evolutionarily unstable situation that could be relaxed upon the evolution of a host protein, termed here "decoy," that specializes in perception of the effector by the R-protein but itself has no function either in the development of disease or resistance.

1.4 Non-host resistance

Potentially phytopathogenic microorganisms incapable of infecting any cultivar of a given plant species are referred to as heterologous pathogens, while plants that are resistant to all isolates of a given pathogen species are called non-host plants (heterologous plant–microbe interaction; basic incompatibility) (Nürnberger et al. 2005). Preformed physical or chemical barriers (passive defense mechanisms) at the plant surface, such as wax layers, cell walls, antimicrobial compounds and other secondary metabolites are the first obstacle a pathogen faces before invading the plant. The second obstacle is the inducible plant defense response (active defense mechanisms), such as de novo synthesis of phytoalexins, antimicrobial reactive oxygen species or several signaling components as well as localized reinforcement of the plant cell wall and programmed cell death (Thordal-Christensen et al. 2003; Nürnberger et al. 2005).

Mysore (2004) proposes that non-host resistance against bacteria, fungi and oomycetes can be classified into two types. During type I non-host resistance no visible symptoms occur and multiplication and penetration of the pathogen into the plant cell is completely abolished. In contrast, within the type II non-host resistance, that is always associated with a HR and is phenotypically more similar to an incompatible gene-for-gene interaction, an elicitor is recognized by the plant and a defense reaction is activated.

Non-host resistance in Arabidopsis against the non-adapted barley pathogen, *B. graminis* f. sp. *hordei* (Bgh) normally involves the rapid production of cell wall appositions (physical barriers) and antimicrobial metabolites at the site of pathogen entry, but no HR. Arabidopsis penetration mutants PEN1 (syntaxin) (Collins et al. 2003), PEN2 (peroxisomal glucosyl hydrolase) (Lipka et al. 2005) and PEN3 (plasma membrane ABC transporter) (Stein et al. 2006) are partially compromised in this response suggesting that cell wall structures play an important role as physical barriers. Syntaxins belong to the superfamily of SNARE (soluble N-ethylmaleimide- sensitive fusion protein attachment protein receptor) proteins representing key mediators of membrane fusion events in yeast and animal cells (Nürnberger et al. 2005). Even though significant similarities exist between non-host and gene-for-gene resistance such as HR, production of reactive oxygen species (ROS), lignification and ubiquitin ligase-associated protein SGT1, there are also differences between the two. Recent studies suggest that non-host cell death requires caspase-like activity (Christopher-Kozjan and Heath 2003). In these experiments, two caspase inhibitors significantly impaired cell death kinetics exclusively in several non-host combinations, but not in incompatible host interactions. Resistance conferred by single dominant R-genes is specific to a particular pathogen race that

can express the corresponding Avr-gene(s). Pathogen Avr-genes can be easily mutated or eliminated and hence protection conferred by R-genes is not durable. By contrast, non-host resistance can be more durable (Mysore et al. 2004).

2 Plant resistance responses

2.1 Early recognition events

When a plant and a pathogen come into contact, close interaction occur, whereby the plant is able to recognize the invading pathogen and to initiate defenses, while successful pathogens cause disease by suppressing host defense (Hammond-Kosack et al. 1997). In the simplest interaction plants contain dominant R-genes that specifically recognize the corresponding Avr-gene within the pathogen in a direct or indirect manner. Specific recognition results in the induction of signaling cascades and defense gene expression. The activation of plant defense leads to immediate responses at the point of infection that include protein phosphorylation, ion fluxes across the plasma membrane, ROS production, nitric oxide (NO), in some cases a HR and accumulation of phenolic compounds (Garcia Brugger et al. 2006; McDowell et al. 2003). The activation of MAPK families but also other PKs like CDPKs (calmodulin (CaM)-like domain protein kinases) is one of the earliest induced events after elicitor perception (Garcia Brugger et al. 2006), but also serves to mediate interaction between pathways (Rojo et al. 2003).
Next to local tissue responses, there are also systemic responses as the synthesis of pathogenesis-related (PR) proteins, accumulation of phytohormones, and cell wall strengthening, that prime uninfected parts of the plant against potential pathogen attack (systemic acquired resistance, SAR) in a long lasting and effective manner.

2.2 Signaling components

Both PTI and ETI are controlled by a complex signaling network that includes three major endogenous signals, the hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (De Vos et al. 2005; Bodenhausen et al. 2007). The ET and JA-dependent defense responses seem to be activated by necrotrophic pathogens such as *Alternaria*, *Botrytis*, *Septoria*, *Phytium*, *Erwinia*, *Plectosphaerella*, whereas the SA-dependent response is triggered by viruses like tobacco mosaic virus (TMV) and biotrophic bacteria and fungi such as *Pseudomonas*, *Peronospora*, *Erisyphe* and nematodes (Thomma et al. 1998, 2002; Rojo et al. 2003) (Figure 2). Most of the studies indicate that ET or JA and SA responses inhibit each other suggesting that events of cross-talk among the pathways exist (Spoel et al. 2003;

Pieterse et al. 2001). But also cases of synergistic interactions between SA and JA or ET in defense responses to pathogens have been reported (Rojo et al. 2003).

Elicitor perception is often followed rapidly by a Ca^{2+} influx and intracellular Ca^{2+} signaling as well as anion effluxes that initiate plasma membrane depolarization which, in turn, activates voltage-dependent Ca^{2+} channels (Romeis et al. 2001; Sanders et al. 2002). Modifications of plasma membrane potential allow signal integration triggering events as oxidative burst and MAPK activation (Ward et al. 1995; Garcia Brugger et al. 2006). ROS, highly reactive and toxic oxygen species, such as superoxide anion (O_2 •–), hydroperoxyl radical (HO_2 •), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) are produced by plant cells because of the enhanced enzymatic activities of plasma-membrane-bound NADPH oxidases, cell-wallbound peroxidases and oxidases in the apoplast (Laloi et al. 2004). In a wide range of incompatible plant–pathogen interactions a biphasic ROS production has been observed, with a first phase peaking after 20 min and a second phase occurring 4 to 6 h later (Lamb and Dixon 1997; Laloi et al. 2004). ROS are thought to be general cell death effectors; they play an important role in modification of the cellular redox state, activation of MAPK as well as in reinforcing plant cell walls via oxidative cross-linking and increasing lignifications (Kawasaki et al. 2006; Laloi et al. 2004). In plants, the redox state regulates NPR1 (NON-EXPRESSOR OF PR1), an essential regulator of SAR (Figure 2). NPR1 accumulates in the cytosol as an inactive oligomer maintained by disulfide bridges. During a SAR response, its reduction releases monomeric units that accumulates in the nucleus and interact with the reduced TGA1 (TGACG-sequence-specific binding-protein1) transcription factor which activates the SA-dependent defense gene expression (Mou et al. 2003; Laloi et al. 2004). In a simplified model, two different R-gene-mediated signaling pathways have been described in *Arabidopsis thaliana* (Hammond-Kosack et al. 2003). The first one involves the TIR-NBS-LRR type of R-genes (e.g. *RPP1* and *RPP5*) and requires *EDS1* (Enhanced Disease Susceptibility) and *PAD4* (Phytoalexin Deficient) function to attain full resistance. The second one involves the CC-NBS- LRR type of R-genes (e.g. *RPM1* and *RPS2*) and requires functional *NDR1* (Non-race specific Disease Resistance), *RAR1* and *SGT1* (Figure 2).