CHAPTER1

Introduction

This work combines approaches of ecology, evolutionary biology and immunology to study the plant immune system. Evolutionary biology describes the heritable change in the genetic structure of successive generations [Thompson, 2008]. Investigating the evolution of the immune system as one of many adaptive traits means to reconstruct the changes of the immune system over time. Analyzing immunity from an ecology point of view, requires to study the present distribution and abundance of organisms and their interactions with their environment, including parasites and pathogens. Immunology, moreover, addresses the physiological functioning of immunity in case of both health and disease [Schulenburg et al., 2009]. Attempts to unify these strategies are scarce, although this combination of approaches may allow to explain physiological variation by its molecular causes and integrate the trait of interest into the adaptive fitness landscape of the organism. The adaptive landscape of an organism consists of multiple complex traits, which may interfere and are shaped by the environment [Fisher, 1930; Orr, 2005]. Therefore immune responses may only be plausibly explained by considering the organism's physiology in its ecological context [de Meaux and Mitchell-Olds, 2003]. Using this integrated approach, variation in immune responses will be investigated and factors described which may contribute to this variation.

1.1. Molecular basis of plant-pathogen interaction

1.1.1. PAMP-triggered immunity

Plants employ a sophisticated immune system which detects and defends pathogens in order to enhance the host's probability of survival and reproduction. Beyond physical barriers such as waxy layers and the cell wall, reliable recognition of potential pathogens is the first crucial

step for successful defense. Molecular signatures of self or non-self are distinguished by pattern recognition receptors (PRR) [Boller, 2005]. PRRs are membrane-bound proteins, often composed of an extracellular leucine-rich repeat (LRR) domain and an intracellular kinase domain. Due to these properties they are referred to as LRR-receptor kinases (LRR-RK). PRRs detect pathogen-associated molecular patterns (PAMPs) which are structural components that are typical of whole classes of microbial pathogens, i.e. fungal chitin or bacterial peptidoglycan and flagellin. These molecular signatures are perceived by invertebrates, vertebrates and plants using structural similar perception systems [Ausubel, 2005; Nürnberger et al., 2004; Zipfel and Felix, 2005].

The detection of PAMPs by PRRs results in PAMP-triggered immunity (PTI). PTI comprises a multitude of immune responses which can be grouped according to their time of occurrence [Boller and Felix, 2009]: immediate signaling and very early immune responses occur within 1 min to 5 min upon PAMP perception and include the alteration of ion flux at the plasma membrane, oxidative burst, activation of mitogen-associated protein kinase (MAPK) cascades and changes in protein phosphorylation [Bauer et al., 2001; Nühse et al., 2000]. Early immune responses are ethylene biosynthesis, salicylic acid accumulation, receptor endocytosis and transcriptional reprogramming [Bauer et al., 2001; Navarro et al., 2004; Robatzek et al., 2006] and occur within 5 min to 30 min. The latest responses, which affect the organism for hours to days, result in callose deposition at the cell wall, accumulation of antimicrobial metabolites and inhibition of seedling growth [Gómez-Gómez et al., 1999; Zasloff, 2002]. Although particular contributions of individual PAMP-triggered defense responses are unknown, sufficient disease resistance is established to allow survival despite constant microbial challenge.

1.1.2. Bacterial countermeasures and effector-triggered immunity

Some microbes, however, evolved strategies to evade or counteract PTI. Adapted pathogens evade recognition by PRRs due to altered PAMP structures, a process called camouflage. The causative agent of tomato wilt, *Ralstonia solanacearum*, as well as *Agrobacterium tumefaciens*, the cause of crown gall disease, exhibit markedly altered flagellin sequences which do not elicit the plant immune response [Felix et al., 1999; Pfund et al., 2004]. A second strategy to interfere PAMP-responses is the perturbation of signaling cascades or subsequent immune reactions within the host. Pathogenic bacteria such as *Pseudomonas syringae* inject effector molecules by their type III secretion system directly into the host's cytoplasm [Hueck, 1998] which disturb PAMP-triggered immunity. The microbial effector AvrPto and AvrPtoB delivered by *Pseudomonas syringae* pv. *tomato* DC3000 directly interfere with PRR function [Göhre et al., 2008; Shan et al., 2008] while the effector HopAI1 irreversibly blocks PRR signaling by

dephosphorylation of the mitogen-activated protein kinases MPK3 and MPK6 which prevents callose accumulation at the cell wall [Li et al., 2005; Zhang et al., 2007]. A third strategy, again presented by *P. syringae*, is the delivery of toxins into the plant cell. Coronatine, for instance, mimics jasmonate and counteracts PAMP-induced reduction of stomatal aperture which facilitates bacterial entry [Melotto et al., 2006].

The host plant in turn counteracts pathogen delivered effectors by a second surveillance system leading to effector-triggered immunity (ETI). This system includes the action of intracellular resistance or *R* genes. These *R* genes often encode nucleotide binding-leucine rich repeat proteins (NB-LRRs) which recognize pathogen-specific effector molecules. Theoretically this interaction leads to an arms race scenario with highly specific defense and pathogen infection systems, respectively [Chisholm et al., 2006]. Recent progress has been made to unravel molecular mechanisms of the interaction of *R* gene products and their cognate microbial effectors [Dodds et al., 1993; van der Hoorn and Kamoun, 2008].

1.1.3. Examples of *R* gene evolution

A well characterized example of *R* gene and effector gene coevolution is the gene-for-gene interaction of the downy mildew effector ATR13 and its cognate *R* gene product RPP13 in *A. thaliana* [Allen et al., 2004]. Both loci encode an extreme amino acid sequence diversity which is maintained in the respective population by frequency-dependent (or balancing) selection. Selection at the host locus RPP13 is driven by the rapid evolution of the oomycete effector ATR13. The pathogen attempts to escape recognition by PRR13 and alters presented effector molecules. The host in turn counteracts by adjustment of receptor function to preserve its ATR13 recognition ability. Obviously this interaction between the two loci leads to the stable maintenance of diverse *R* gene and effector alleles, respectively. This cycle of attack and defense evolution may also provoke adaptive divergence, meaning the consecutive emergence of new effector and receptor variants [Allen et al., 2004].

Other *R* gene loci have maintained different resistant and susceptible alleles, for instance *Cf-2* in *Solanum pimpinellifolium* [Caicedo and Schaal, 2004] presents such a presence/absence polymorphism. Shen et al. [2006] analyzed nine *R* gene loci for their presence/absence of the respective *R* gene and suggested that balancing selection acts at those loci. The *R* gene *RPS5* exhibits a presence/absence polymorphism in the population of *A. thaliana* [Tian et al., 2002]. RPS5 presence/absence has been analyzed for consequences on disease development and host fitness by Gao et al. [2009]. Plants gained a fitness benefit from RPS5-mediated resistance when exposed to the pathogen *P. syringae*. The benefit of RPS5-mediated immunity must be higher than its associated costs (estimated to 8%) to keep the functional allele present in the genome.

Complex selective forces but no clear evolutionary scenario could be outlined for *RPW8*, a disease resistance locus conferring broad-spectrum resistance against powdery mildew in *A. thaliana* [Orgil et al., 2007]. Ancient diversity at two homologous genes could be related to disease phenotypes and single mutations identified that confer phenotypic variations. However, the high average nucleotide diversity opposed the scenario of a putative selective sweep. Moreover, expression of RPW8 incurs fitness benefits and costs on *A. thaliana* in the presence and absence of the pathogens, respectively, which overall contribute to the complex evolutionary scenario without clear traces of selection.

1.2. Recognition of bacterial PAMPs

1.2.1. Bacterial flagellin

Bacterial flagellin is among the best-studied PAMPs in plants and animals and its perception systems are well characterized [Gómez-Gómez and Boller, 2000; Hayashi et al., 2001]. It is the main building block of the flagellum, the motility organ of bacteria. Up to 30,000 flagellin subunits assemble the flagellum which renders flagellin as one of the most ubiquitous and abundant proteins in bacterial fractions [Delmotte et al., 2009]. The flagellin protein consists of several domains which are either exposed to the environment and are therefore structurally more diverse or form the inner tube of the flagellum. The latter contribute to protein interactions between flagellin units and are functionally more constrained [Beatson et al., 2006].

Sun et al. [2006] demonstrated intra- and interstrain variation for flagellin in *Xanthomonas campestris* pv. *campestris*. This variation allows some strains of *Xanthomonas campestris* pv. *campestris* to evade detection by plants. A correlation of increased pathogen virulence and flagellin variants could not be confirmed in isogenic *Xanthomonas campestris* pv. *campestris* lines expressing the detected or nondetected flagellin. The ecological relevance of flagellin variants in natural *Xanthomonas campestris* pv. *campestris* populations residing on *A. thaliana* remains to be demonstrated. Yet, the causative agent of tomato wilt, *Ralstonia solanacearum*, as well as *Agrobacterium tumefaciens*, the cause of crown gall disease, exhibits markedly altered flagellin sequences compared to flg22. Furthermore, several post-translational modifications of flagellin such as glycosylation [Moens et al., 1995], phosphorylation [Kelly-Wintenberg et al., 1993], methylation [Ambler and Rees, 1959], and sulfatation [Wieland et al., 1985] have been reported. Glycosylation of flagellin of *Pseudomonas syringae* pv. *tabaci* 6605 contributes to variation in elicited immune responses in *Nicotiana tabacum*. The post-translational modification reduces the dissociation of flagellin units within the flagellum which presumably helps to evade detection by the host Taguchi et al. [2009].

1.2.2. FLS2-mediated perception of flagellin

All higher plants perceive a highly conserved domain at the N-terminus of bacterial flagellin [Beatson et al., 2006; Felix et al., 1999]. The derived consensus sequence of this plant-sensed domain is a 22-amino-acid peptide, referred to as flg22, which acts as a potent elicitor of immune responses at subnanomolar concentrations [Felix et al., 1999]. Flg22 is perceived by FLAGELLIN SENSING 2 (FLS2) which has been extensively studied in *A. thaliana* and therefore is the currently best characterized PRR in plants. The LRR receptor kinase FLS2 triggers a plethora of short- to long-term PTI responses and subsequently restricts pathogen growth [Zipfel et al., 2004]. *FLS2* is a single copy gene in *A. thaliana* and null-mutants do not bind flg22 resulting in an enhanced disease susceptibility [Zipfel et al., 2004]. FLS2 function upon flg22 binding requires complex formation with its co-receptor BRI1-associated receptor kinase 1 (BAK1), a LRR receptor kinase which also plays a role in brassinosteroid signaling [Chinchilla et al., 2007].

Species-specific differences in flagellin perception were documented in cell cultures of different plant species. The flg22 peptide variant flg15 was nearly as active as flg22 in tomato but exhibited an approximately 100-fold lower activity than flg22 in *A. thaliana*. Furthermore, tomato and *A. thaliana* perceive slightly different parts within the flg22 epitope [Chinchilla et al., 2006]. Moreover, flagellin perception induces typical PAMP triggered responses in *A. thaliana* but surprisingly provokes local cell death (hypersensitive response) in tobacco which is usually a characteristic of effector triggered immunity [Takemoto et al., 2005].

1.2.3. Comparison of flagellin perception in plants and mammals

In mammals, flagellin perception is mediated by the Toll-like receptors 5 which directly binds monomeric flagellin. The membrane localized receptor perceives a highly conserved motif within the D1 domain of flagellin [Andersen-Nissen et al., 2005], a site which is structurally distinct from the epitope recognized by FLS2 [Felix et al., 1999; Smith et al., 2003]. This illustrates convergent evolution of the same principle and emphasizes the evolutionary and ecological relevance of flagellin perception. However, mammals additionally possess a cytosolic flagellin receptor, IPAF (pro-caspase-1-activating protein), which belongs to the class of NOD-like receptors [Miao et al., 2007]. Whether plants also perceive flagellin through an additional cytosolic recognition system remains to be investigated.

1.2.4. EFR-mediated perception of EF-Tu

A second well characterized PRR in plants is EFR (EF-Tu receptor) which recognizes the bacterial elongation factor EF-Tu [Zipfel et al., 2006]. The epitope perceived by EFR is a 18 amino acid peptide, referred to as elf18. EFR belongs to the XII subfamily of LRR receptor like kinases that also includes FLS2 [Shiu and Bleecker, 2001]. Both proteins are structurally highly related and trigger almost identical immune responses upon perception of their respective ligands flg22 and elf18 which suggests the employment of common signaling components. The evolution of FLS2 and EFR, however, followed distinct paths. Flagellin perception is common in all major groups of higher plants [Boller and Felix, 2009] suggesting that a PRR for bacterial flagellin is evolutionarily ancient. Indeed, homologs of FLS2 have been investigated in tomato, tobacco and rice [Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008] and share a high degree of amino acid conservation [Boller and Felix, 2009]. In contrast, perception of EF-Tu is limited to the Brassicaceae [Kunze et al., 2004; Zipfel et al., 2006], presenting an evolutionary novelty. Transient expression of EFR in *N. benthamiana* introduced responsiveness to elf18 indicating that downstream elements of PRR activation are conserved in *A. thaliana* and *N. benthamiana* [Boller and Felix, 2009; Zipfel et al., 2006].

1.3. The evolutionary ecology of immunity

The arena of natural infection is highly variable [Lazzaro and Little, 2009]: parasite epidemics vary geographically and temporally, abiotic factors such as temperature and availability of nutritions may highly fluctuate. The outbreak of disease, therefore, does not solely depend on the host's and pathogen's genetics but also on their environment including all interactions among the three [Barrett et al., 2009; Thompson, 2005]. As a consequence, biotic and abiotic ecological factors can potentially mold the evolution of immune systems. Thus, the full array of demands affecting the optimization of immune responses need to be considered to explain patterns of variation in immune responses [Schulenburg et al., 2009; Sheldon and Verhulst, 1996; Van Valen, 1973].

Given the importance of the immune system for Darwinian fitness, selection should favor hosts with a high potential to prevent or limit infections and purge all susceptibility alleles. Still, variability in immune function is maintained and susceptibility observed in different host-pathogen interactions and environmental settings [Barrett et al., 2009; Lazzaro and Little, 2009; Sadd and Schmid-Hempel, 2009]. Ecological immunology studies invoke two scenarios to explain variation in immune responses in natural populations: specificity in host-pathogen interactions and costs of immunity [Jokela et al., 2000; Schmid-Hempel and Ebert, 2003].