Innate immunity mediated by the flagellin receptor FLS2 in Arabidopsis and tomato: A molecular approach to characterize ligand binding and function, using receptor chimeras

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Summary

Flagellin, the major subunit of the bacterial motility organ flagellum is an archetypical elicitor molecule perceived by a variety of plant species (Felix et al., 1999). Flg22, a synthetic peptide comprising the highly conserved amino acid residues of the flagellin N-terminus, has been shown to be the active epitope of flagellin which is recognized by plants and sufficient to activate plant defense responses (Felix et al., 1999). Flagellin/flg22 recognition has been attributed to a single protein, FLS2. FLS2 is a leucine rich repeat (LRR) receptor like kinase (RLK), consisting of 28 extracellular LRRs, a single transmembrane domain and an intracellular ser/thr kinase domain, was first identified in the model plant *Arabidopsis thaliana* (Gómez-Gómez and Boller, 2000) and shown to directly bind flg22 (Chinchilla et al., 2006). Meanwhile, orthologues of FLS2 have been identified in a variety of species from different families, among them tomato (*Lycopersicon esculentum*), *Nicotiana benthamiana*, *Ricinus communis* and *Populus trichocarpa*, to name just a few (Robatzek et al., 2007b). Although all these plants recognize flg22 as an elicitor, distinct species specific differences were identified. In this work, the molecular differences between the flagellin recognition systems of Arabidopsis (AtFLS2) and tomato (LeFLS2) are analyzed in depth. It was shown that full length flg22 is required for activity in *Arabidopsis* while tomato requires only the 15 aa peptide flg15 for full stimulation of defense responses (Meindl et al., 2000). Receptor activation of FLS2 by flg22 occurs according to the address-message concept with binding of the address as a first step, and message-induced receptor activation as a second step (Meindl et al., 2000). By using a variety of flg22-derivatives, we analyze how Arabidopsis and tomato flagellin receptors discriminate between different variants of flg22 in terms of binding and receptor activation. By using the species specific differences of Arabidopsis and tomato flagellin perception, we identify areas within the LRR domain of the respective flagellin receptors which are responsible for interaction with the ligand. To achieve this, we constructed a series of chimeric receptors by swapping different parts of the LRR domain from LeFLS into the AtFLS2 sequence. These chimeric receptors were transformed into *Nicotiana benthamiana* and *Arabidopsis thaliana* and the transformed plants were tested for receptor function using various bioassays such as ethylene production and growth inhibition and we performed binding assays using immunoprecipitated receptors. Based on these experiments we show that the LeLRR 1 to 10 are sufficient to bind the minimum peptide flg15-Δ7, the shortest flg22-derivative perceived by tomato consisting only of the central 8 amino acids of flg22. We

show that the initial ten N-terminal LRRs between the amino acids 32-337 and within this area, especially the amino acids 236-337 are import for the higher affinity of LeFLS2 to flg22 and N-terminally truncated flg22-derivatives. We further show that an additional region between the LRR 19 to 24 of LeFLS2 is involved in the recognition of the C-terminus of flg22. Because the C-terminus of flg22 has been shown to be part of the “message”, which activates receptor signaling (Meindl et al., 2000; Chinchilla et al., 2006), we propose the region of LRR 19 to 24 to be play an important role for activation of FLS2. Additionally a chimeric receptor between AtFLS2 and LeFLS2 is presented which shows the characteristics of a constitutive active FLS2 allele when transformed into Arabidopsis. Interestingly, the constitutive signaling of this chimeric receptor can only be triggered via the artificial extracellular LRR domain, since the complete intracellular receptor part, e.g. transmembrane- juxtamembrane- and kinase-domain is not affected from the LRR domain swapping. Together, this study provides new insight towards the understanding of FLS2-ligand interaction and an interesting tool to further study receptor activation.

INNATE IMMUNITY MEDIATED BY THE FLAGELLIN RECEPTOR FLS2 IN ARABIDOPSIS AND TOMATO: A MOLECULAR APPROACH TO CHARACTERIZE LIGAND BINDING AND FUNCTION, USING RECEPTOR CHIMERAS

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General Introduction

In nature, all organisms are in constant contact with their abiotic and biotic environment. A vast array of potentially pathogenic bacteria, fungi, oomycetes and viruses are ongoingly probing the defense condition of all free living organisms. However, despite a plethora of threats, disease is surprisingly not the rule, but rather the exception and most organisms are resistant to most pathogens.

Besides physical barriers, complex molecular processes determine if an organism is a potential host or non-host for a certain pathogen. In a ongoing arms-race, the attacked organism is trying to mount defense strategies against the attacker while the attacker tries to avoid or actively neutralize the defense reactions encountered. Perception of and defense against pathogens by any species is the outcome of highly coordinated and sophisticated immune networks. In the end, the result will determine about the host or non-host status of the attacked organism. Mechanisms to protect higher eukaryotic organisms from microbial infections are generally termed “immunity”. For all organisms, there are three cornerstones of immunity: i) - Detection of the pathogenic organism, e.g. perception of a specific signal, ii) - Transduction of the perceived “danger” message across the membrane and onset of appropriate signaling, and iii) - Initiation of various defense-related responses. If any of these steps fail to take place, the attacked organism is not able to control the invading pathogen, which inevitably results in developing disease.

Concepts of immunity

Immunity is classified into two general systems: the “innate” or “natural” immune system, found in all classes of plant, animal and fungal life and the “acquired” or “adaptive” immune system, found only in higher vertebrates.

Principles of innate immunity

Innate immunity is thought to be an evolutionarily older defense strategy than adaptive immunity. Innate immunity does not confer a long lasting or protective immunity of the host. However, in vertebrates, the innate immune system plays an important role for the activation of the adaptive immunity through a process called “antigen presentation” (Fearon and Locksley, 1996).
In contrast to the adaptive immune system, innate immunity does not depend on specialized cells, but can be triggered by all cells in an autonomous manner. This allows a faster activation of defense reactions when the organism is confronted with previously unmet threats. Forms of innate immune systems are found in all classes of plant and animal life. Non-vertebrate animals, plants and fungi entirely rely on this concept of defense. Receptor-proteins of the innate immune system are encoded in the germ line. The receptor-genes do not undergo a rearrangement process like the immunoglobulin or T-cell receptors (TCR) genes of the adaptive immune system.

Due to the germ line fixed nature of the receptor-genes, the variability of perception systems used by the innate immune system is much lower than the variability seen in the adaptive immune system of vertebrates. Receptors of both the animal and plant innate immune system recognize molecular structures called MAMPs (Microbe-Associated-Molecular-Patterns) or PAMPs (Pathogen-Associated-Molecular-Patterns). MAMPs are characteristically invariant among entire classes of microbes and essential for the survival of the pathogen. Therefore, they are evolutionarily not easily exchangeable. Additionally, MAMPS have to be distinguishable from "self" in order to enable the host to differentiate between “non-infectious self” and “infectious non-self”. MAMPS are perceived via so called pattern recognition receptors (PRRs). PRRs can be located at the cell surface or intracellularly (Palm and Medzhitov, 2009). Upon MAMP perception, PRRs trigger antimicrobial responses of the host cell by activating a multitude of intracellular signaling pathways. Several classes of MAMPs are recognized by both plants and animals alike, for example bacterial flagellin, lipopolysaccharide of gram-negative bacteria and fungal chitin (Boller and Felix, 2009; Palm and Medzhitov, 2009).

Principles of adaptive immunity

Adaptive immunity is thought to be by far the younger evolutionary concept and can be found only in jawed vertebrates (Gnathostoma). Hallmark of adaptive immunity is an extremely high adaptability to counter invading pathogens. Adaptive immune responses are carried out on the basis of highly specialized white blood cells, the T- and B-lymphocytes. The two cell types provide two different classes of responses. B-cells, responsible for antibody mediated responses, produce on the basis of recombination (Schatz, 2004) and somatic hypermutation (Odegard and Schatz, 2006) a virtually unlimited number of immunoglobulins (antibodies) carrying different antigen specificities. When a naïve B-cell encounters its cognate antigen, it undergoes clonal expansion and differentiates into a plasma cell. Plasma cells massively secrete antibodies containing the respective epitope specificity against the noxious molecule. Antibodies circulate in the bloodstream and by binding to their cognate epitope mark the invading structure/pathogen for destruction by phagocytic cells of the immune system.

The second class of adaptive immune responses is mediated by T-cells that react directly against antigens presented on the surface of infected or dysfunctional host-cells. TCR differentiation is determined by recombination and, similar to B-cell immunoglobulins, a virtually unlimited array of receptor specificities can be produced. T-cells bound to their cognate epitope become activated, differentiate to effector-T-cells, and undergo clonal expansion to produce a multitude of cells equipped with the cognate receptor set against the threat. When the activated effector-T-cell is bound to an infected or dysfunctional host-cell, the effector-T-cell releases cytotoxins in order to perforate the target cell’s membrane and finally causes its burst or lysis.

After infection, most of the activated B- and T-cells will die; however, some further differentiate into B- and T-memory cells. Throughout the entire lifetime of the organism, these memory cells form a toolbox of effective B- and T-cells against a certain type of pathogen invasion. Upon interaction with a previously encountered antigen, the cognate memory cells are activated and the second exposure to such a previously encountered threat will produce a much stronger and faster immune response. The “memory-feature” is the true meaning of "adaptive", because the system can prime itself for future challenges.

The adaptive immune system produces with a relatively small number of genes a virtually unlimited diversity of different receptor-variants. Drawback of this unlimited variability of the adaptive immune system is that only a few B- and T-

cells at a given time point recognize a previously unknown threat. These few cells must rapidly proliferate in order to produce enough cells to mount an efficient immune response against this newly encountered danger. This typically takes several days and, during this time, the pathogen could cause considerable harm. It is here, where the innate immune system comes into play.

Pattern Recognition Receptors in vertebrates

The innate immune system of vertebrates constitutes the first line of host defense during an infection with a pathogen (Medzhitov and Janeway, 2000). The innate immune response of vertebrates relies, like plants or fungi, on the perception of conserved structures of pathogens, so called MAMPs. In vertebrates, extracytoplasmic and cytoplasmic MAMPs are perceived via TLRs (Toll-Like Receptors) and cytoplasmic proteins named NLRs (Nod-Like Receptors) and RLRs (RIG1-Like Receptors).

The TOLL-LIKE RECEPTORS

Toll-like-receptors (TLRs) recognize MAMPs expressed by infectious organisms and mediate the production of antimicrobial compounds such as cytokines which are necessary for the development of an immune response (Medzhitov and Janeway, 2000). They are highly conserved from insects to mammals and share several structural and functional similarities. TLRs were originally discovered based on their homology to the Drosophila melanogaster TOLL protein. In Drosophila, TOLL plays a developmental stage dependent dual role as a dorso-ventral pattern regulator of the larvae-embryogenesis and as an immune receptor involved in the antifungal defense response of the adult Drosophila fly (Hashimoto et al., 1988; Lemaitre et al., 1996).

The family of human TLRs is the biggest and most intensively studied class of PRRs in vertebrates. Up to date, 10 TLRs have been identified in humans. They each recognize different MAMPs derived from diverse classes of microbial pathogens, including viruses, bacteria and fungi.

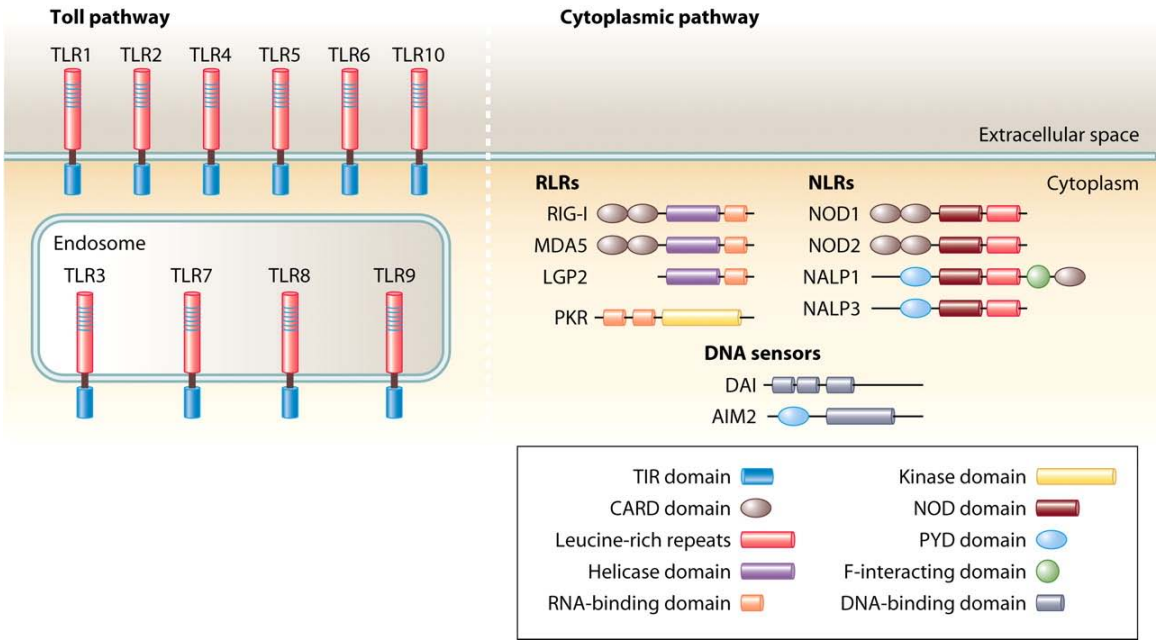

Figure 1: Vertebrate cellular PRRs.
TLRs are membrane bound receptors localized at the cellular or endosomal membranes. MAMP recognition occurs via the LRR domain and cytoplasmic signaling via the TIR domain. RLRs bind RNA and induce CARD-dependent signaling. NLRs are characterized by a central NOD domain and a C-terminal LRR domain (which serves as pattern recognition domain). Signaling is initiated via the N-terminal domains which include CARD and PYD (pyrin) domains.
(Picture from:(Mogensen, 2009))

TLRs are type I glycoproteins and structurally defined by an extracellular or luminal ligand binding domain containing Leucine Rich Repeat (LRR) motifs, a transmembrane domain and a cytoplasmic TIR (Toll/Interleukin-1 Receptor homologue) domain (O'Neill and Bowie, 2007). The LRR domain of TLRs is composed of 16 to 28 LRRs. TLRs can be classified into two groups based on their cellular localization. Group one includes TLR1, 2, 4, 5, 6 and 10 which have been shown to be localized at the plasma membrane. The second group includes TLR3, 7, 8 and 9 and localizes to the membranes of intracellular compartments such as endosomes. Cytoplasmic signaling of TLRs occurs via recruitment of adaptor proteins though the TIR-domains of ligand-activated TLRs. It is assumed that TLRs are present as, depending on the specific TLR, pre-assembled homo- or heterodimers that are complexed in a low affinity state prior to ligand binding (O'Neill and Bowie, 2007). Upon ligand binding, a conformational change is thought to occur that brings the TIR domains of the receptors in close vicinity. This TIR-TIR complex finally creates a signaling platform that recruits adapter proteins which initiate cytoplasmic signaling (O'Neill and Bowie, 2007).

TLRs and their ligands

TLR4 was the first TLR to be identified and is involved in the recognition of lipopolysaccharides (LPS) from gram-negative bacteria (Poltorak et al., 1998; Qureshi et al., 1999). LPSs are outer-membrane glycolipids and are well-known inducers of the innate immune response (Erridge et al., 2002). However, TLR4 does not directly bind LPS. Several intermediate steps are necessary for the activation of TLR4. As a first step the LPS is recognized by a lipid binding protein (LPB) (Schumann et al., 1990). The LBP shuttles the LPS to the CD14 receptor, which in turn delivers the LPS to MD2. MD2 is considered the coreceptor of TLR4. MD2 binds to the TLR4 ectodomain and is essential for LPS signaling (Shimazu et al., 1999; Viriyakosol et al., 2001). It is thought that binding of LPS to MD2 causes a conformational change of MD2, which is relayed to TLR4 and finally causes activation of TLR4 downstream signaling (Jerala, 2007).

TLR2 recognizes a structurally diverse range of MAMPs (Figure 2).This diversity of ligand recognition is enabled by the TLR2 ability to heterodimerize with TLR1 and 6. This complex formation is thought to strongly influence ligand specificity of the particular complex (Akira, 2009). In particular, dimers of TLR2/1 and TLR2/6 can discriminate between triacyl- and diacyl-lipopeptides (Takeuchi et al., 2001; Takeuchi et al., 2002).

TLR5 is the vertebrate sensor for bacterial flagellin (Hayashi et al., 2001). Exogenously expressed TLR5 and flagellin can be co-immunoprecipitated. This suggests a direct interaction between flagellin and TLR5 (Mizel et al., 2003). Furthermore, flagellin could be shown to bind to a soluble, monomeric form of the extracellular domain thus suggesting that indeed the extracellular LRR domain is responsible for interaction with the ligand (Mizel et al., 2003). However, compared to plants, animals recognize a different epitope of the flagellin protein than the flagellin receptor FLS2 (Flagellin sensing 2) the TLR5 counterpart in plant immunity (Smith et al., 2003).

TLR3, 7, 8 and 9 are localized to intracellular compartments. They have been shown to be sensors for mainly microbial derived nucleic acids. TLR3 detects ds (double stranded) RNA (Alexopoulou et al., 2001) while TLR7 and 8 recognize ss (single stranded) RNA (Diebold et al., 2004; Heil et al., 2004). The last member of the intracellular TLRs, TLR9 has been shown to be essential for the recognition of DNA that incorporates unmethylated CpG-rich motifs (Bauer et al., 2001a). Unmethylated CpG-rich DNA sequences are a hallmark of microbial derived DNA and therefore serve as a “non-self indicator” molecule.

So far, TLR10 is the orphan member of the TLR family. It is most highly expressed in lymphoid tissues and has been shown to not only homodimerize but also heterodimerize with TLR1 and 2 (Hasan et al., 2005).

Ligand-induced activation and signaling of the TLR family proteins

Upon binding of ligands to TLRs, a conformational change is thought to occur that brings the two TIR domains on the cytoplasmic face of each receptor into close proximity (O'Neill and Bowie, 2007). As a result, it is thought that the TIR-TIR complex constitutes a new platform on which the signaling complex is assembled.

Cytoplasmic signal transduction is mediated by a family of at least four, TIR domain containing adaptor molecules: MyD88 (Myeloid Differentiation primary-response gene 88), TIRAP (Toll-Interleukin 1 Receptor domain containing Adaptor Protein), TRIF (TIR-domain-containing Adaptor Protein inducing IFN-β) and TRAM (TRIF-Related Adaptor molecule). Upon ligand recognition, each TLR recruits a specific combination of adapters to activate different transcription factors, allowing an appropriate and effective immune response (Figure 2) (O'Neill and Bowie, 2007).

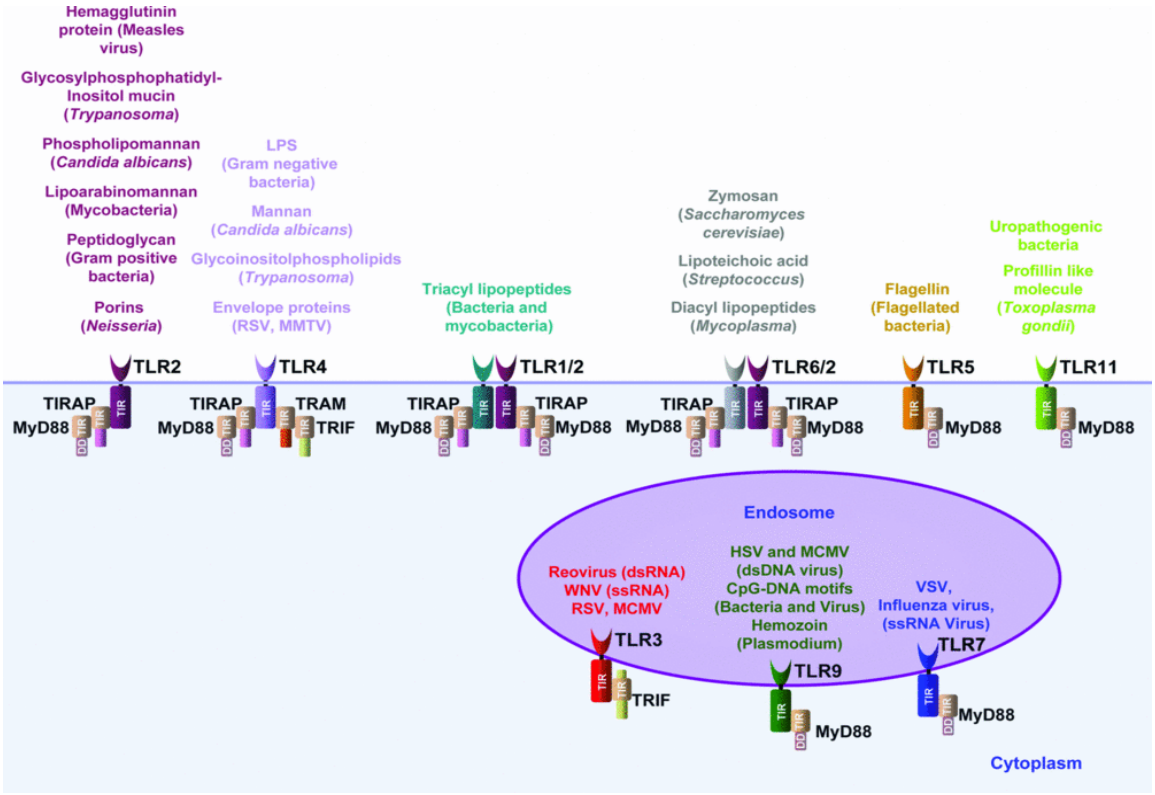

Figure 2: TLRs, the respective recognized ligands and required adaptors.
Toll-Like-Receptors 1, 2, 4, 5, 6 localize to the plasma membrane. TLRs 3, 7, 9 localize to the membranes of intracellular compartments. TLR1, TLR2, TLR4 and TLR6 recruit TIRAP and MyD88. MyD88 also contains the DD. In addition to TIRAP and MyD88, TLR4 recruits TRAM and TRIF. TLR5, TLR7, TLR9 and TLR11 recruit MyD88, whereas TLR3 recruits only TRIF.
(Picture from: (Kumar et al., 2009))

Generally, TLR signaling can be very broadly divided into two signaling pathways: the MyD88-dependent pathway and the TRIF-dependent pathway.

MyD88 is the central adapter molecule that mobilizes inflammatory pathways in innate immunity; it is shared by all TLRs with the exception of TLR3. Briefly, association of the TLR TIR-domain and MyD88 stimulates the recruitment of members of the IRAK (IL-1 Receptor Associated Kinase) family. Once phosphorylated, IRAKs dissociate from MyD88 and interact with TRAF6, an E3 ligase member of the TRAF (TNF-Receptor Associated Factor) family. This finally leads to activation of MAP kinases and the nuclear translocation of the transcription factor NF-κB (Necrosis Factor κB), which controls the expression of inflammatory cytokines. For TLR2/6, TLR2/1 and TLR4, the adaptor molecule TIRAP is additionally needed to link MyD88 to the TLR-TIR domain in order to activate the pathway (Kawai and Akira, 2007).

It has been shown that MyD88-deficient mice fail to produce inflammatory cytokines in response to ligands specific for TLR2, 5, 7 and 9 as well as TLR2/1 and TLR2/6 heterodimers (Yamamoto et al., 2002). However, cytokine production could be observed after treatment with TLR3 and TLR4 ligands (Yamamoto et al., 2002). This observation suggested the presence of a MyD88-independent pathway for TLR3 and TLR4 signaling. In this context the adaptor molecules TRIF and TRAM were identified to play key roles in a MyD88-independent pathway in order to produce inflammatory cytokines. TLR3 has been shown in a yeast-to-hybrid experiment to directly interact with the TRIF adaptor via its TIR domain (Oshiumi et al., 2003; Yamamoto et al., 2003a). In the case of TLR4, the adaptor TRIF is linked to the TLR-TIR via the additional adaptor molecule TRAM. TRAM has been shown to function exclusively in the TLR4 pathway (Fitzgerald et al., 2003; Yamamoto et al., 2003b).

NLRs (Nod-Like Receptors) and the Inflammasomes

Unlike membrane bound TLRs, which sense MAMPs on the cell surface or in endosomes, NLRs are thought to recognize MAMPS in the host cytoplasm. NLR proteins are structurally defined by three parts: a variable, N-terminal protein–protein interaction domain (effector domain), defined by the CARD (Caspase Recruitment Domain), PYD (Pyrin Domain) or the BIR domain (Baculovirus Inhibitor Repeat) followed by a central NOD domain (Nucleotide-binding Oligomerization Domain) that promotes self oligomerization during activation. A C-terminal LRR sensor domain is responsible for detecting and binding of MAMPs. 23 NLR genes have been identified in humans (Franchi et al., 2006a). Among these 23, NOD1 and NOD2, both carrying CARDs as effector domains, are the most intensively studied members of the NLR family. NOD1 and NOD2 mediate the perception of different structural peptidoglycan (PGN) motifs which are components of bacterial cell walls (Inohara et al., 2001; Franchi et al., 2006a). NOD1 recognizes peptidoglycan fragments containing *meso*-diaminopimelic acid (meso-DAP). DAP an unusual amino acid unique to the PGN of most gram-negative bacteria and certain Gram-positive bacteria (Chamaillard et al., 2003). NOD2 detects muramyl-dipeptide (MDP), a conserved structure found in nearly all gram-positive and gram-negative bacteria (Girardin et al., 2003). Single residue deletion experiments have demonstrated that both NOD1 and NOD2 sense their ligands via their LRR domain (Tanabe et al., 2004). NOD1 and NOD2 have been shown to induce NF-κB production and activation of MAPK (Mitogen Activated Protein Kinase) in a TLR-independent fashion (Shaw et al., 2008).

The NLR family members NALP1, NALP3 and Ipaf are involved in the assembly of multiprotein complexes called 'inflammasomes'. Three inflammasomes named after the NLR involved (NALP1, NALP3 and Ipaf) have been characterized so far (Franchi et al., 2009). The assembly of n inflammasome, is induced through the oligomerization of the NLRs after ligand binding. It is responsible for the activation of caspase-1, a protease that has been shown to be responsible for the conversion of proinflammatory cytokines pro-IL-1β and pro-IL-18 into their active forms (Martinon and Tschopp, 2004; Franchi et al., 2006b; Franchi et al., 2009). Common to all the three inflammasomes is the function of ASC as the adaptor protein that links the NLRs to pro-caspase-1, the precursor form of the biologically active caspase-1 protein via the CARD (Srinivasula et al., 2002).

Of special interest is the NLR Ipaf. Infection of macrophages by *Salmonella typhimurium* results in Ipaf dependent activation of caspase-1 (Mariathasan et al., 2004). Both pathogens have been shown to replicate within a membrane-bound compartment in the cytoplasm. Interestingly, *S. typhimurium* mutants that do not express flagellin do not activate caspase-1 production during macrophage infection (Franchi et al., 2006b; Miao et al., 2006). In this context, Ipaf has been demonstrated to be activated by bacterial flagellin that is secreted into the cytoplasm during *S. typhimurium* infection (Franchi et al., 2006b; Miao et al., 2006). However, no data about what epitope is recognized or the molecular mechanisms of flagellin recognition by Ipaf is available to date (Miao et al., 2007).

RLRs (RIG1-Like Receptors) - Intracellular viral MAMP sensors

TLR3, TLR7, TLR8 and TLR9 recognize different forms of viral derived nucleic acids in endosomal compartments. Although the importance of anti-viral TLRs is obvious, the key viral sensors in many cell-types are proteins called RLRs (Kato et al., 2005). RLR-proteins are a class of cytoplasm-localized PRRs that bind specific RNA molecules from different RNA viruses. Upon recognition, a signaling cascade is triggered that finally leads to the production of immune responses against viral infections.

As examples, RIG-I (Retinoic acid Inducible Gene 1), the first discovered RLR and the namesake of the whole class, and MDA5 (Melanoma Differentiation-Associated gene 5) are described.
Both are DExD/H-box RNA helicases with an RNA-binding domain and were found to play an important role in virus recognition and the subsequent defense strategy. They have been shown to be cytoplasmic sensors of dsRNA (Andrejeva et al., 2004; Yoneyama et al., 2004; Takeuchi and Akira, 2008). The length of the

recognized dsRNA is of importance for differential dsRNA recognition by RIG-I and Mda5 (Kato et al., 2008). RNA viruses having a shorter RNA length (approx. 1.2–1.4 kb) are recognized by RIG-I, while viruses with longer dsRNA (longer than 3.4 kb) are recognized by MDA5 (Kato et al., 2008).

The plant immune system

Plants are sessile organisms and therefore are especially exposed to abiotic and biotic stresses. Primary mechanisms of plants to avoid attaching and invasion of pathogens are physical barriers like the cuticula or cell-walls or chemical compounds like a vast array of secondary metabolites (Dixon, 2001). However, after a pathogen has overcome the primary barriers, plants rely on inducible defense strategies that are exclusively based on innate immune mechanisms. The current model on plant immunity is hallmarked by two main mechanisms: PTI (MAMP/PAMP mediated immunity) and ETI (effector-triggered immunity). Jones and Dangl proposed this model in 2006 by the introduction of the “ZigZag-model” in their keystone review article “The plant immune system” depicted in figure 3. The model found immediate acceptance in the plant research community and is today seen as a comprehensive reference model for plant-pathogen interaction (Jones and Dangl, 2006).

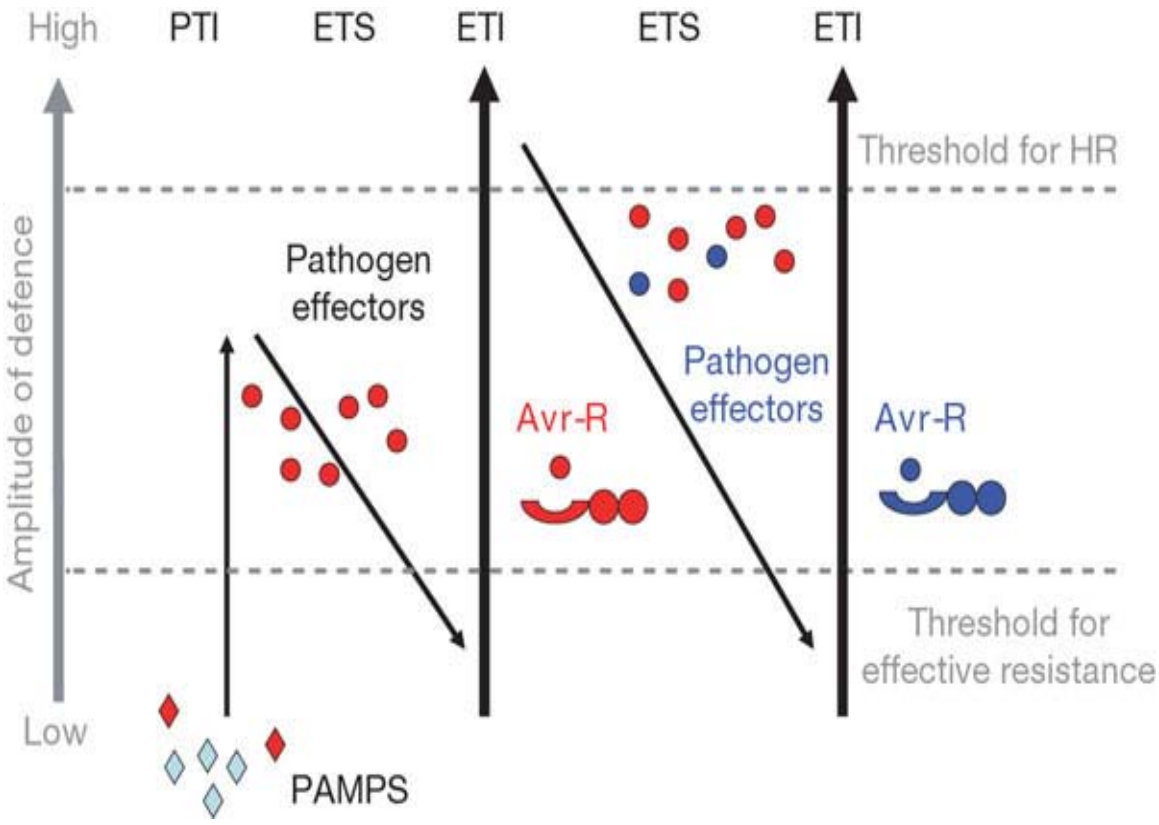

Figure 3: The ZigZag-model of the plant immune system.
The recognition of MAMPS (red diamonds) by PRRs triggers PAMP-triggered-immunity (PTI). Via appropriate effector-proteins (red/blue circles) evolved by the pathogen, PTI is suppressed and effector-triggered-susceptibility (ETS) is observed. If such an effector-protein is recognized by a cytoplasmic R-protein, Effector-triggered-immunity (ETI) is observed. ETI constitutes a stronger form of PTI that crosses the threshold for hypersensitive response (HR) and finally leads to cell-death. Evolutionary pressure can lead to mutual adaptation of the plant and then pathogen in a way that new effectors and new receptors compete each other in a constantly ongoing interplay or "arms race". (Picture from: (Jones and Dangl, 2006)

Accordingly, MAMPs/PAMPs are recognized by conserved PRRs in order to stimulate defense responses. PTI (PAMP-triggered immunity), formerly called basal defense or primary innate immunity provides basal resistance that is sufficient to prevent infection by a wide range of microbes. PTI is involved in both non-host and host resistance (Chisholm et al., 2006; Jones and Dangl, 2006).
PTI is associated with various defense related processes. Among the first, taking place within the first minute after perception, a rapid change of ion fluxes over the plasma membrane can be observed. Changes include an increased influx of protons and calcium ions and a concomitant efflux of potassium ions. Within a few minutes, the production of reactive oxygen species (ROS), induction of MAP kinase signaling and changes in protein phoshorylation status can be observed

(Nuhse et al., 2007). Also within minutes, the production of the stress-hormone ethylene is strongly increased. Further, late-PTI typical responses include the transcriptional induction of pathogenesis related genes (PR-genes), the deposition of callose to reinforce the cell wall at sites of penetration and the production of antimicrobial compounds (phytoalexins) (Boller and Felix, 2009). Interestingly, the pattern of gene regulation in response to the well studied MAMPs flagellin and EF-Tu is almost identical; indicating that signaling through the respective receptors converges at an early step (Felix et al., 1999; Kunze et al., 2004; Zipfel et al., 2006).

However, during evolution, certain pathogens have gained the ability to counteract the onset of defense by developing and delivering so called effector-molecules into plants. Effector molecules are released by the pathogen into the extracellular space or injected into the cytoplasm. Gram negative bacteria often use a type III secretion system (TTSS), encoded in the hrp (hypersensitive response and pathogenicity) gene cluster for translocation of effector-molecules into the cytoplasm of an infected cell (He et al., 2004). In recent years, it became more and more evident that effector molecules specifically suppress defense signaling (both ETI and PTI) and thereby enhance the pathogen's virulence (Zhang et al., 2007; Shan et al., 2008; Guo et al., 2009).

The process of modulating and/or lowering the defense state of the attacked plant by the use of effector molecules is referred to as ETS (effector triggered susceptibility). Effector-molecules are, however, double-edged swords. By performing highly specific, virulence promoting actions, they represent an excellent target for the attacked plant to identify the intruding microbe undoubtedly as a dangerous pathogen. Indeed, in turn, plants have developed a sophisticated second layer of immunity as the result of a co-evolution with the foe. This type of resistance was first described by H. H. Flor in the early 1940ties and the underlying genetic mechanisms formed the basis for the so called gene-for-gene concept: Specialized resistance (R-) proteins specifically detect a certain pathogen effector-molecules, which are then called avirulence (Avr) proteins (Chisholm et al., 2006; Jones and Dangl, 2006).

The resulting effector-triggered-immunity (ETI) is a race-cultivar specific interaction. It was sometimes referred to as secondary innate immunity. A great research effort in the last years has revealed two distinct mechanisms of the gene-for-gene concept of plant immunity: The classical, direct interaction between a R-protein and the cognate effector-molecule as ligand and the so

called guard-model (van der Biezen and Jones, 1998; van der Biezen et al., 2000).
The guard model implies that R-proteins monitor presumably important, effector targeted host proteins and activate defense if the guardee disturbed. Two variations of this model can be found in the literature: Either the R-protein is constitutively bound to its guardee host factor or alternatively, the R-protein may bind to its guardee only after the guardee is interacting with a pathogen effector molecule (Dangl and Jones, 2001). Indeed, it seems to emerge that most R-proteins do function according to the guard concept rather than directly interact with pathogen effectors. Actually, direct interaction of an effector and an R-gene has been demonstrated only in a few cases.

ETI, e.g. recognition of the presence of an Avr/effector-molecule by the cognate R-protein initiates a rapid and vigorous resistance response that, in contrast to PTI, often includes a hypersensitive response (HR) (Chisholm et al., 2006; Jones and Dangl, 2006). HR is a very vigorous defense strategy that is characterized by rapid apoptotic cell death and local necrosis of the infected and neighboring cells, most probably in order to prevent spreading of the pathogen. HR is a highly effective strategy against biotrophic pathogens, yet the account in regards to necrotrophic or hemibiotrophic pathogens is unclear.

When comparing PTI and ETI, MAMPs and Avr/effector-molecules trigger partially overlapping defense responses. However, ETI generally confers a much stronger and probably also more targeted defense response against the attacking pathogen (Jones and Dangl, 2006). However, in the light of the ongoing research effort, it seems more and more emerging that PTI and ETI should not be seen as two independent systems of plant immunity but rather as different levels of intensity of one overarching principle.

To date, only few PRRs have been identified in plants. Identified PRRs are members of the RLK- (Receptor-Like-Kinase) and RLP- (Receptor-Like-Protein) protein families. In contrast, identified R-proteins are of a more divergent nature: although the great majority seems to be cytoplasmic proteins harboring a nucleotide binding site and a C-terminal LRR domain (NB–LRR) others, however, include transmembrane proteins of the RLK-, RLP-, or the RLCK (receptor like cytoplasmic kinases) class of proteins.

Pathogen recognition at the surface: RLKs and RLPs

The frontline of the plant immune system is set up by surface receptors detecting molecules in the extracytoplasmic space. Receptors consisting of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular kinase domain are referred to as receptor-like kinases (RLK). Receptor-like proteins (RLPs) are similarly structured as RLKs, but completely lack a cytoplasmic kinase domain. In Arabidpsis, 610 RLKs and 57 RLPs have been identified. RLKs and RLPs are known to be involved not only in plant immunity but also in a plethora of other processes ranging from regulation of development, hormone perception and symbiosis.

Receptor-Like-Kinases

The name “receptor like kinases” is often applied instead of receptor kinases (RKs) because the corresponding ligands have still to be identified. The Arabidopsis genome contains at least 610 genes coding for RLKs. RLKs represent about 2.5% of the protein coding genes of Arabidopsis. In general, RLKs are integral membrane proteins with a C-terminal cytoplasmic serine/threonine kinase domain that resembles the Drosophila PELLE kinase (Shiu and Bleecker, 2001b). 60% of all kinases in Arabidopsis are represented by this family and constitute nearly all transmembrane kinases in Arabidopsis (Shiu and Bleecker, 2003). By definition, RLKs are composed of a predicted signal sequence, an extracellular domain, a single transmembrane region and cytoplasmic C-terminal serine/threonine kinase domain. 193 of the 610 identified RLK in Arabidopsis do not have an obvious receptor configuration as determined by the presence of a signal sequence and/or transmembrane domain (Shiu and Bleecker, 2001b). This subclass is referred to as RLCK (receptor like cytoplasmic kinases). Nonetheless, the remaining 417 RLKs (75%) do have a configuration where both signal sequences and transmembrane regions are present, and show the typical hallmarks for type I membrane proteins. 44 different subfamilies of RLKs can be distinguished based on the kinase domain phylogeny (Shiu and Bleecker, 2001b). Interestingly, an alternative classification based on the extracellular domains tends to be consistent with the classification based on the kinase domains (Shiu and Bleecker, 2001b). RLKs vary greatly in their sequence identity and domain architecture of the extracellular domains. The extracellular domains of RLKs can be of various shape and structure. They are classified into at least 14 distinguishable different subtypes: CRINKLY4-like, C-type-lectin, CrRLK1-like, DUF26, extensin, legume-lectin, LRK10-like, LRR, LysM, PERK-like, RKF3-like, S-domain, thaumatin and WAK-like (Shiu and Bleecker, 2001b).

Among these 14 subtypes, the by far most frequent extracellular motif is the LRR domain. For example, in Arabidopsis, the LRR-containing RLKs (LRR-RLK) represent with 216 out of the 417 receptor configured RLK members the largest group. The LRR-RLKs represent 13 families (LRR-I to LRR-XIII) (Shiu and Bleecker, 2001b). The families are classified according to the structural architecture of their LRRs and the organization of introns in the extracellular domains of the individual LRR-RLKs. The second most frequent extracellular domain-types are various sugar binding motifs such as lectins and the lysine motive (LysM) domains. Especially the LysM domain is thought have a general peptidoglycan binding function (Shiu and Bleecker, 2001a).

Functions of plant receptor-like kinases

RLKs play fundamental roles in a plethora of processes during the plants life cycle. RLKs have been shown to be involved in developmental processes and hormone perception as well as biotic and abiotic stress responses (Shiu and Bleecker, 2001b). Some examples are given below:

Brassinosteroid (BR) is an intensively studied plant hormone. BR regulates growth processes for plant growth and development such as cell expansion and cell elongation (Gendron and Wang, 2007). The LRR-RLK BRI1 binds and is essential for brassinosteroid (BR) signaling in interaction with other LRR-RLKs including BAK1 (BRI1-associated receptor kinase 1), another LRR-RLK (He et al., 2000). Phytosulphokine (PSK) is a five-residue peptide which functions as the growth factor that induces dedifferentiation of plant cells and subsequent callus growth. PSK triggers cell proliferation by binding directly to the LRR-RLK receptor PSKR (phytosulphokine receptor) (Matsubayashi et al., 2002). The LRR-RLK ERECTA has been shown to play a role in organ elongation (Torii et al., 1996). Recently, the presence of ERECTA has been found to have an influence on plant immunity, and a specific function for ERECTA in regulating cell wall-mediated disease resistance distinct from its role in development has been proposed (Sanchez-Rodriguez et al., 2009).

Of most interest for this work are RLKs which are involved in plant immunity. Bacterial flagellin, for instance, is recognized by the LRR-RLK Flagellin Sensing 2 (FLS2) and the bacterial Elongation-Factor-Tu protein (EF-Tu) by the LRR-RLK EF-Tu receptor (EFR) (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). The LRR-RLK proteins FLS2 and EFR represent so far the only known bona-fide

PRRs in Arabidopsis and the only confirmed bona-fide RLKs involved in MAMP perception in plants. These two cornerstone receptors, amongst others, are described in detail in the following.

Flagellin perception by the LRR-RLK FLS2

The flagellum is the main bacterial motility organ. It enables bacterial motility and thus has a strong impact on bacterial virulence (Penn and Luke, 1992). The single flagellum is a complex structure. Its filament is composed of repeated subunits of the protein flagellin. It is anchored in the bacterial plasmamembrane and cell wall by a basal body and hook structure (Fig. 4, A) (Macnab, 2003). Flagellin protein has been shown to induce immunogenic responses in both plants and animals (Fig. 4, B) (Felix et al., 1999; Wyant et al., 1999). Flagellin is an archetypal MAMP: It is of pivotal importance for bacterial fitness and survival and it contains highly conserved sequence patterns among various classes of gram-negative bacteria, esp. in the N- and C-terminal ends (Wilson and Beveridge, 1993). In plants, the synthetic 22 amino-acid peptide QRLSTGSRINSAKDDAAGLQIA, called flg22, representing the most highly conserved part of the N-terminus of flagellin, is sufficient for the induction of defense responses in several plant species including *Arabidopsis thaliana*. The synthetic peptide flg22 acts as a potent elicitor of plant defense at subnanomolar concentrations (Felix et al., 1999).

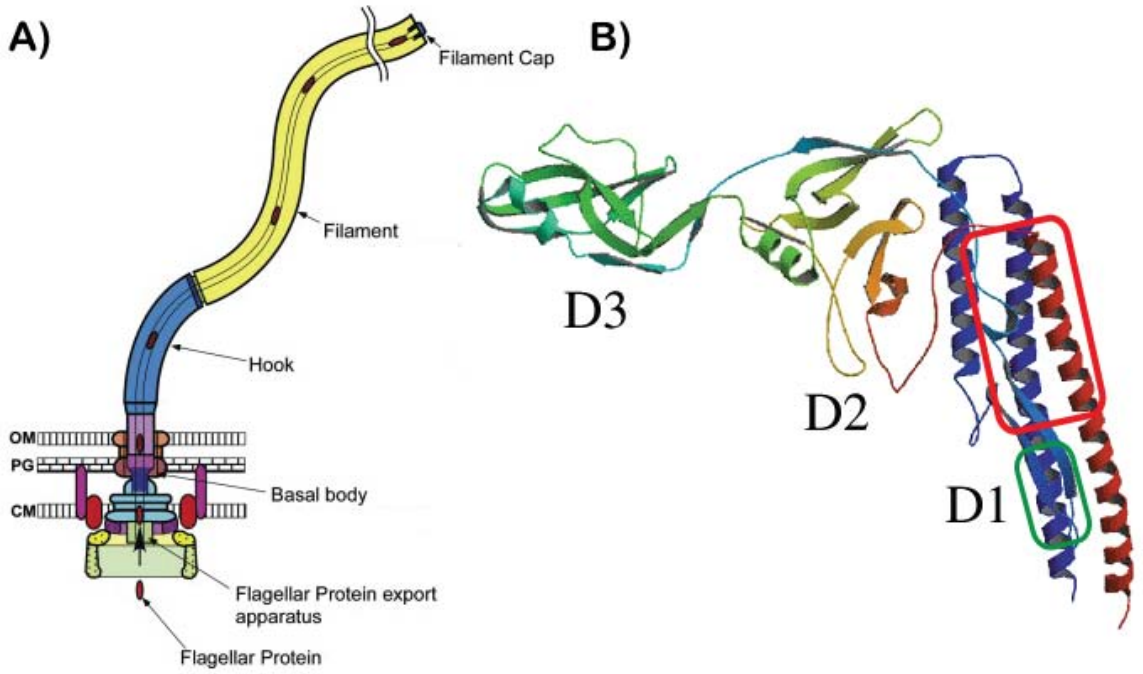

Figure 4: Schematic drawing of the bacterial flagellum and the flagellin structure.
A) Schematic diagram of the flagellum which consists of three parts: the basal body, which acts as a reversible rotary motor; the hook, which functions as a universal joint; and the filament, which acts as a helical screw. (OM, outer membrane; PG, peptidoglycan layer; CM, cytoplasmic membrane). **B)** Flagellin structure. The square in red shows the position of vertebrate TLR5 recognition and in green the conserved N-terminal part of flagellin (flg22) that is recognized by the LRR-RLK FLS2 in plants. Modified from (Minamino et al., 2008)

The flagellin receptor in *Arabidopsis thaliana* is FLS2 (Flagellin-sensing 2). FLS2 includes 28 LRRs in its extracellular domain (Gomez-Gomez and Boller, 2000) and has been shown to be an integral transmembrane protein that is localized to the plasma membrane (Robatzek et al., 2006). It belongs to the subfamily XII of RLK according to the nomenclature of Shiu and Bleecker (Shiu and Bleecker, 2003).

Flg22 perception leads to several PTI typical defense reactions in most plants. In Arabidopsis, flg22 triggers the alkalinization of extracellular media of suspension cells at subnanomolar concentrations, increases ethylene biosynthesis and production of ROS and induces the formation of callose deposition in cell walls. Furthermore flg22 activates a MAP kinase based signaling cascade (MEKK1, MKK4/5, MPK3/6) (Asai et al., 2002b). Additionally, flg22 perception induces the expression of about 1000 PR-genes, including a high number of RLKs (Navarro et al., 2004; Zipfel et al., 2004). Biologically most important, flg22 treatment of wildtype Arabidopsis, but not *fls2*$^{-}$ mutant plants, leads to a strongly enhanced

disease resistance to the pathogen *Pseudomonas syringae* DC3000 (Zipfel et al., 2004).

Upon flg22 perception, FLS2 has been shown to rapidly associate and form a heteromeric complex with the LRR-RLK BAK1 (Chinchilla et al., 2007). Recent new findings indicate that BAK1 as well as FLS2 get phosphorylated upon stimulation with flg22 (Schulze et al, in press). BAK1 belongs to a group of five SERKs (somatic embryogenesis-related kinases) and is also referred to as SERK3. BAK1 has long been known to act as a coreceptor of BRI1 mediated brassinolide signaling. Additionally to the finding that BAK1 is involved in flagellin signaling, it emerges more and more that BAK1 is an important general regulator of many receptor-mediated signaling pathways. However, important for this study, BAK1 seems not to be involved in flg22 binding (Chinchilla et al., 2007). Despite their clear loss of sensitivity to flg22, *bak1*$^{-}$ Arabidopsis mutants are not more susceptible to bacteria than wildtype Arabidopsis (Kemmerling et al., 2007). In contrast, *N. benthamiana* silenced for *NbBAK1* were shown to be more susceptible to bacterial pathogens than wildtype plants (Heese et al., 2007). However, this discrepancy might be explained by co-silencing of closely related BAK1 paralogs or other members of the SERK family in *N. benthamiana* that might partially substitute the BAK1 loss in Arabidopsis.

Up to date, no further interacting elements of FLS2 besides BAK1 have been identified that link the FLS2/BAK1 complex to the downstream signaling cascade. However, ultimately flg22 binding has been shown to lead to receptor endocytosis from the plasma membrane into intracellular mobile vesicles (Robatzek et al., 2006). This internalization requires probably both the kinase activity and the ubiquitination related (PEST) motif in the C-terminal kinase domain of the Arabidopsis FLS2 (Robatzek et al., 2006).

Responsiveness to flagellin has been observed in all major classes of higher plants. This suggests that flagellin perception is an ancient mechanism of plant immunity. Indeed, orthologues of the FLS2 protein have been identified in tomato, tobacco and rice (Hann and Rathjen, 2007; Robatzek et al., 2007b; Takai et al., 2008). Furthermore, proteins with a high degree of conservation can be indentified in silico from outputs of genome sequencing projects of grapevine, ricinus, maize, poplar and other plant species. This suggests that FLS2 is an evolutionary ancient and successful PRR.

There is strong evidence that FLS2 directly binds flg22 via its LRR domain and that this LRR domain is responsible for ligand specificity (Chinchilla et al., 2006).

Ligand specificity of FLS2 proteins differs from one plant species to the other (Meindl et al., 2000; Chinchilla et al., 2006). This is illustrated by the difference in ligand specificities of tomato FLS2 (LeFLS2) and Arabidopsis FLS2 (AtFLS2): The flg15 peptide, a 7 amino acid truncated derivative of flg22, is fully active in tomato, whereas it is an about 100 fold less active agonist in Arabidopsis. Flg22-Δ2 (QRLSTGSRINSAKDDAAGLQ--), a C-terminal two amino acid truncated derivative of flg22, acts as an agonist in tomato whereas in Arabidopsis, this peptide acts as a receptor antagonist. Equally interesting is flg22ΔA17 (QRLSTGSRINSAKDD-AGLQIA), a flg22 derivative that lacks the alanine at position 16 of the flg22 sequence. This peptide has an agonistic activity in Arabidopsis while it acts as a receptor antagonist in tomato (Meindl et al., 2000; Chinchilla et al., 2006).

In observations is that, in contrast to flg22, native flagellin from certain bacteria can induce strong HR in nonhost plants. It has been shown that flagellin of *Pseudomonas syringae pv. tomato (Pst)* induces HR in their nonhost tobacco plants, while flagellin of *Pseudomonas syringae pv tabaci (Psta)* does not induce HR in its host tobacco plants. Similarly, flagellin from *Pseudomonas syringae pv. gycinea* (*Psgl*) induces HR in the nonhost tobacco but not in the host soybean (Taguchi et al., 2003). Interestingly, and adding additional weight to these findings, silencing experiments using fragments of the *Nicotiana benthamiana* FLS (NbFLS2) have shown that the observed HR responses in tobacco correlates with the presence of FLS2 (Dagmar Hann, personal communications).

A second, similar finding is of special interest because the amino acid sequences of flagellins of *Psta* and *Psgl* are completely identical. However, studies have shown that they are differentially glycosylated (Takeuchi et al., 2003). This indicates that posttranslational modification of flagellin is correlated with the ability of flagellin to cause HR. Moreover, inoculation of mutant *Psgl* carrying deletions in a glycosylation island on non-host tobacco plants resulted in the development of prominent disease symptoms, strong reduction of oxidative burst and failure of HR-induction. In contrast, the host-plant soybean showed strongly reduced disease symptoms when treated with glycosylation-island mutated *Psgl.* It appears that the glycosylation moieties of *Psgl* flagellin is specifically detected by the non-host tobacco and used as a trigger for HR whilst in soybean the glycosylation moieties are not recognized as a trigger for HR and maybe even mask the flagellin from detection (Taguchi et al., 2003).

Incorporating these findings, the FLS2 receptor could actually play a dual role both as an inducer of PTI and as an inducer of ETI with the outcome depending

on the presence of specific glycosylation moieties on the ligand. Whole flagellin, depending on its glycosylation moieties, might therefore serve as both a “specific elicitor” that provokes ETI in specialized plants as well as a “general elicitor” in other plants. These findings indicate that PTI and ETI should not to be seen as separate mechanisms but rather as interlocking steps of one overarching principle. In the future, it will be interesting to learn about studies which examine if glycosylation of flagellin is a way for pathogens to hide the elicitor-active epitope of their flagellin from recognition by FLS2.

Elongation Factor - Tu perception by the LRR-RLK EFR

The Elongation factor Tu (EF-Tu) is the most abundant protein occurring in a bacterial cell. As a principal function, EF-Tu binds an aminoacylated tRNA molecule and catalyzes the covalent transfer of the amino acid onto the growing polypeptide via a GTP dependent process in the ribosome. Because of its pivotal role in protein biosynthesis, the EF-Tu protein has been extensively studied at the biochemical and structural level (Kawashima et al., 1996; Nilsson and Nissen, 2005).

EF-Tu acts as a MAMP in Arabidopsis thaliana and other *Brassicaceae* but no elicitor activity has been shown so far for other plant families (Kunze et al., 2004). This might indicate that perception of EF-Tu as a MAMP is an exclusive innovation of the *Brassicaceae* family and therefore is, in contrast to flagellin perception, an evolutionary rather recent MAMP perception system. The elicitor active epitope of EF-Tu weas identified as the N-terminal 18 to 26 amino acids of EF-Tu (Kunze et al., 2004). Similar to flg22, a synthetic peptide called elf18 is able to trigger the MAMP specific responses at subnanomolar concentrations (Kunze et al., 2004). In Arabidopsis, EF-Tu is recognized by the LRR-RLK EFR (EF-Tu receptor). Arabidopsis plants lacking EFR have been shown to loose the ability to bind elf18 and failed to initiate defense responses upon treatment with elf18 (Zipfel et al., 2006). In contrary, when EFR is expressed in *N. benthamiana*, a plant that has no perception system for elf18, it confers elf18-binding and associated defense signaling. These findings make it likely that EFR is indeed the bona-fide receptor of EF-TU and suggest that the downstream elements of activation both FLS2 and EFR are conserved between Arabidopsis and *N. benthamiana* (Zipfel et al., 2006).

EFR belongs to the same family XII of LRR-RLKs as FLS2 and contains an extracellular signal peptide, 21 LRRs, a transmembrane domain and a

cytoplasmic Ser/Thr kinase domain (Zipfel et al., 2006). With respect to the kinase domain, the Arabidopsis genome encodes 5 genes that are closely related to EFR, referred to as EFR-likes. Four encode also LRR-RLKs with 21 extracellular LRR domains. However, interestingly, the most closely related gene encodes for a protein that completely lacks an extracellular LRR domain. The function for none of these proteins has been elucidated so far.

Chitin perception in Rice and Arabidopsis

Chitin is the main building block of fungal cell walls. Chitin serves as a major MAMP in both plants and animals. In rice, a RLP called CEBiP was identified as a high-affinity binding site for chitin (Kaku et al., 2006). Knockdown experiments of CEBiP in rice cell cultures resulted in a strong suppression of chitin induced reactive oxygen species (ROS) generation while ROS generation induced by LPS (Lipopolysaccharides) was not affected (Kaku et al., 2006).
CEBiP contains an extracellular LysM-domain and a single transmembrane domain but lacks any obvious intracellular domains. The obvious lack of a cytoplasmic domain suggests that additional factors are required for signalling. Interestingly, another gene encoding for a LysM-RLK, CERK1 (chitin elicitor receptor kinase 1) was shown to be required for chitin signaling in Arabidopsis (Miya et al., 2007). *Cerk1* knockout mutants were completely insensitive to chitin treatment and did not show defense responses upon treatment with chitin. Biologically significant, disease resistance of *cerk1* knockout plants to the incompatible, necrotrophic fungus *Alternaria brassicicola* was partly impaired (Miya et al., 2007). However, direct binding of chitin to CERK1 has not yet been demonstrated. Similar to CEBIP, CERK1 is a plasma membrane protein with an extracellular LysM-domain. However, in contrast to CEBiP, CERK1 has a cytoplasmic Ser/Thr kinase domain and is therefore an RLK.

Interestingly, a recent study by the group of John Rathjen showed that CERK1 also plays an essential role in restricting bacterial growth on plants. Arabidopsis-mutants that do not accumulate the CERK1 protein were shown to be more susceptible to bacterial infection by *Pseudomonas syringae* pv. *tomato DC3000* in about the same magnitude as *fls2*-mutant Arabidopsis plants (Gimenez-Ibanez et al., 2009). Additionally, CERK1 has been shown to be a target of the bacterial type III effector molecule AvrPtoB, which blocks all defense responses through this receptor (Gimenez-Ibanez et al., 2009).

These findings put up the question whether CERK1 indeed perceives chitin-similar, carbohydrate-based structures which are present in Pseudomonad bacteria or whether CERK1 may rather act as a coreceptor of multiple yet unknown defense related PRRs, similar to the requirement of BAK1 for activation of FLS2 (Gimenez-Ibanez et al., 2009).

Receptor Like Proteins in plant immunity

Receptor-like proteins (RLPs) are cell surface receptors that typically consist of an extracellular sensor domain, a transmembrane domain, and a short cytoplasmic tail but no cytoplasmic kinase. In total, 57 RLP genes have been identified in the Arabidopsis genome. RLPs are involved in growth, development and, mostly, in plant defense (Shiu and Bleecker, 2003).

The tomato Cf- and Ve-locus genes

The majority of RLPs with known functions in immunity have been considered classical R-genes that activate ETI in a gene-for-gene specific manner. This is exemplified in the following by the *Cf* s and the *Ve* resistance genes in tomato.
The *Cf* resistance genes provide resistance against the leaf mold fungus *Cladosporium fulvum*, a semi-biotrophic pathogen which infects tomato species. The cloned *Cf* resistance genes all encode RLP proteins with an extracellular LRR domain and a single transmembrane domain. Among them, Cf9 was the first RLP that was functionally identified. Cf9 mediates resistance against strains of *C. fulvum* that carry the avirulence/effector gene *Avr9* (Jones et al., 1994). Meanwhile, several *Cf* resistance genes have been cloned from tomato and all belong to the RLP family and have the same architecture. The *Cf* resistance genes are organized in gene clusters that are grouped into two loci (Thomas et al., 1998). Both clusters contain members with currently unknown function an*d Cf r*esistance genes that recognize the presence or activity of specific *C. fulvum* effector-molecules.

Besides Cf9, the described *Cf*-gene products Cf2, Cf4, Cf4E and Cf5 confer resistance against strains of *C. fulvum* secreting Avr2, Avr4, Avr4E, Avr5 and Avr9 effector-molecules (de Wit et al., 2002). However, only very scarce information is available about the effector functions of these proteins and the mechanisms how Cf-proteins confer resistance. Only in recent studies light has been shed on the molecular mechanisms underlying Cf2–dependent resistance. Cf2 has been shown to depend on the presence of Rcr3, an extracellular Cys-

protease to be activated (Dixon et al., 2000). Cf2 has already then been proposed to be the guard protein of Rcr3 (Dixon et al., 2000). Indeed, Avr2 could be shown to bind and inhibit Rcr3. However, solely inhibition of Rcr3 is not enough for triggering activation of Cf2. In fact it is rather suggested that Cf2 is specifically activated upon recognition of the Rcr3/Avr2-complex (Rooney et al., 2005). This renders Cf2 a typical guard R-protein according to the guard model. Additionally, a recent study has revealed that Avr2 not only inhibits Rcr3, but also other extracellular Cys-proteases. Plant proteases have been shown to play important roles during defense against pathogens (van der Hoorn, 2008; van Esse et al., 2008). No information about how Cf4 recognizes the presence of Avr4, directly or indirectly, is currently available. Avr4 has been shown to bind to chitin present in fungal cell walls and that, through this binding, it can protect these cell walls against hydrolysis by plant chitinases (van Esse et al., 2007).

For Cf9/Avr9, various experimental procedures were performed to investigate whether Cf9 has a direct binding affinity for the Avr9 effector-molecule or not (Luderer et al., 2001). It has been shown that Avr9, whose target molecule in the plant is yet unknown, encodes a small cysteine-rich peptide that is secreted into the plant apoplast during infection (Vankan et al., 1991). However, all experiments to demonstrate any direct interaction of Cf9 and Avr9 were not successful (Luderer et al., 2001). Omitting technical issues, this implies that at least one third partner is required for perception of Avr9 by Cf9 (Luderer et al., 2001). Interestingly, introduction of the Cf9 gene in tomato, tobacco and potato has been shown to be sufficient to transfer responsiveness to Avr9 (Hammond-Kosack et al., 1998). This indicates that such a third partner is already present in these species.
Additionally, the tomato RLP gene family comprises two genes that have been shown to provide resistance against soil-borne vascular wilt pathogens of the genus *Verticillium,* including *Verticillium dahliae* and *Verticillium albo-atrum*. The genes *Ve1* and *Ve2* are located in the *Ve* locus (Kawchuk et al., 1994; Kawchuk et al., 2001). The corresponding proteins Ve1 and Ve2 are typical RLPs that share an amino acid identity of 84% and are composed of an N-terminal signal peptide, a leucine-rich repeats domain, a single transmembrane and a C-terminal cytoplasmic domain with a potential endocytosis signal (Kawchuk et al., 2001). Biologically important, transfer of the *Ve1* or *Ve2* gene into susceptible potato plants conferred resistance against *V. albo-atrum* (Kawchuk et al., 2001). However, no information about potential ligands is available up to date.

Interestingly, silencing experiments with NbSERK3/BAK1 fragments transferred into tomato plants compromised *Verticillium* resistance in tomato plants (Fradin et

al., 2009). Although this finding is rather preliminary, it might suggest that NbSERK3/BAK1 might be involved in the pathway of signal transduction of this race specific disease resistance.

Xylanase perception in tomato by LeEIX1/2

Fungal Xylanase has been known for a long time to be a strong elicitor of defense responses in plants. In tomato, xylanase from *Trichoderma viride*, termed EIX (Ethylene Inducing Xylanase), is recognized by the gene products of a single, dominant locus in tomato and tobacco (Bailey et al., 1993).

The *EIX*-locus locus comprises three homologous *LeEIX* genes of which two, *LeEIX1* and *LeEIX2*, have been cloned and belong to the tomato RLP gene family (Ron and Avni, 2004). LeEIX2 has an extracellular LRR domain, a transmembrane domain, and a short cytoplasmic tail with a putative endocytosis signal (Ron and Avni, 2004). LeEIX1 has the same architecture LeEIX2, but lacks the putative endocytosis signal. A kinase domain is completely lacking in both LeEIX1 and LeEIX2. Both LeEIX1 and LeEIX2 were shown to bind EIX independently. However, when transiently expressed in the EIX-nonresponding tobacco cultivar *N. tabacum* cv SR1 only LeEIX2 could transduce a signal that activated HR. Additionally, the putative endocytosis motif present in the LeEIX2 sequence proved to be essential for EIX induced HR mediated by LeEIX2, suggesting a role of LeEIX2- endocytosis for EIX signaling (Ron and Avni, 2004). However, although EIX induces HR, it should be considered as a MAMP due to the conservation of the recognized epitope among xylanases from different fungal species (Furman-Matarasso et al., 1999; Rotblat et al., 2002). EIX being a MAMP renders, by definition, the corresponding receptors to be PRRs. PRRs, by the current definition, are involved in PTI whereby the outcome of EIX perception is clearly more similar to ETI due to the strong HR.

Intracellular immune receptors

Unlike in animal systems, no intracellular PRRs have been identified so far in plants. However, many identified effector-molecules act inside the plant cell and many long-time known R-genes reside in the cytoplasm. The pathogens therefore must be able to deliver molecules across the plant cell wall and plasma membrane. Typically, this is achieved through the Type III Secretion System (TTSS). The TTSS forms a needle like structure, also known as the *hrp* –pilus. It

acts as a channel for the secretion of molecules across the plant cell wall into the cytoplasm (He et al., 2004).

Plant intracellular immune receptors are classical R-proteins which directly or indirectly perceive specific pathogen effector molecules to trigger ETI in a gene-for-gene specific manner. In Arabidopsis, the largest class of intracellular plant R-genes encodes proteins characterized by a central nucleotide-binding (NB) site domain and a C-terminal LRR domain. 149 predicted members of the NB-LRR gene family have been identified in the Arabidopsis genome. NB-LRR proteins reside in the cytoplasm and the LRR domain is generally thought to constitute the interaction interface (Kobe and Kajava, 2001). At the N-terminus, NB-LRR proteins carry either a region with similarity to the N-terminus of the Toll and Interleukin 1 receptor (TIR-NB-LRR proteins), a leucine-zipper (LZ-NB-LRR proteins) or a coiled-coil motif (CC-NB-LRR proteins) (Caplan et al., 2008). Especially the TIR-NB-LRR receptors share remarkable structural and functional similarities to the TOLL immune receptor in *Drosophila* and Toll-like receptors (TLR) in mammals. However, the stunning similarity between insect, mammalian, and plant NB-LRRs is thought to be an excellent example of convergent evolution.

NB-LRR R-proteins often function according to the guard model, e.g. as sentinels of presumably important host proteins to ensure proper presence and function of the guardee. Thereby, they perceive modification or loss of the guardee as a signal to induce ETI. However, few examples exist where direct interaction of effectors with R-proteins are described.

Direct interaction: PITA and the flax/flax-rust pathosystem

The first example of direct interaction between an Avr/effector-molecule was the one of the Pita CC-NB-LRR in rice and the AvrPita effector from the fungus *Magnaporthe grisea* (Jia et al., 2000). The LRR domain of Pita directly interacts with the AVR-Pita effector. A single amino acid substitution in the LRR can abolish this interaction, resulting in loss of resistance (Bryan et al., 2000).

However, the most detailed studies on direct recognition were carried out in the flax/flax-rust pathosystem. More than 30 closely linked TIR-NB-LRR genes clustered at five genetic loci (K, L, M, N and P) recognize approximately 30 flax rust effectors. Using yeast two-hybrid systems, a strong correlation between the direct association of a flax NB-LRR with its corresponding effector and activation of ETI could be demonstrated (Dodds et al., 2006; Ellis et al., 2007).

Indirect Interaction: The effectors AvrPto and AvrPtoB, the RLCK Pto and the NB-LRR Prf

In tomato, host resistance to *Pseudomonas syringae pv. tomato* (*Pst*), the causative agent of bacterial speck disease, occurs upon interaction of the type III effectors AvrPto or AvrPtoB with the RLCK Pto and the NB-LRR Prf (Ronald et al., 1992; Salmeron et al., 1996; Kim et al., 2002) .

The type III secreted *Pst* effector molecules AvrPto and AvrPtoB have been shown to interfere with PTI signaling in Arabidopsis (Jamir et al., 2004; Wulf et al., 2004; He et al., 2006; Shan et al., 2008). When expressed in Arabidopsis protoplasts, both molecules have been shown to interrupt flg22 signaling. AvrPto has been shown to interact with FLS2 and EFR when overexpressed in Arabidopsis protoplasts (Xiang et al., 2008). However, under weaker expression conditions, AvrPto has been shown to primarily interact with BAK1 and to prevent complex formation of FLS2 and BAK1 (Shan et al., 2008). This inhibition of complex-formation with the PRR leads subsequently to a suppression of FLS2-mediated PTI-signaling. Thus, under biological relevant conditions, AvrPto and AvrPtoB might rather target the integrating coreceptors BAK1 in order to suppress PTI instead of the PRRs themselves (Boller and Felix, 2009).

Pto has been shown to directly interact with both *Pst* effectors AvrPto and AvrPtoB (Tang et al., 1996; Kim et al., 2002). However, because AvrPto and AvrPtoB are structurally completely unrelated, it appears that the interaction between these effectors and the Pto is rather driven on part of the pathogen and not on part of the host (Mucyn et al., 2006).

It has been shown that Pto requires the NB-LRR protein Prf for successful onset of resistance against *Pst* (Salmeron et al., 1996). Prf and Pto have been shown to constitutively interact *in vivo* (Mucyn et al., 2006). According to the guard model, this indicates that Prf is an NB-LRR protein that 'guards' Pto and detects modification by and/or complex-formation of Pto with AvrPto/AvrPtoB (and maybe other bacterial effector-molecules), and subsequently activates defense. However, it is yet not clear why Pto is targeted by the effectors AvrPto and AvrPtoB. It remains to be elucidated if Pto plays a role, like BAK1 and maybe CERK1 as an important, integrating defense-related molecule, that renders it a worthy target to be shut down by the invading bacteria in order to suppress PTI or other yet unknown defense mechanisms.

The leucine rich repeat domain– molecular interaction platforms

Repetitive sequences are an often met component of tertiary protein structures. Among them, the LRR structure is probably the most intensively studied example. LRR structures have been identified in a plethora of proteins of organisms from all kingdoms, including viruses. Members include intra- and extracellular proteins and have been shown to be involved in a plethora of processes ranging from stress responses to developmental functions to hormone perception. Despite their apparently unrelated functions, LRR containing proteins share a common structural architecture.

Proteins with two or more tandem LRRs form the LRR-superfamily. The superfamily of LRR proteins across all kingdoms can be subdivided into at least seven families, characterized by different lengths and consensus sequences of the repeats (Kobe and Kajava, 2001). This classification is robust because repeats from different families never occur simultaneously in the same protein and therefore have probably evolved independently (Kobe and Kajava, 2001).
Each LRR typically consists of 20 to 30 amino acids and contains the 11 residue core consensus sequence *LxxLxLxxNxL* (whereby *x* can be any amino acid) (Kajava, 1998). Tandem repeats of LRRs form the LRR domain. A LRR domain can be generally characterized as a solenoid, horseshoe-shaped structure. Each repeat is one turn of the solenoid. Residues belonging to the core consensus sequence form a curved parallel β-sheet lining the inner concave side of the solenoid. Within this β-sheet, five solvent-exposed residues extend out of the concave face of each repeat (L**xx**L**x**L**xx**N). These solvent-exposed residues in the β-sheets are thought to be involved in the interaction with ligands and as determinants of ligand specificity. Moreover, it is becoming apparent that LRR domains are a platform on which binding sites can readily evolve and that LRR-domains are an important factor for R-gene evolution (Bakker et al., 2006). The remaining 10-20 residues form a range of secondary structures which form the backbone and the connecting loops/turns of the solenoid structure. The secondary structure of the backbone depends largely on the amount of residues per turn.

Because the first crystallized LRR, the ribonuclease inhibitor protein (RNI) was shown to consist of a convex side that is composed completely of α-helices which are linked by a loop structure to the β-sheet of the following repeat, all LRR proteins and domains routinely were classified as α/β-folds (α-helices alternating with β-strands) structures (Kobe and Deisenhofer, 1993). However, this is not correct. Many of the LRR structures determined by crystallography up to date have very little or no α-helical conformation. Generally, only LRRs with longer

repeats (27 to 29 residues), such as RNI, form a true α-helix running through the convex backbone between two consecutive β-strands (Bella et al., 2008). However, the typical length of an LRR is in the average 24 residues. Such repeats with intermediate lengths (22 – 26 residues) can adopt a variety of different secondary structures lining their concave backbone, including α-helices. However, the published structures of these medium-length repeats clearly show a prevalence for 3_{10} helices as the predominant secondary structure forming in the convex backbone (Bella et al., 2008). Very short repeats (20 to 21 residues) on the other hand use segments of the polyproline-II helix. The polyproline-II helix structure allows a more extended helix chain conformation in order to form and maintain the concave backbone (Bella et al., 2008).

The plant specific extracellular LRR motif

In plants, extracellular LRRs (eLRR) are composed of 23 to 25 amino acids and follow the consensus sequence **xxLxxLxx<u>LxxLxLxxN</u>xL^{T}/$_{S}$GxIP**. The unique and well conserved motif **L^{T}/$_{S}$GxIP** following the β-strand motif led to their classification as a unique LRR-family (Kajava, 1998) .

The largest group of eLRR-containing cell surface receptors is formed by the RLKs and the second largest group of eLRR-containing proteins form the RLPs.
From the 103 determined LRR structures deposited in the protein data bank, only two are of plant origin. The eLRR containg polygalacturonase inhibiting protein 2 (PGIP2) from *Phaseolus vulgaris* was the first and so far only plant eLRR-protein that was crystallized (Di Matteo et al., 2003).

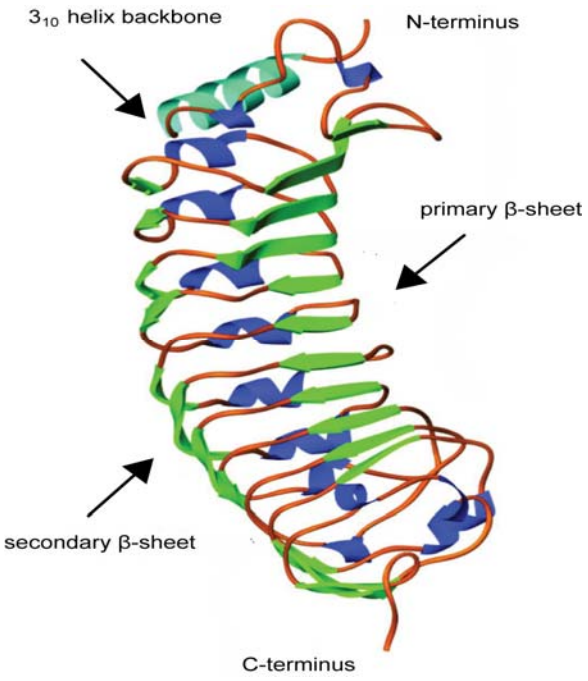

Figure 5: Crystal structure of PGIP2.
Polygalacturonase inhibiting protein 2 binds endopoly-galacturonases secreted from fungal pathogens. PGIP2 consists of 10 consecutive LRRs. The 3_{10} helix backbone is shown in blue and individual β-strands forming the β-sheet in green. The primary β-sheet is the concave side that is made up of the LRR core consensus sequence xxLxLxx. Hydrophobic leucine residues face towards the core of the solenoid, while the variable amino acid residues remain solvent exposed. (Modified from (Di Matteo et al., 2003))

PGIPs are plant cell wall proteins that bind endopolygalacturonases (PG) secreted by pathogenic fungi and thereby protect plants from fungal invasion (Fig. 4) (Cervone et al., 1987; De Lorenzo and Ferrari, 2002). The central LRR domain of PGIP2 is composed of 10 tandem repeat units, each made up of 24 residues. The crystal structure revealed that the overall structure of PGIP2 shows the typical horseshoe shaped, solenoid structure known for LRR proteins. A parallel β-sheet formed by all ten repeats lines the inner concave side of the structure and nine 3_{10}-helices on the convex side form the backbone of the solenoid. Interestingly, however, the plant characteristic motif **$L^T/_S$GxIP** is involved in the formation of a second β-sheet that is rarely seen in LRR structures. This second β-sheet is situated in the loop/turn region that connects the convex front and the concave back of the structure (Fig. 4). The highly conserved glycine residue allows bending of single β-strand in order to figure a as half-turn/loop (Di Matteo et al., 2003). The secondary β-sheet is considered a special feature of plant eLRR domains that has been so far not detected in animal LRR containing proteins (Di Matteo et al., 2003).

The LRR domain is usually flanked by small domains containing conserved Cys residues. Both the N- and C-terminal regions usually contribute to cap the hydrophobic core of the protein solenoid and are therefore of high structural importance for protein function. In the PGIP crystal structure the N- and C-terminal Cys-residues form four disulfide bridges that flank the LRR domain: two bridges are located in the N-terminal region (Cys-3–Cys-33 and Cys-34–Cys-43), and two in the C-terminal region (Cys-281–Cys-303 and Cys-305–Cys-312) (Di Matteo et al., 2003). The Cys-residues have been proposed to be involved in intramolecular bridging (van der Hoorn et al., 2005).

Ligand recognition and specificity

In LRR-domains, solvent-exposed residues have been shown to extend out from the concave side within the primary β-sheet of each repeat (bold x in L**xx**L**x**L**xx**N). The solvent exposed residues have in many cases been predicted to be involved in interaction with ligands. Indeed, several three-dimensional structures of complexes between LRR proteins or domains in complex with their ligands do show the ligand surrounded by the concave surface of the parallel β-sheet from the LRR domain, therefore suggesting the concave β-sheet as the preferred structure for interaction on the LRR domain (Kobe and Deisenhofer, 1995; Fan and Hendrickson, 2005; Tan et al., 2007). Two examples of studied LRR receptor-ligand pairs are given below: The well studied example of PGIP2 with fungal PGs and the still enigmatic interaction of Cf9 and Avr9.

PGIP2 inhibits fungal PGs through the formation of complexes. Complex formation of PG with PGIPs is thought to completely cover the active site of PG, and thus preventing access of PG to the substrate. Domain-swap and site-directed mutagenesis experiments showed that the concave β-sheet surface of PGIP contains the residues that determine specificity for different polygalacturonases (Leckie et al., 1999; Federici et al., 2001). It has furthermore been shown that the interaction between PG of *Fusarium moniliforme* (FmPG) and PGIP2 involves at least two positively charged residues of the FmPG (Arg-267 and Lys-269). These two residues are located at the edge of the active site of FmPG and are involved in substrate binding (Federici et al., 2001). An analysis of the electrostatic potential surface of PGIP2 revealed a negative pocket formed by the charged residues Asp-131, Asp-157, and Asp-203 and the polar residues Ser-133, Thr-155, and Thr-180 which are located in the center of the primary β-sheet (Fig. 6). Especially the three aspartic residues have been shown to be highly conserved among all PGIPs (Di Matteo et al., 2003). The

negative pocket is thought to be large enough to accommodate the positively charged PG residues and might thus cover the enzymes active site and prevent PG from access to its substrate. Interestingly, another residue has been identified to confer specificity of PGIP2 to FmPG. The residue Gln-224 of PGIP2 has been shown to be also located in the primary β-sheet just above the negative pocket that is putatively involved in PG binding. It is hypothesized that this residue interacts with a yet unidentified partner of FmPG in order to correctly lock the positive residues Arg-267 and Lys-269 into the negative pocked. All in all, the insights gained from the crystal structure of PGIP2 strongly consolidate the importance of the LRR β-sheet for ligand interaction and specificity.

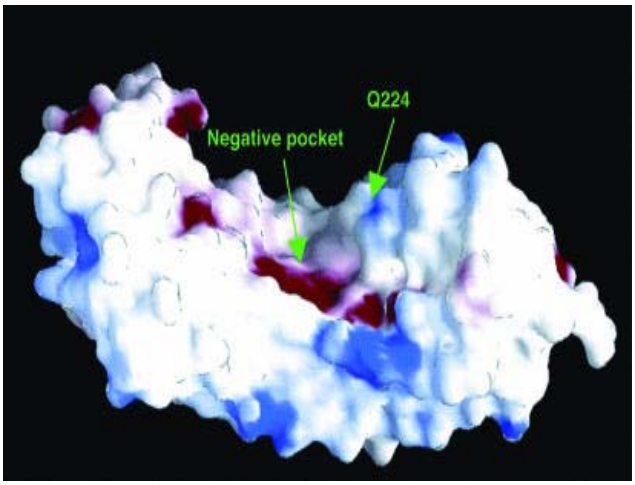

Figure 6:

Potential electrostatic surface of PGIP2.
Regions of negative potential are shown in purple. The negative pocket, putatively involved in PG recognition, is located in the middle of the inner concave surface of the protein. Additionally, the residue Gln-224 is crucial for PGIP2 specificity. (From: Di Matteo et al, 2003)

The tomato resistance genes Cf4 and Cf9 confer specific recognition of Cladosporium strains carrying the Avr-genes *Avr4* and *Avr9*. Cf9 and Cf4 encode proteins that share 91% amino acid residue identity (Thomas et al., 1997). Compared with Cf9, Cf4 lacks two LRRs and differs in 78 amino acid residues. Domain-swap experiments between Cf-4 and Cf-9 showed that the specificity of Cf-4 and Cf-9 is determined by a few solvent-exposed residues in the ß-sheets of the concave side of the LRR domain (Van der Hoorn et al., 2001; Wulff et al., 2001).

Additionally, sequential replacement of LRRs in blocks of five LRRs indicated that Cf9 specificity required the Cf9-specific residues that are distributed over distant LRRs, whereas the exchange of Cf4-specific residues did not abolish Cf9 function. The replacement of Cf4-specific residues present in LRRs 1 to 10 by those of Cf 9 did not affect Cf4 function while the Cf4-specific residues residing in

LRRs 13 to 16 are required for Cf4 function (Van der Hoorn et al., 2001; Wulff et al., 2001).

In studies performed to investigate the perception of flagellin by FLS2, Dunning and coworkers have outlined an approach towards identifying LRR functional sites. To identify the functionally important LRRs within FLS2, Dunning and coworkers performed a targeted alanine-scanning mutagenesis and comparison of FLS2 variants from taxonomically related *Brassicaceae* species including various Arabidopsis ecotypes. An Alanine scanning mutagenesis strategy of the solvent exposed residues of each of the 28 LRRs of FLS2 was used to identify LRRs that contribute to the function of FLS2. In this study, the alanine-scanned alleles with alterations in LRRs 9, 12, and 15 showed a moderate reduction in flg22 sensitivity, whereas ala-scanned alleles with alterations of solvent exposed residues in the LRRs 11, 13, and 14 showed significantly reduced sensitivity. This finding suggested that the region between LRRs 9 to 15 might play role for flg22-FLS interaction in *Arabidopsis thaliana* (Dunning et al., 2007). Furthermore, comparison of various Arabidopsis ecotypes and *Brassica* accessions revealed significant variation in the LRRs of *Brassica* FLS2 homologs, but not within FLS2 of Arabidopsis ecotypes. However, comparative studies of the LRR domains of the different *Brassica* accessions revealed two islands of conservation within the xxLxLxx motif of the LRRs 9-15 and LRRs 24-27 (Dunning et al., 2007). In conclusion, these studies suggested that LRRs 9-15 play an important role for flg22 binding of FLS2 (Dunning et al., 2007).

Comparison between the immune system of animals and plants

“Plants don’t have a circulatory system and no specialized immune cells to track down bacteria, fungi and viruses.” -At first glance, plant immunity is far simpler than animal immunity. However, when delving deeper into the subject, it soon becomes evident that things are not that simple! Obviously, members of both the animal and the plant kingdom are very well capable of successfully defending their selves against pathogen attacks, and plants are not suffering at all from a depauperate defense against biotic stresses: Therefore, speaking of a primitive immune system when speaking about plant immunity would be a fundamentally wrong starting point.

Plants do have the basic innate immune system in common with mammals. While in mammals, antibodies that specifically target microbes rise up after the innate arm of the immune system carries out the initial immune response to a pathogen, plants must entirely rely on their innate immune system to defend themselves against pathogens. Interestingly, in recent years, it emerged more and more that there are striking similarities between the animal innate and the plant immune system. In fact, both systems use surprisingly similar molecules to detect invading pathogens: In both groups PRRs initiate the immune responses upon perception of their specific “danger”-signals.

This raises an intriguing question: Were the basic components of the innate immune system evolved by a primordial ancestor common to plants and animals and began to differ once plants and animals split up (divergent evolution)? Or did plants and mammals evolve their innate immunity independently but ended up with similar mechanisms (convergent evolution)? In this respect it is interesting to observe that, although similar proteins are used for detection, they don’t function in the same way. For example, both plants and mammals have similar cell-membrane bound receptors which detect bacterial flagellin - FLS2 (in Arabidopsis) and TLR5 (in mammals). However, despite striking similarities in their extracellular domains (both are composed of LRRs) the two receptors detect different epitopes of the flagellin protein. This finding might rather be an indication pointing towards a convergent evolution.

But why are plants not continuously threatened by disease when they only have germ line fixed receptors available as detection systems? A look at the more and more emerging whole genomes of both mammals and plants reveals that plants encode a much larger array of putative PRRs than mammals (Shiu and Bleecker, 2001b, 2003).

In mammals, TLRs as well as the NLR and RLR proteins described so far respond only to highly conserved MAMPS. For them, the evolution of the adaptive immune system might have eliminated the need for the evolvement of a broad array of innate detection systems. From this perspective, the presence of classical R-genes in plants, with receptors recognizing specifically pathogen strain specific molecules and thus providing specific immunity against certain microbes might indicate that ETI mediated defense may function as the plants “innate” analog to the adaptive immune system of mammals. PTI, in contrast would then stand for the initial onset of defense, comparable to what is referred to as “classic innate immunity” in mammals.

Materials and Methods

General Materials and Methods

General chemicals, enzymes, kits and technical devices

Chemicals have been purchased, if not otherwise indicated, at high purity grade at Sigma-Aldrich AG (St. Louis, USA), Merck AG (Darmstadt, Germany) or Duchefa (Haarlem, The Netherlands). PCR reactions have been accomplished in a Biometra T-1 or an Eppendorf Mastercycler Gradient. Restriction digests, ligation and digestion reactions have been accomplished in PCR- reaction tubes in a MJ-Research PTC-100. Electroporation of A. tumefaciens have been accomplished in a Gene-Pulser System from BioRad Laboratories, Inc (Hercules, USA). Plant breeding has been accomplished in Snyder scientific phytochambers (long-day conditions), in-house growth rooms (short-day conditions) or a greenhouse (for flowering). Culturing of bacteria was accomplished in a Multitron shaker-incubater from Infors AG (Bottmingen, Switzerland). Molecular biology kits were obtained from Macherey-Nagel AG (Düren, Germany) and, for gateway-cloning, Invitrogen Corp. (San Diego, USA). Restriction enzymes and DNA-Ligase were purchased from New England Biolabs (Beverly, USA) and Roche Diagnostics (Rotkreuz, Switzerland), respectively.

Peptides and radiolabel

Flg22 and the flg22-derived peptide flg22-Δ2 were obtained from EZ-Biolabs (Carmel, USA). PaRm22 was obtained from GeneScript Inc. (Piscataway, USA). Flg22-ΔA17 was obtained from Peptron Inc. (Daejaeon, South-Korea). Flg15, flg22$^{E.coli}$, flg15$^{E.coli}$ and PaRm15 were obtained from the in-house peptide-synthesis unit of the Friedrich-Miescher-Institute (Basel, Switzerland). Peptides were dissolved and diluted in a solution containing 0.1% BSA and 0.1 M NaCl. Tyr-flg22 was labeled with ^{125}I by Anawa Trading AG (Wangen, Switzerland) to yield ^{125}I-Tyr-flg22 with a specific radioactivity of >2000 Ci/mmol.

Commonly used bacterial strains

The bacterial strain *Escherichia coli* DH5α was used for amplification and subcloning of plasmids. *Agrobacterium tumefaciens* GV3101 strain was used for transformation of *A. thaliana* and *N. benthamiana*.

Commonly used bacterial growth media

E. coli cultures were grown in LB-medium (1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0) either as liquid cultures or on agar-solidified (1.5% (w/v) bacto-agar) plates. Immediately after chemical transformation, *E. coli* were grown in SOC-medium (2% (w/v) bacto-tryptone, 0.5% (w/v) yeast-extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, pH 7). *A. tumefaciens* cultures were grown in YEB-medium (0.5% (w/v) beef-extract, 0.1% (w/v) yeast extract, 0.5% (w/v) bacto-tryptone, 0.5% (w/v) sucrose, pH 7.2) either as liquid cultures or on agar-solidified (1.5% (w/v) agar) plates..

Commonly used plant growth medium

Arabidopsis thaliana was grown on MS-medium (0.43% (w/v) Murashige and Skoog medium, 1% (w/v) sucrose, 0,05% (w/v) MES, pH 5.6 (KOH) either in liquid or on agar-solidified (0.8% (w/v) agar) plates

Plant material and breeding

For this study the wild-type plants *Arabidopsis thaliana ecotype Columbia-0* (Col-0), *Solanum esculentum var. Roter Gnom* and *Nicotiana benthamiana* was used. For stable transformations or Arabidopsis, the fls2-nullmutant T-DNA insertion line described by Zipfel et al was used (Zipfel et al, 2004). *Arabidopsis thaliana* was grown as follows: After a three- four days vernalization period at 4°C in the dark, seeds were germinated either on MS-plates under constant light at 21°C or in soil in single pots in phytochambers. In the phytochambers, the following conditions were applied: 8h light period at 22°C with a mean PAR of 100-120 µmol/m^2/s and a 16h dark period at 18°C. Relative humidity was kept at 65%. The soil used was a standard garden soil mixture supplemented with vermiculite, sand and Osmocot slow release fertilizer. Induction of flowering was done either in a controlled environment greenhouse (summer) or in a long-day phytochamber using the following conditions: 16h light period at 22°C with a mean PAR of 100-120 µmol/m^2/s and a 8h dark period at 18°C. Relative humidity was kept at 65%.
Nicotiana benthamiana were cultivated in controlled growth chambers: Seeds were sown in single pots and placed in a phytochamber using the following settings: 16h light period at 25°C with a mean PAR of 150-180 µmol/m^2/s and a 8h dark period at 22°C. Relative humidity was kept at 50%
Solanum esculentum var. Roter Gnom was grown in a controlled environment greenhouse.

Competent *Escherichia coli* cells for chemical transformation

Chemically competent *E.coli* cells were produced exactly according to the Hanahan-method described in the Maniatis laboratory manual (Hanahan, D, 1985; Maniatis, Fritsch and Sambrook, 1982).

Competent *Agrobacterium tumefaciens* cells for electroporation

100ml YEB-medium (without antibiotics) was inoculated with 5ml of an overnight culture of A. tumefaciens GV3101. The culture was grown under constant shaking at 200rpm and 28°C until an OD_{600} of 0.5 was reached. Subsequently, the culture was decanted into two 50ml Falcon-tubes and centrifuged at 4000g at 4°C for 15min. The supernatant was discarded and the pellets were resuspended in 25ml 1mM HEPES, pH 7.5. This procedure was repeated four times whereas the last two times the HEPES-buffer was supplemented with 10% glycerol. Subsequently, the pellets were resuspended in 400µl of 1mM HEPES + 10% glycerol and aliquoted in 2ml Eppendorf tubes. The tubes were shock-frozen in liquid nitrogen and stored at -80°C for further usage.

Chemical transformation of *Escherichia coli*

For transformation of plasmid DNA into *E. coli*, one batch of competent bacteria (50µl) was slowly thawed on ice. Not more than 5µl of ligation mixture or plasmid was added to the bacteria and the mixture was incubated on ice for 30min. Subsequently, the cells were heat-shocked at 42°C for exactly 90s and kept for another 5 minutes on ice. Following the incubation, 1ml of SOC medium was added and the bacteria were kept shaking at 200rpm for 1h at 37°C. Subsequently, the tube was centrifuged at 3000g for 3min and the supernatant was discarded. The resulting pellet was resuspended in 100µl of fresh SOC and dispersed in portions of 10µl and 90µl on selective LB-plates containing the appropriate selection antibiotic/s.

Electroporation of *Agrobacterium tumefaciens*

Electroporation of competent *A. tumefaciens* GV3101 was done using a GenePulser System from BioRad Laboratories, Inc (Hercules, USA). Approx. 100ng of plasmid DNA and 50µl of competent *A. tumefaciens* were transferred into a prechilled 0.2cm micopulser cuvettes. Electroporation was done using the following settings: 2.5ΣV / 25µF / 125µF / 400Ω. Immediately after electroporation, 1ml YEB medium was added directly to the c25µF cuvette, mixed and transferred into a 2ml Eppendorf tube. The cells were kept shaking at

200rpm for 2h at 28°C. After centrifugation at 3000g for 3min, the supernatant was discarded and the pellet was resuspended in 100µl fresh YEB-medium. Subsequently, the solution was dispersed in portions of 10µl and 90µl on YEB-plates containing 100µg/ml rifampicine (chromosomal resistance), 25µg/ml gentamycine (helper plasmid resistance) and the respective selective antibiotic for plasmid selection at appropriate concentration. The plates were kept growing for two days at 28°C.

Glycerol stocks and storage of bacteria

Bacteria were grown in liquid LB or YEB-medium either overnight (*E. coli*) or for two days (*A. tumefaciens*). Subsequently, 750µl of the bacterial culture was mixed with 250µl of pure glycerol and shock-frozen in liquid nitrogen. Glycerol stocks were kept at -80°C for future usage.

Quantification of DNA

For quantitation and verification of purity of DNA, a NanoDrop 2000 from Thermo-Scientific (Wilmington, USA) was used according to the manufacturer's instructions.

Agarose gel analysis of DNA

DNA fragments were commonly separated on 1% agarose gels containing 0.01µl/ml ethidiumbromide. Samples were analyzed using a BioRad Gel-Doc 2000 darkroom documentation system. If required, the fragments from the gel were excised and purified using the NuceloSpin Extract II Kit from Macherey-Nagel according to the manufacturer's instruction.

Isolation of plasmid DNA

10ml of LB-medium with appropriate antibiotics were inoculated with a respective transformed *E.coli* strain and grown overnight at 37°C. 5ml of the culture was used for plasmid DNA isolation. The plasmid DNA was isolated and purified using the NucleoSpin plasmid kit from Macherey-Nagel according to the manufacturer's instruction.

Ethanol precipitation of DNA

The DNA solution was mixed with 0.1 volume of 3M sodium acetate and 3 volumes of ethanol (puriss) and kept at -20°C for at least 1h. Subsequently, the sample was centrifuged at 25'000g for 30min at 4°C. The supernatant was discarded and the pellet was washed twice with 70% ethanol. The DNA was resuspended in ddH_2O and stored at -20°C for further usage.

Phenol-Chloroform purification of DNA

One volume of phenol/chloroform/isoamyalcohol was added to the DNA solution. The mixture was gently vortexed and centrifuged at 10'000g for 1min. The upper, DNA containing, aqueous phase was transferred into a new 1.5ml Eppendorf tube and an equal amount of chilled chloroform was added for washing. The mixture was gently shaken for 2min and centrifuged at 10'000g for 1min. This washing procedure was repeated twice. Subsequently, the aqueous DNA containing solution was ethanol-precipitated and resuspended in ddH_2O for further usage.

Polymerase chain reaction

Polymerase chain reactions (PCR) were routinely performed using 20 to 300ng of template DNA. Primers were designed and used at a concentration of 0.2 µM. For fragments that were amplified for subsequent cloning and plasmid construction procedures, the Phusion High-Fidelity DNA Polymerase (Finnzymes) was used. For checking and selection PCRs, Taq-DNA polymerase from NEB was used. The total reaction volume varied between 10 and 50µl. Cycling conditions were optimized individually depending on the primers, template and length of the PCR product. An extension time of 1min / 1kb for Taq-DNA polymerase and 20sec/1kb for the Phusion DNA-polymerase was used according to the manufacturer's instructions.
PCR reactions were performed in a Biometra T1 thermocycler (Biometra biomedizinische Analytik GmbH, Goettingen, Germany) or an Eppendorf Mastercycler Gradient (Eppendorf AG, Hamburg, Germany).

Colony PCR

Bacterial colonies putatively containing recombinant constructs were confirmed by PCR. The PCR was directly performed on bacterial cells recovered from selective plates. Primers were applied at a concentration of 0.2 µM. Taq-DNA polymerase (NEB) was used following the manufacturer's instructions. Bacteria

were directly lysed during thermal cycling. The following conditions were used: 94°C for 3min, 94°C for 20sec; 55°C for 30sec; 72°C for 1min/1kb x 28, 72°C for 5 min.

Restriction endonuclease digest

1-5µg of DNA was incubated together with up to 20U of restriction endonuclease and the respective buffer from NEB (Beverly, USA) in PCR tubes. The mixture was supplemented with ddH_2O to yield a final volume between 10µl and 50µl. The digest was accomplished at the optimal reaction temperature of the respective enzyme according to the manufacturer's instruction. Products were purified either using the NuceloSpin Extract II Kit from Macherey-Nagel according to the manufacturer's instructions or by phenol-chloroform purification and ethanol precipitation.

DNA ligation

Insert and vector DNA were mixed in varying molar ratios in PCR-tubes together with T4-DNA ligase and buffer (Roche AG, Rotkreuz, Switzerland) in a final volume of 10µl according to the according to the manufacturer's instructions. Ligation was performed at 16°C overnight or at 4°C for 24h.

Sequencing of double stranded DNA

500ng of template dsDNA were mixed together in standard PCR tubes with the sequencing-primer at a concentration of 0.5µM and 4µl of BigDye Ready Reaction Mix (Applied Biosystems Inc, Foster City, USA). ddH_2O was added up to a final volume of 10µl and the following conditions were used for the PCR: 96°C for 30sec, 50°C for 20sec; 60°C for 4min. The ramping rate of the thermocycler used was adjusted to not exceed 1°C/sec.
After the PCR reaction, the samples were ethanol precipitated and purified and the resulting pellet was air-dried for approx. 15min. Subsequently the pellet was resuspended in 35µl of Template Suppression Reagent (TSR). The resuspended sample was denatured for approx. 2min at 95°C and sequenced using the in-house ABI Prism 310 DNA Genetic Analyzer System (Applied Biosystems Inc, Foster City, USA). Resulting raw sequence data were analyzed using the Lasergene v5.0 DNA and sequence analysis software toolkit (DNAStar Inc, Madison, USA)

Agrobacterium tumefaciens mediated transient transformation of *Nicotiana benthamiana*

A. tumefaciens harboring the desired binary expression vector were grown for one to two days in 10mL YEB and the appropriate antibiotics in 50mL Falcon tubes in a shaking incubator at 28°C and 200-250 rpm.
Subsequently, the bacteria were centrifuged at room temperature at 4000g for 15 min and resuspended in 10mL induction medium (1.05% (w/v) K_2HPO_4, 0.45% (w/v) KH_2PO_4, 0.1% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $C_6H_5Na_3O_7x2H_2O$, 1% (w/v) glucose, 1% (w/v) fructose, 0.4% (v/v) glycerol, 1mM $MgSO_4$, 10mM MES, 50µg/ml 4′-hydroxy-3′,5′-dimethoxyacetophenone, pH 5.6). The bacteria were incubated in induction medium for 4h, 200rpm, 28°C. Subsequently, the bacteria were harvested by centrifugation at room temperature at 4000g for 5min. The supernatant was discarded and the pellet resuspended in 5ml infiltration medium (10mM $MgCl_2$, 10mM MES, 150µg/ml 4′-hydroxy-3′,5′-dimethoxyacetophenone, pH 5.3). For subsequent infiltration into *N. benthamiana* leaves, OD600 was adjusted to 0.8. This injection-ready solution was left slowly shaking in 50mL falcontubes at room temperature for half an hour to further induce *A. tumefaciens*. Subsequently, the bacteria were pressure-infiltrated into leaf parenchyma using 1mL syringes. To ensure T-DNA transfer into the *N. benthamiana* genome and protein expression, plants were kept for two days in a phytochamber set up at *N. benthamiana* growth conditions.

Stable transformation of *Arabidopsis thaliana* by floral dip

Plants were transformed at the early flowering stage. The method used was according to the method published by Steven Clough and Andrew Bent (Clough and Bent, 1998). Briefly, transformed *A. tumefaciens* harboring the plasmid of interest were inoculated with 250ml of YEB-medium charged with the appropriate selection antibiotics. The culture was grown for two days in a shaker-incubator at 28°C and 200rpm. The suspension was centrifuged at 4°C at 5000rpm for 30min. and the supernatant was discarded. OD_{600} was adjusted with a 5% sucrose solution to a value of 0.8. 200µl of Silwet L-77 were mixed with 400ml of the bacterial solution using a magnetic stir bar. Subsequently, the inflorescences were dipped into the solution for approx. 10s. After a few minutes of drying the plants were placed for 3 days under a dome in a phytochamber at long-day conditions. After maturation of the plants, the seeds were harvested and selection of positively transformed progeny was done using selective MS plates. Seeds were ethanol sterilized and sown under sterile conditions on MS-plates and kept in the dark at 4°C for approx. 3 days. Seeds were allowed to germinate in a permanent light-chamber under sterile conditions and grown for about 7

days. Seedlings and were used for further experiments or transferred into soil and placed in a phytochamber at short-day conditions to mature and/or produce seeds.

Protein extraction from plant tissue

Plant material was shock frozen in liquid nitrogen and ground in a mortar or a ball mill until a free floating powder was yielded. Plant powder was mixed in a 1:1 mixture ratio (w/v) of powder and extraction buffer (50mM TRIS-HCl, 50mM NaCl, 3mM $MgCl_2$, pH 8.0). Additionally, 1µl protease inhibitor cocktail (Sigma, St. Louis, USA) per 30mg plant tissue was added. Proteins were extracted by vigorous mixing of the sample using a drill and pestle.

SDS-polyacrylamide gel electrophoresis

The reagents and SDS-polyacrylamide gel preparation methods were according to Laemmli using the Mini PROTEAN II gel system (BioRad, Hercules, USA) (Laemmli, 1970). Tanks were filled with Tris-glycine electrophoresis buffer (25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS), pH 8.3). Samples were commonly loaded onto 7% SDS-polyacrylamide gels (7% acrylamide mix, 375mM TRIS-HCl, pH 8.8, 0.1% SDS, 0.1% 10%APS, 0.08% TEMED) covered with a 1% SDS-polyacrylamide stacking gel (1% acrylamide mix, 130mM TRIS-HCl, pH 6.8, 0.01% SDS, 0.1% 10%APS, 0.08% TEMED, trace of bromphenolbue)
Prior to loading into the wells, the prepared samples were mixed with 2x SDS reducing buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 0.1% (w/v) bromophenolblue, and 50mM β-mercaptoethanol, added freshly), and boiled at 95°C for 5 min. A subsequent quick-spin in a tabletop centrifuge pelleted the insoluble debris and the supernatant was used for loading the wells. All gels included a molecular size marker. Commonly, the the 7– 175kD Broad Range Prestained Protein Marker from NEB (Beverly, USA) was used. Electrophoresis was powered by an EBV5000 power device from BioRad by applying a voltage of 150 V for 1 h or until the bromophenol stain migrated out of the gel.

Western blotting

PVDF Immobilon-P transfer membrane (0.45 µm pore size) was activated in pure MeOH, rinsed with ddH_2O and equilibrated in buffer anode-II (25mM TRIS-HCl, pH 10.4 + 10% MeOH freshly added). Three Whatman-papers were soaked with either buffer anode-I (300mM TRIS-HCl, pH 10.4 + 10% MeOH freshly added),

buffer anode-II or buffer cathode (25mM TRIS-HCl, 40mM aminohexanoic acid, pH 9.4 + 20% MeOH freshly added) and arranged in a Trans-Blot semi-dry transfer cell (BioRad, Hercules, USA). The transfer cassette was arranged in the following manner (from anode to cathode): Whatman-anode-I / Whatman-anode-II / PVDF-Membrane / gel / Whatman-cathode. During the stacking process, all layers were exactly aligned to prevent air-bubbles.
Transfer from the gel onto the membrane was powered by an EBV5000 power device from BioRad by applying a current of 75 mA per gel for 1h. For verification of the transfer, the membrane was stained in Ponceau-red solution (1% (w/v) Ponceau S, 5% (w/v) glacial acetic acid) for 5 min. After verification, the PVDF membrane was destained in buffer PBS (140mM NaCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, and 2.7mM KCl, pH 7.3) and used for further immunological detection of transferred proteins.

Immunoprecipitation

Polyclonal rabbit antibodies raised against the C-terminal peptide KANSFREDRNEDREV of FLS2 were used (Chinchilla et al, 2005). Antibodies were used on protein gel blots using goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma Aldrich) to detect and stain for immunoreactive proteins. For immunoprecipitation, anti-FLS2 antibodies were incubated with protein A–Sepharose (Amersham Biosciences) at 4°C on a rotary shaker. After 1h of incubation, the solubilzed proteins were added and the mixture was incubated on a rotary shaker at 4°C for 1h. The pellet was washed three times with 1ml with detergent buffer and twice with binding buffer. The samples were further analyzed either by boiling in Laemmli buffer for 5 min and protein gel blot analysis or by measurement of flg22 and flg22-derivatives binding activity.

Binding assay

Binding of the ^{125}I-Tyr-flg22 to crude plant extracts and immunoprecipitates was done as described previously (Chinchilla et al, 2006).
Briefly, aliquots of crude plant extracts or immunoprecipitates were resuspended in binding buffer [25 mM 2-(*N*-morpholino)-ethane-sulfonic acid/KOH, pH 6.0, 3 mM $MgCl_2$, and 10 mM NaCl] in a total volume of 100µL with ^{125}I-Tyr-flg22 (60 fmol in standard assays) for 30 min either alone or with different concentrations of unlabeled competing flagellin-derived peptides as indicated. Cells or immunoprecipitates were collected by vacuum filtration on glass fiber filters (Macherey-Nagel; 2.5 cm diameter, preincubated with 1% BSA, 1% bactotrypton, and 1% bactopepton in binding buffer) and washed with 10mL of ice-cold binding

buffer. Radioactivity retained on the filters was determined by γ-counting (Kontron Instruments).

Growth Inhibition assay

After germination and six days of growth in constant light on solid MS-plates, sterile seedlings were transferred into 24-well tissue culture plates. Each well contained one or two seedlings and 500µL liquid MS-medium. Flg22 or flg22-derivatives were or were not added to the wells. After a growing period of 14 days in constant light, the fresh weight was determined.

Measurement of reactive oxygen species

Leaves were cut in approx. 1mm x 1mm squares and incubated in ddH_2O over night. Production of reactive oxigen species (ROS) upon elicitor treatment was detected in a luminol-dependent chemiluminescence reaction by quantifying light emission of oxidized luminol in the presence of peroxidase. Single squares were transferred into 96-well M Lumitrac-200 microplates. Each well contained 100µL ddH_2O supplemented with 10µg horseradish peroxidase as the catalyst and 100µM luminol as substrate for oxidation by ROS. Oxidation of the luminol by ROS was quantified in a multiwell-luminometer. Relative light units (RLU) were commonly recorded for at least 30min. The indogenous leaf ROS production was measured for 10min before elicitation with flg22 or flg22-derivatives.

Ethylene accumulation assay

Plant leaves were cut in strips of approx. 10mm x 1mm and incubated with ddH_2O over night. When cutting transiently transformed *N. benthamiana,* large leaf venation areas were omitted. Two to four randomly picked strips were transferred into glass tubes containing 500mL ddH_2O. Flg22 and/or flg22-derivatives were or were not added in various concentrations to the samples. The tubes were closed with rubber lids and incubated slightly shaking at room temperature for three to four hours. The ethylene accumulation was analyzed by gas chromatography via FID detection using a GC-14A from Shimadzu Corporation (Kyoto, Japan).

Peptides and radiolabel

Flg22 and the fl22-derived peptide flg22-Δ2 were obtained from EZ-Biolabs (Carmel, USA). PaRm22 was obtained from GeneScript Inc. (Piscataway, USA). Flg22-ΔA17 was obtained from Peptron Inc. (Daejaeon, South Korea). Flg15, flg22$^{E.coli}$, flg15$^{E.coli}$ and PaRm15 were obtained from the peptide-synthesis unit of the Friedrich-Miescher-Institute, Basel. Peptides were dissolved and diluted in a solution containing 0.1% BSA and 0.1 M NaCl. Tyr-flg22 was labeled with ^{125}I by Anawa Trading AG (Wangen, Switzerland) to yield ^{125}I-Tyr-flg22 with a specific radioactivity of >2000 Ci/mmol

Plant Material

Arabidopsis thaliana and *Nicotiana benthamiana* plants were grown in single pots at 21°C/18°C and 25°C/22° and an 8 hr photoperiod, or on plates containing MS medium (Duchefa), 1% sucrose, 0.05% MES and 0.8% agar under continuous light. Transgenic *Arabidopsis thaliana* were selected on MS-plates using 35µg/ml kanamycin.

Construction of the chimeric receptors

Construction of chimeric receptors between AtFLS2 and LeFLS2 was done using the pCAMBIA2300 vector as backbone. All constructs were verified by sequencing. For all cloning related PCR amplifications the Phusion high-fidelity DNA polymerase from Finnzymes (Espoo, Finland) was used according to the manufacturers instructions. Restriction enzymes were purchased at NEB (Ipswich, USA) and used according to the manufacturers instructions. The cloning-strategy was to make use of conserved amino acid pairs occurring in AtFLS2 and LeFLS2 to which the recognition sites of the restriction enzymes used for cutting are corresponding when re-ligated and translated. The previously published (Robatzek et al, 2006) FLS2p:AtFLS2-3xmyc-GFP construct in pCAMBIA2300 from CAMBIA (Canberra, AUS) as starting material.

The AtFLS2-3xmyc-GFP was excised via BamHI and SalI and a new multiple cloning site (**GGATCC**ACGCGT**CCTAGG**TTAATTAA**GGCGCGCC**GTCGAC), incorporating the restriction enzyme sites BamHI, MluI, AvrII, PacI, AscI and SalI was introduced via BamHI and SalI. Subsequently, the eGFP-sequence was PCR-amplified and cloned via the AscI and SalI sites (underlined) using the following primers:

5’-TTAAGGCGCGCCTATGGTGAGCAAGGGCGAG-3’
5’-TTGCAAGTCGACTTACTTGTACAGCTCGTC-3’

Subsequently, the AtFLS2 signal sequence was PCR-amplified and cloned via the BamHI and AvrII sites (underlined) using the following primers:
5'-ACTCTTCTAAAGTCGGATCCATGAAGTTACTCTCAAAGAC-3'
5'-GATCCCTAGGCTGGTTCGAAGCTCTGTTTCGCTAGTGCAATGC -3'
The translated nucleotide sequence of the restriction enzyme BstBI (double underlined) correlates to the conserved Phe and Glu at position 27 and 28 of AtFLS2 and LeFLS2. The resulting construct FLS2p:AtFLS2sig-GFP was termed pOrigin3.

Construction of AtFLS2:GFP

The AtFLS2 sequence was PCR amplified and cloned via the BstBI and AscI sites (underlined) into pOrigin3 using the following primers:
5'-CAGAGCTTCGAACCAGAGATCGAAGCTTTGAAATCC-3'
5'- AATTCTATGGCGCGCCGGCAACTTCTCGATCCTCGTTACGA -3'

Construction of Le1-6:GFP, Le1-18:GFP, Le1-24 :GFP and Le1-28 :GFP

First, the respective LeFLS2-sequence-parts were PCR amplified and cloned via the BstBI and AvrII sites (underlined) into pOrigin33 using the following primers:
5'-CAAGATTCGAAGTTGAAGTTGCTGCTTTGAAAGC-3'
5'-AATTCCTAGGTATAGGTCCAGATAACTGG-3'
5'-CAAGATTCGAAGTTGAAGTTGCTGCTTTGAAAGC-3'
5'-TCGACCTAGGAATGGCACCTGAGAAACTGTTTTT-3'
5'-CAAGATTCGAAGTTGAAGTTGCTGCTTTGAAAGC-3'
5'-GGGACCTAGGAATGCTGCCTGACAGATTATTATTTG-3'
5'-CAAGATTCGAAGTTGAAGTTGCTGCTTTGAAAGC-3'
5'-GCTACCTAGGAATGTGACCTTCAAGTTGGTTG-3'
Subsequently, the respective AtFLS2-sequence-parts were PCR amplified and cloned via the AvrII and AscI sites (underlined) using the following primers:
5'-AATACCTAGGGATTTTGGAAATCTCTTGAACTTACAG-3'
5'- AATTCTATGGCGCGCCGGCAACTTCTCGATCCTCGTTACGA -3'
5'-AATCCCTAGGGAGATGTCGAATCTCACTCTCCTCCAGG -3'
5'- AATTCTATGGCGCGCCGGCAACTTCTCGATCCTCGTTACGA -3'
5'-TATTCCTAGGTCTTTACAGGCCTGCAAAAATGTG-3'
5'- AATTCTATGGCGCGCCGGCAACTTCTCGATCCTCGTTACGA -3
5'-TGTTCCTAGGTCCGGGGTGTTCAAAAACATCAACGC-3'
5'- AATTCTATGGCGCGCCGGCAACTTCTCGATCCTCGTTACGA -3

Construction of Le1-10:GFP

First, the respective LeFLS2-sequence-part was PCR amplified and cloned via the BstBI and AvrII sites (underlined) into pOrigin3 using the following primers:
5'-CAAGATTCGAAGTTGAAGTTGCTGCTTTGAAAGC-3'
5'-GCTACCTAGGGCAGACTAGTTAAGGATCCCAACTGGGGAGGAATATT -3'
Subsequently, the respective AtFLS2-sequence-part was PCR amplified and cloned via the SpeI and AscI sites (underlined) using the following primers:
5'-TGACACTAGTCTTGAAGTCCTCACACTTC-3'
5'-TCGTAACGAGGATCGAGAAGTTGCCGGCGCGCCATAGAATT-3'

Construction of Le11-24:GFP and Le19-24:GFP

The AvrII-site of Le1-24:GFP was mutated by site directed mutagenesis using the following primer: 5'-CTGTCAGGCAGCATTCCCAGATCCCTAGAACGCTGC-3'
First, the respective AtFLS2-sequence-part was PCR amplified and cloned via the BstBI and AvrII sites (underlined) into pOrigin3 using the following primers:
5'-CAGAGCTTCGAACCAGAGATCGAAGCTTTGAAATCC-3'
5'-GTCACCTAGGAAGACTAGTAAGAAAACCGATTTCTTCTGATATCG-3'
5'-CAGAGCTTCGAACCAGAGATCGAAGCTTTGAAATCC-3'
5'-TCTCCCTAGGGATTCTCCCTGTGAAACCATTAGAG-3'
Subsequently, the respective sequence-parts were PCR amplified from the AvrII-mutated Le1-24:GFP and cloned via the SpeI and AscI sites (underlined) and AvrII and AscI sites, respectively, using the following primers:
5'-CTTAACTAGTCTAGAAGTGCTTACCCTTCACTCC-3'
5'- AATTCTATGGCGCGCCGGCAACTTCTCGATCCTCGTTACGA -3'
5'-CATTCCTAGGGAAATTTCAATGCTTTCAAACCTTC-3'
5'- AATTCTATGGCGCGCCGGCAACTTCTCGATCCTCGTTACGA -3'

Construction of Double:GFP

First, the respective Le1-10:GFP sequence-part was PCR amplified and cloned via the BstBI and AvrII sites (underlined) into pOrigin3 using the following primers:
5'-CAAGATTCGAAGTTGAAGTTGCTGCTTTGAAAGC-3'
5'-GCTACCTAGGGCAGACTAGTTAAGGATCCCAACTGGGGAGGAATATT -3'
Subsequently, the respective Le19-24:GFP-sequence-part was PCR amplified and cloned via the SpeI and AscI sites (underlined) using the following primers:
5'-TGACACTAGTCTTGAAGTCCTCACACTTC-3'
5'-TCGTAACGAGGATCGAGAAGTTGCCGGCGCGCCATAGAATT-3'

Bacteria used

All plasmids were transformed in *Escherichia coli* DH5α for subcloning and storage using the standard chemical transformation protocol. Final constructs were electrotransformed into *Agrobacterium tumefaciens* GV3101 and used for agrobacterium mediated transient and stable transformation of *Arabidopsis thaliana* and *N. benthamiana*.

Transient transformation of *Nicotiana benthamiana*

Agrobacterium mediated transient transformation of Nicotiana benthamiana leaves was performed as previously described (Robatzek et al, 2007).

Stable transformation of *Arabidopsis thaliana*

Stable transformation of Arabidopsis thaliana was performed by floral dip, as described by Clough, SJ and Bent, AF, 1998.

Protein Extraction

Plants were homogenized in extraction buffer (25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 10 mM $MgCl_2$) and supplemented with the protease inhibitors (cocktail P9599 from Sigma-Aldrich). For immunoprecipitation assays, proteins were solubilized from plant extracts with a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl and 1% (w/v) octylphenoxypolyethoxyethanol (Nonidet P-40) as detergent. After 1h incubation at 4°C with slight shaking, the solution was centrifuged and the supernatant containing the solubilized proteins was used for

immunoprecipitation. The broad range prestained protein marker (NEB) was used routinely as molecular mass standard for proteins.

Sequence data and accession numbers

Sequence data of AtFLS2 from this work can be found in the GenBank/EMBL libraries under accession number At5g46330. The sequence data for LeFLS2 can be found in (Robatzek et al., 2007b)

Results

Two distinct regions on the LRR-domain of LeFLS2 confer specificity of the tomato flagellin receptor LeFLS2.

Abstract

Both Arabidopsis and tomato harbor highly sensitive perception systems for bacterial flagellin. The respective receptors, AtFLS2 and LeFLS2 have been identified and characterized. However, the molecular basis for flagellin recognition is largely unknown. Here, we demonstrate that Arabidopsis and tomato flagellin receptors discriminate between different variants of flg22, the synthetic peptide shown to comprise the eliciting epitope of flagellin. We used these species-specific differences to map regions on LeFLS2 that are responsible for species-specific characteristics of flagellin binding and receptor activation. We constructed and transformed a series of chimeric receptors between AtFLS2 and LeFLS2 in Nicotiana benthamiana and Arabidopsis thaliana and performed binding assays using immunoprecipitated receptors. Based on this we show that the initial ten N-terminal LRRs between the amino acids 32-337 and within this area, especially the amino acids 236-337 are of pivotal importance for the previously described higher affinity of LeFLS2 to flg22 and N-terminally truncated flg22-derivatives. We further show that an additional region between the LRR 19 to 24 of LeFLS2 is involved in the recognition of C-terminally altered flg22-derivatives. These data provide new insight towards the understanding of FLS2-ligand interaction

Introduction

Flagellin sensing is a widespread mechanism for detection of bacterial invasion in a broad variety of plant species (Felix et al., 1999). The Leucine Rich Repeat-Receptor Like Kinase (LRR-RLK) Flagellin Sensing 2 (FLS2) has been described as the bona fide receptor for flagellin in *Arabidopsis thaliana*. It directly binds flg22, the elicitor active epitope of the bacterial MAMP flagellin (Chinchilla et al., 2006). AtFLS2 is a transmembrane protein consisting of 28 extracellular LRRs, a single transmembrane domain and a cytoplasmic serine/threonine kinase domain.

Little is known about how AtFLS2 physically binds its ligand and how it becomes activated in order to transduce the extracellular, ligand binding event to the cytoplasmic serine/threonine kinase. From a molecular structure-function point of view, so far only the AtFLS2 extracellular LRRs 9–15 were identified as to contribute significantly to flg22 binding (Dunning et al., 2007). The LRR-RLK BAK1 has been shown to rapidly interact with AtFLS2 upon binding of flg22 and to form a complex with AtFLS2 (Chinchilla et al., 2007). However, BAK1 does not seem to be involved in binding of flg22 to AtFLS2 (Chinchilla et al., 2007).

Orthologues of AtFLS2 have been identified in tomato (Robatzek et al., 2007b) and, functionally, in *Nicotiana benthamiana* (Hann and Rathjen, 2007). The tomato FLS2 (LeFLS2) and AtFLS2 share 55% amino acid identity in the LRR domain and 59% in their kinase domain (Robatzek et al., 2007b). Like AtFLS2, LeFLS2 consists of 28 extracellular LRRs, a single transmembrane domain and a cytoplasmic serine/threonine kinase domain. LeFLS2 has been proven to confer tomato specific ligand-specificity when heterologously expressed in *Nicotiana benthamiana* (Robatzek et al., 2007b).

Comparative studies between the Brassicaceae *Arabidopsis thaliana* and the Solanaceae *Solanum lycopersicum* revealed distinct differences in the specificity for derivatives of the flg22 peptide (Meindl et al., 2000; Bauer et al., 2001b; Chinchilla et al., 2006). Flg15, a derivative of flg22 is truncated in the N-terminal seven amino acids. It is highly active in tomato whilst in Arabidopsis, flg15 elicits defense responses only at higher concentrations, thus indicating a lower affinity of AtFLS2 for this peptide (Meindl et al., 2000; Bauer et al., 2001b; Chinchilla et al., 2006). Flg22-Δ2, a flg22 derivative lacking the C-terminal two amino acids, also elicits defense responses in tomato but acts as a competitive antagonist for flg22 binding in Arabidopsis (Chinchilla et al., 2006). Truncation by seven amino acids at both the N- and the C-terminus of flg22 results in the peptide flg15-Δ7. This particular derivative has been shown to act as a competitive antagonist for flg22 induced elicitation of tomato cell cultures whereas no effects of this peptide

could be observed when tested on Arabidopsis (Meindl et al., 2000; Chinchilla et al., 2006). In general, the minimal flagellin epitope that is required to bind and activate to LeFLS2 does not require a large portion of the N-terminal amino acids and the C-terminal amino acids can be more variable than in Arabidopsis. However, the biological significance of differential flagellin recognition by Arabidopsis and tomato FLS2 remains unknown. Species specific differences for flagellin perception have also been described for the mammalian flagellin receptor Toll-Like Receptor 5 (TLR5). TLR5 is a type I transmembrane proteins with 22 LRR motifs in the extracellular domain but no cytoplasmic kinase domain (Hayashi et al., 2001). Comparative studies between the human TLR5 (hTLR5) and mouse TLR5 (mTLR5) revealed that mTLR5 detects flagellins derived from *Escherichia coli and other bacteria* at lower doses than hTLR5. Chimeric receptors between mouse and human TLR5 showed that the central 228 amino acids (residues 174-401) of the TLR5 extracellular domain are responsible for species-specific flagellin recognition (Andersen-Nissen et al., 2007).

Binding and activation of the flagellin receptor in tomato and Arabidopsis has been proposed to occur following the address-message concept: Part of the ligand molecule is required for binding, conferring selectivity for the receptor (address), and part for the biological action, by mediating initiation of signal transduction (message) (Meindl et al., 2000). The N-terminal- and especially the core amino acids *RINSAKDDA* of flg22 have been shown to be important for the initial interaction with the receptor (Meindl et al., 2000).

We tested tomato, Arabidopsis and *Nicotiana benthamiana* with a variety of flg22-derivatives to identify flg22-derivatives that are perceived differently in these three species. We could identify species-specific patterns for flg22 derivatives that are able or not to elicit defense responses in Arabidopsis, tomato and *N. benthamiana*. We also constructed chimeric flagellin receptors between AtFLS2 and LeFLS2. Parts of the LRR domain of AtFLS2 were consecutively replaced by the respective LeFLS2-LRRs. We expressed these chimeric receptors in *Nicotiana benthamiana* and screened for tomato specific flg22-derivatives in order to identify regions on the LRR that confer tomato specificity. In addition, we analyzed the binding properties of the chimeric receptors to further characterize differences between LeFLS2 and AtFLS2. In summary we show that the LeFLS2 LRRs 7-10 are key LRRs for the higher affinity of LeFLS2 for flg22 and derivatives and that the region between LeFLS2 LRRs 19-24 plays a role for tomato type perception of the C-terminal region certain flg22-derivatives.

Results

Tomato and Arabidopsis show distinct species-specific differences in the eliciting activity of various flg22-derivatives

Both tomato and Arabidopsis have a highly sensitive perception system for bacterial flagellin and the flagellin based synthetic peptide flg22 (Felix et al., 1999). Both the tomato and the Arabidopsis receptors for flagellin sensing, AtFLS2 and LeFLS2 have been identified and characterized (Gómez-Gómez and Boller, 2000; Robatzek et al., 2007a). From previous work it is known that both plant species are stimulated to elicit defense responses such as medium alkalinization, induction of ethylene production or synthesis of reactive oxygen species (ROS) upon treatment with sub-nanomolar concentrations of flg22.
It has been shown that tomato and Arabidopsis have distinct differences in the perception of modified flg22 peptide derivatives. Here we tested both Arabidopsis and tomato leaf material with an array of various flg22-derivatives to investigate if these peptides were able to elicit defense in planta (Fig 1A). Induction of ethylene production was used as a highly sensitive bioassay for elicitor activity. Both plant species reacted clearly and strongly with an increase of ethylene production upon addition of flg22, the respective epitope from the bacterium *E.coli* flagellin, $flg22^{E.coli}$ and flg15, a peptide lacking the initial seven amino acid residues at the N-terminus of flg22 at a concentration of 1µM (Fig 1B). Flg15, has been shown previously to be ~100-fold less active than flg22 in pH alkalinization assays using cell culture suspensions (Meindl et al., 2000; Bauer et al., 2001b). However, when using a concentration of 1µM, no significant difference between flg22 and flg15 could be observed in the ethylene bioassay using plant leaf material (Fig 1B). In contrast, when using the 15mer peptide derived from the flagellin sequence of *E. coli*, $flg15^{E.coli}$, a species-specific, significant difference could be observed between Arabidopsis and tomato (Fig 1B). Arabidopsis plants were completely insensitive to $flg15^{E.coli}$ when treated with 1µM but tomato plants showed a clear and significant increase of ethylene biosynthesis. It is an interesting observation that, at a concentration of 1µM, Arabidopsis did not show a significant difference of ethylene biosynthesis when the two peptides flg22 and flg15 were added, while a clear difference for $flg22^{E.coli}$ and $flg15^{E.coli}$ could be detected (Fig 1B).

To assess the importance of the C-terminal region of the flg22 peptide we tested peptides which were altered in the C-terminal region. PaRm22 and PaRm15 are hybrid peptides composed of the initial 17 (PaRm22) or 10 (PaRm15) amino acid residues of the N-terminus of flg22 and the C-terminal 5 amino acid residues of

the inactive flg22 peptide from *Agrobacterium tumefaciens/Rhizobium meliloti* (Fig 1A) (Meindl et al., 2000). Using these peptides we observed that tomato plants were clearly elicited while Arabidopsis leaf tissue remained completely insensitive for both PaRm22 and PaRm15 (Fig 1B). We further tested flg22-derivatives that were known to act as competitive antagonists for flg22 mediated medium pH alkalinization in either Arabidopsis or tomato cell culture suspensions. The peptide flg22-Δ2, a flg22-derivative lacking the C-terminal two amino acid residues (Fig 1A), was acting as a tomato specific elicitor of ethylene accumulation while no elicitation activity was observed for Arabidopsis (Fig 1B) (Chinchilla et al., 2006). Conversely, the peptide flg22-ΔA17 ,(Fig 1A), was acting as an agonist for induction of ethylene biosynthesis in Arabidopsis, while no in increase of ethylene production could be detected for tomato plants (Fig 1B) (Chinchilla et al., 2006). In summary, by testing different flg22-derivatives, we could confirm in planta, that tomato and Arabidopsis exhibit a distinct species-specific pattern in the recognition of flg22-derivatives.

Arabidopsis and tomato have different binding affinities for the flg22-derivatives flg22-Δ2, PaRm22 and PaRm15

We further tested the binding affinity of crude extracts of Arabidopsis and tomato leaf tissue. The specificity of binding was tested in competitive binding assays. In competitive binding experiments the binding of a single concentration of labeled ligand is measured in the presence of various concentrations of unlabeled ligand. Ideally, the output of a competitive binding experiment is a sigmoid shaped curve. The concentration of unlabeled ligand that produces radioligand binding half way between the upper and lower plateaus of the curve is called the IC50 (Inhibitory Concentration 50%). For this experiment we used increasing concentrations of various flg22-derived peptides as competitors of binding of ^{125}I-Tyr-flg22, a radiolabeled flg22 variant. 30min binding assays at 4°C of ^{125}I-Tyr-flg22 in the presence of different amounts of flg22-Δ2, PaRm22 and PaRm15 are shown in (Fig. 2). All of the used competitor peptides clearly induced increased ethylene biosynthesis in tomato at a concentration of 1µM but only flg22 did so in Arabidopsis (Fig. 1B). The overall emerging picture was that tomato did not significantly differentiate between the binding of the peptides flg22, flg22-Δ2 and PaRm22. These three peptides were competing with a similar efficiency with ^{125}I-Tyr-flg22 for binding sites and the calculated IC50 values were 1nM for flg22 and PaRm22 and 2nM for flg22-Δ2, respectively (Fig. 2A/B/C, circles). However, PaRm15 was clearly less efficient when competing with ^{125}I-Tyr-flg22 for binding sites. The IC50 value for PaRm15 in tomato tissue was measured at around 75nM (Fig. 2D, circles).

However, the examined peptides each strongly differed in their ^{125}I-Tyr-flg22 competitor ability when tested with *Arabidopsis thaliana (*Col-0) tissue. The best competitor was, as expected, the flg22 peptide with an IC50 of 20nM (Fig. 2A, triangles). But also the three non-ethylene inducing peptides were able to compete with ^{125}I-Tyr-flg22 for binding sites, although only at higher concentrations. Flg22-Δ2 reached IC50 at a concentration of 1µM (Fig. 2B, triangles). The PaRm22 peptide had an IC50 value of 200nM while PaRm15 virtually did not show competing activity unless used in extremely high concentrations, which is demonstrated by the IC50 of 50µM when competing for binding sites with ^{125}I-Tyr-flg22 (Fig. 2C/D, triangles). Our results suggest that the peptide PaRm22 has a stronger antagonistic activity than flg22-Δ2 when competing with flg22 for binding sites.

AtFLS2/LeFLS2 chimeric receptors are functional in *Nicotiana benthamiana*

We made use of the species-specific pattern of flg22 and flg22-derivative recognition of tomato and Arabidopsis in order to map areas on the LRR domains which are responsible for either tomato or Arabidopsis type of binding. For this, we constructed a series of chimeric flagellin receptors by swapping increasingly more LRR subunits of LeFLS2 into AtFLS2 (Fig. 3). All constructs were cloned in frame with an N-terminal GFP sequence into a modified pCAMBIA2300 vector backbone. Expression of the constructs was driven by the AtFLS2 promoter region (1000bp upstream ATG).

We first assayed *Nicotiana benthamiana* for its endogenous pattern of flg22-derivative recognition. We wanted to find out whether additional peptides besides flg15$^{E.coli}$ (Robatzek et al., 2007b, a) are uniquely specific for tomato type of recognition. Using the ethylene bioassay, we screened *Nicotiana benthamiana* leaves for induction of ethylene production upon treatment with various flg22-derivatives. As for tomato and Arabidopsis, flg22, flg15 and flg22$^{E.coli}$ applied at a concentration 1µM elicited a clearly detectable ethylene accumulation in *Nicotiana benthamiana* (Fig. 4A). However, a specific pattern emerged when we further tested the flg22-derivatives PaRm22, PaRm15, flg22-Δ2 and flg22-ΔA17: In contrast to Arabidopsis and similar to tomato, both PaRm22 and flg22-Δ2 were eliciting an ethylene accumulation in *Nicotiana benthamiana* leaves (Fig. 4A). This indicated specificity more closely related to that of tomato. This was to be expected since the two plants both belong to the *Solanaceae* family and thus are closely related. However, when we treated with flg22-ΔA17, unlike tomato and similar to Arabidopsis, a clear ethylene accumulation could be observed (Fig.

4A). Finally, the peptides flg15$^{E.coli}$ and PaRm15 did not induce ethylene production in *N. benthamiana* and proved to be flg22-derivatives that elicit ethylene accumulation only in tomato plants (Fig. 4A and Fig1B).

To test the chimeric receptors for tomato specific recognition of flg15$^{E.coli}$ and PaRm15, we used the well established system of Agrobacterium-mediated transient transformation of *N. benthamiana* leaves. Western blot analysis using transiently transformed *Nicotiana benthamiana* leaf extracts and antiGFP-antibodies showed that all proteins were expressed whereas no signal was detected when plants were transformed with an empty vector construct (Fig. 4B). The detected bands for the GFP-tagged proteins indicated a molecular mass of ~200 kDa. However, we observed repeatedly that that LeFLS2:GFP had a slightly lower molecular weight than AtFLS:GFP or the GFP-tagged chimeric receptors (Fig. 4B).

We tested the leaves of the transiently transformed *Nicotiana benthamiana* for ethylene accumulation upon treatment with 1µM of flg22 and the tomato specific flg22-derivatives flg15$^{E.coli}$ and PaRm15 (Fig. 4C/D). In all cases, we could see a clear accumulation of ethylene when leaves were treated with 1µM flg22, thus indicating the viability of our samples. We subsequently confirmed that samples transformed with AtFLS2:GFP do not respond to 1µM flg15$^{E.coli}$ (Fig 4C) (Robatzek et al., 2007b). Additionally, no elicitation could be observed when we treated the leaves with 1µM PaRm15 (Fig 4C). Samples transformed with LeFLS2:GFP showed a clear increase in ethylene production upon treatment with both peptides and thus were displaying the expected tomato specificity (Fig. 4C).

We next tested the chimeric FLS2 receptors. No tomato typical elicitation activity of the flg15$^{E.coli}$ or PaRm15 peptides could be observed in leaves transformed with Le1-6:GFP (Fig. 4D). However, Le1-10:GFP transformed leaves showed a clear ethylene accumulation when treated with flg15$^{E.coli}$ but not with PaRm22 (Fig. 4D). We observed the same for Le1-18:GFP transformed leaves (Fig. 4D). However, when leaves were transformed with Le1-24:GFP and the construct containing the full tomato LRR, Le1-28:GFP, a clear accumulation of ethylene was observed for both tomato specific flg-derivatives, flg15$^{E.coli}$ and PaRm15 (Fig. 4D). Thus, we showed that the chimeric receptors are functional. We also showed that they were capable of transferring the tomato specific perception of flg15$^{E.coli}$ and PaRm15 into the non-responding plant *N. benthamiana*.

Chimeric flagellin receptors containing the first ten LLR subunits of LeFLS2 have a higher affinity for flg22 and flg22-derivatives

To further probe the differences between AtFLS2 and the chimeric receptors, we assayed binding of ^{125}I-Tyr-flg22, a radiolabeled derivative of flg22 previously used in binding studies (Meindl et al., 2000). However, due to the endogenous NbFLS2 of *N. benthamiana*, we could not simply perform binding studies using crude plant tissue. We could circumvent this issue by immunoprecipitating our chimeric receptors using the well characterized antiFLS2 antibodies which does not precipitate or coprecipitate NbFLS2 (Chinchilla et al., 2006; Wyrsch, 2010).

IPs from detergent-solubilized extracts of transiently AtFLS2:GFP transformed *N. benthamiana* leaves showed strong binding of the radiolabel (Fig. 5A). By adding increasing amounts of unlabeled flg22 and keeping the samples on ice for 30 min, binding of ^{125}I-Tyr-flg22 to the binding sites was competed in a concentration-dependent manner, and inhibition of the radioligand binding by 50% (IC50) could repeatedly be detected at a concentration of ~2 nM flg22 (Fig. 5A). IPs of Le1-28:GFP showed a higher affinity for flg22 than samples did with AtFLS2:GFP, as shown by the lower IC_{50} value that was calculated for Le1-28:GFP at ~0.5 nM (Fig. 5B).
We further tested the chimeric receptors Le1-6:GFP and Le1-10:GFP. No change in the IC_{50} compared to AtFLS2:GFP was observed when IPs of Le1-6:GFP were tested (Fig. 5C). However, when IPs of Le1-10:GFP transformed leaves were tested, the IC_{50} ranged in the same dimension as for Le1-28:GFP (Fig. 5D). Thus, the determinative residues responsible for the higher affinity of Le1-28:GFP for flg22 are located within the amino acids 32-337. Additionally it can be concluded that the amino acids 236-337 are needed for the higher affinity of LeFLS2 for flg22.

Our findings show that this region is playing a crucial role for the binding of flagellin. Flg15 has also been shown to be an efficient competitor of binding of ^{125}I-Tyr-flg22 in tomato cell cultures at a concentration of 100nM (Chinchilla et al., 2006). However, no significant competition of flg15 for ^{125}I-Tyr-flg22 binding could be observed for Arabidopsis cell cultures at a concentration of 100nM (Chinchilla et al., 2006), indicating that the N-terminal seven amino acids of flg22 are of particular importance for recognition by AtFLS2 but not necessarily for LeFLS2.
Hence, we tested flg15 as a competitor for ^{125}I-Tyr-flg22 binding (Fig. 6). We used IPs of AtFLS2:GFP and the chimeric receptors. IPs of AtFLS2:GFP and Le1-6:GFP were not significantly competed by flg15 at the concentration of 100nM. Neither were IPs of Le11-24:GFP nor Le19-24:GFP. However, the full LeLRR spanning chimera Le1-28:GFP showed a clear and significant

competition of ^{125}I-Tyr-flg22 binding by flg15 at a concentration of 100nM. Additionally, all the subsequently tested chimeric receptors that contain the first ten LeLRRs also showed the same clear competition of flg15 with ^{125}I-Tyr-flg22 for binding sites (Fig. 6). This result suggested that the area between the previously identified amino acids 236-337 of LeFLS2 indeed play a crucial role for the tomato-typical higher affinity of binding of N-terminally truncated flg22-derivatives to LeFLS2 than to AtFLS2.

We next tested ^{125}I-Tyr-flg22 binding performance in competitive binding assays with the peptide flg15-Δ7 (Fig. 7). Flg15-Δ7 is a peptide that spans only the evolutionary most conserved core *RINSAKDD* of the flg22 sequence, without the variable N- and C-termini. Flg15-Δ7 has been shown to have an antagonistic effect for flg22-induced medium alkalinization in tomato cell cultures (Chinchilla et al., 2006). Flg15-Δ7 has also been shown to compete binding of ^{125}I-Tyr-flg22 in tomato cells (Meindl et al., 2000). In *Arabidopsis,* in contrast, this particular peptide is completely inactive, e.g. neither acting as an agonist nor antagonist *when tested in a* medium alkalinization bioassay (Chinchilla et al., 2006). These findings suggest that flg15-Δ7 contains the "core-binding elements" that are necessary for binding to LeFLS2 but not the additionally necessary N-terminal or C-terminal residues which are required to establish a stable interaction with AtFLS2.

No specific competition of ^{125}I-Tyr-flg22 binding to IPs of AtFLS2:GFP could be detected when we were applying flg15-Δ7 up to a concentration of 10µM for 15 min. on ice (Fig 7A). This result confirmed earlier data obtained with cell culture experiments by (Chinchilla et al., 2006). Likewise, when IPs of Le1-6:GFP were tested, we could never detect any flg15-Δ7 competition of ^{125}I-Tyr-flg22 binding up to the tested concentration of 10µM (Fig. 7C). However, when we tested IPs of Le1-28:GFP we could repeatedly observe a striking binding competition of flg15-Δ7 with ^{125}I-Tyr-flg22 for binding sites resulting in an IC_{50} of flg15-Δ7 at around 5nM (Fig. 7B). Interestingly, when IPs of Le1-10:GFP were tested we could also observe also competition of flg15-Δ7 with^{125}I-Tyr-flg22 for binding sites resulting in an IC_{50} value of flg15-Δ7 at 5nM (Fig 7D). Notably, this value is the same as we obtained when testing Le1-28:GFP.
Hence, we can also conclude for the "core"-peptide flg15-Δ7, like for flg22 and flg15, that the amino acids 32-337 harbor important residues of LeFLS2 which are responsible for the binding of flg15-Δ7 to LeFLS2. We could not detect binding of Le1-6:GFP for flg15-Δ7, while Le1-10:GFP was able to bind flg15-Δ7 with a similar affinity as the full LeLRR spanning construct Le1-28:GFP. Accordingly, it can be concluded that within the area of amino acids 32-337, the

amino acids 236-337 of LeFLS2 must be of importance for the higher affinity of LeFLS2 for this peptide when compared to AtFLS2.
We could show that Le1-28:GFP is much less dependent of the N- and C-terminal amino acids of flg22 to establish binding of the RINSAKDD core-motive than AtFLS2 and that this tomato typical specificity is located within the N-terminal ten LRRS of LeFLS2.

Lower reversibility of binding of flg22 to LeLFS2 than AtFLS2

Immunoprecipitates of AtFLS2:GFP, Le1-28:GFP, Le1-6:GFP and Le1-10:GFP were tested to study the reversibility of binding of flg22 to the receptor. First, a standard binding assay was performed. Total binding of ^{125}I-Tyr-flg22 was measured after 30min incubation on ice. Subsequently, binding-buffer containing 10µM non-radioactive labeled-flg22 was added to the samples and the samples were incubated at 4°C for 1 h. During the incubation time, receptor-released ^{125}I-Tyr-flg22 was replaced by the excess of non-radioactive labeled-flg22. The remaining amount of ^{125}I-Tyr-flg22 bound to the receptors was measured. The resulting difference between the CPM value of the first, total binding assay and the remaining amount of radioactivity after 1h of competitive incubation with 10µM non-radioactive labeled-flg22 gives an indication of the reversibility of flg22 binding to the receptors (Fig. 8).

All tested samples showed clear capacity to efficiently bind flg22. However, after 1 hour of incubation with 10µM non-radioactive labeled-flg22 containing binding-buffer, a clear difference between IPs of AtFLS2:GFP and Le1-6:GFP and Le1-28:GFP and Le1-10:GFP could be observed. While samples of both AtFLS2:GFP and Le1-6:GFP retained only about 15% of the bound ^{125}I-Tyr-flg22, Le1-28:GFP and Le1-10:GFP retained both around 75% of the radiolabel measured in the total binding assay (Fig. 8). This result shows that the tomato LRR binds flg22 in a more irreversible way than AtFLS2 and that the responsible area for this irreversibility is located within the N-terminal ten LRR subunits of LeFLS2.

The LeLRRs 19-24 recognize PaRm-type C-terminally altered flg-derivatives

Our previous findings showed that the high affinity of LeFLS2 for the core residues RINSAKDD of flg22 is directed by the LeLRRs 7-10. However, we additionally could observe a change in ligand specificity in the ethylene production bioassay when we tested chimeric receptors including LeLRRs 19-24 in *N. benthamiana* (Fig 4C/D).

Consequently, we hypothesized that this region is also involved in interaction with the ligand. Chimeras not comprising the initial ten LeLRRs but only LeLRRs 19-24 might therefore also show an altered binding performance of the C-terminally altered flg22-derivatives PaRm22 and flg22-Δ2 when compared to AtFLS2 or Le1-28:GFP. Competitive binding assays using IPs of AtFLS2:GFP, Le1-28:GFP, Le11-24:GFP and Le19-24:GFP were performed. The results depicted in Fig. 9 demonstrate that AtFLS2:GFP clearly differentiates between these two peptides in terms of ligand affinity while Le1-28:GFP does not (Fig 9A/B).

For IPs of AtFLS2:GFP, the IC_{50} value of PaRm22 and flg22-Δ2 was calculated at around a concentration of 20nM and 100nM, respectively (Fig. 9A). Compared to the previously determined IC_{50} values for flg22, receptor-ligand affinity appeared to be reduced by a factor of 10 for PaRm22 and 50 for flg22-Δ2, thus showing a the importance of the C-terminus of flg22 and flg22-derivatives in the binding process. The chimeric receptor Le1-28:GFP, e.g. the LeFLS2 extracellular domain, on the other hand, did not distinguish between the two peptides PaRm22 and flg22-Δ2 in terms of binding affinity (Fig. 9B). The IC_{50} value for both peptides was 1nM and thus the affinity compared to flg22 was only decreased by a factor of 2. We next tested Le11-24:GFP and Le19-24:GFP. In both cases we could observe competitive binding of both PaRm22 and flg22-Δ2 (Fig. 9C/D). Parm22 showed in both cases an IC_{50} value of around 9nM, indicating that binding affinity of this peptide is enhanced by two-fold compared to AtFLS2 (Fig. 9C/D, triangles). Interestingly, both chimeric receptors gave similar values for IC_{50} of PaRm22, indicating that LeLRRs 19-24 are sufficient for the observed effect. The results of this binding study stands in line with our previous, functional data showing that ligand specificity is altered when LeLRRs 19-24 are added in chimeric receptors that already contain the preceding, N-terminal LeLRRs. However, the effect of these swaps for flg22-Δ2 perception is less clear. Although an increase in binding affinity can be observed for both Le11-24:GFP and Le19-24:GFP, the IC_{50} values of 70nM and 80nM, respectively, still rather correspond to the AtFLS2-type of binding than Le1-28:GFP (Fig. 9C/D, circles).

Chimeric receptors in *fls2 Arabidopsis thaliana* (Col-0)

In order to assess ligand specificity changes we stably transformed *fls2*$^-$ *Arabidopsis thaliana* (Zipfel et al., 2004) with the chimeric receptors via floral

dipping (Clough and Bent, 1998). However, we were neither able to detect protein expression nor significantly increased ethylene production upon elicitation with flg22 in transgenic Arabidopsis harboring chimeric receptors that comprised the full LeLRR N-terminal domain. Plants were examined for three generations both in the hetero- or homozygous state without yielding reliable results (data not shown).

We subsequently tried to transform *fls2* Arabidopsis with the hybrids Le19-24:GFP, Le11-24:GFP and an additional chimeric receptor, Le1-24b.:GFP. Le1-24b:GFP, in contrast to Le1-24:GFP, contains additional to the signal peptide sequence of AtFLS2 the full AtFLS2 LRR-N-terminal domain (Fig. 3). Using these three chimeric receptors, we were able to detect expression of the transgenes as well as an increased ethylene biosynthesis upon treatment of the plants with various flg22 derivatives (Fig. 10). Expression was verified by western blot using anti-FLS2 antibodies. We tested homozygous transgenic plants and could clearly detect expression of the transgenes, although to a lesser amount than the endogenous AtFLS2 in WT-Col-0 plants (Fig. 10A). However, when we tested the homozygous T3 generation plants in the ethylene bioassay for flagellin responsiveness, a significant increase of ethylene biosynthesis comparable to WT-Col-0 plants could be observed (Fig. 10B). We subsequently tested several of the flg22-derivatives that we used previously for the characterization of Arabidopsis, tomato and *N. benthamiana* to further characterize regions on the LRR responsible for tomato specific flg22-derivative perception (Fig. 10B). We could observe increased ethylene biosynthesis for WT-Col-0 and all transgenic Arabidopsis upon treatment with 1µM flg22. This indicated that all expressed chimeric receptors were functional in Arabidopsis.

We next tested the C-terminally altered peptide PaRm22 at a concentration of 1µM. In the WT Col-0 control, this peptide was, as expected, not eliciting an increase in ethylene biosynthesis. In contrast to WT-Col-0, all tested transgenic Arabidopsis showed a strong increase in ethylene biosynthesis upon addition of PaRm22 (Fig. 10B). Together with our previous finding that chimeric receptors incorporating the LeLRRs 19-24 have a higher affinity for this flg22-derivative that AtFLS2, it appears that this change in binding affinity is linked to the ability of the chimeric receptors to get activated by PaRm22 and initiate ethylene biosynthesis. We next tested the PaRm15 peptide (Fig 10B). Here as well, no eliciting activity could be observed for the WT-Col-0 control. In addition, no eliciting activity was observed in transgenic Arabidopsis harboring the Le19-24:GFP and Le11-24:GFP chimeric receptors. However, when we tested transgenic Arabidopsis harboring the Le1-24b:GFP chimeric receptor, a significant induction of ethylene

biosynthesis was observed upon addition of PaRm15 at a concentration of 1µM. This result indicated that for a proper perception of this particular, both N-terminally truncated and C-terminally altered tomato specific flg22-derivative, the region of LeLRR19-24 alone is not sufficient.

The next peptide tested was flg22-Δ2 (Fig. 10B). We could not detect eliciting activity in WT-Col-0 plants. However, we could detect clear induction of ethylene biosynthesis in the transgenic Arabidopsis plants harboring Le1-24b:GFP and Le11-24:GFP. However, surprisingly, no eliciting activity of flg22-Δ2 could be observed in transgenic Arabidopsis plants harboring the Le19-24:GFP chimeric receptor, indicating that the region between LeLRR ten and LeLRR19 is important for receptor activation by this peptide. We next tested the Arabidopsis-specific flg22-derivative flg22-ΔA17 (Fig 10B). We could observe clear induction of ethylene biosynthesis in the WT Col-0 plants. Interestingly and unexpectedly, we also observed a clear, AtFLS2 typical, induction of ethylene biosynthesis for all transgenic Arabidopsis. This is surprising; especially for the Le1-24b:GFP chimeric receptor as is comprises most of the tomato LRRs except the last four.

A chimeric receptor containing LeLRR 1-10 and LeLRR 19-24 cannot confer perception of tomato specific flg22-derivatives

Following the finding that the LeRRs 19-24 alone are not sufficient to mediate responsiveness to the PaRm15 peptide but to PaRm22, we hypothesized that a chimeric receptor consisting of the LeLRRs 1-10 and LeLRRs 19-24 might be able to do so. We have previously shown that the region between LRRs 1-10 is important for the tomato specific higher affinity of LeFLS2 for both flg22 and N-terminally truncated flg22-derivatives. We therefore agroinfiltrated and tested another chimeric receptor incorporating the LeLRRs 1 to 10 and LeLRRs 19 to 24 in *N. benthamiana* with the peptides flg15$^{E.coli}$ and PaRm15 at a concentration of 1µM (Figure 10). The chimeric receptor Le1-10/19-24:GFP (Fig. 10A), was expressed, as shown by western blot (Fig. 11C). However, we could only observe induction of ethylene biosynthesis when the flg15$^{E.coli}$ peptide was applied. No increase in ethylene biosynthesis was ever observed when we applied the PaRm15 peptide(Fig. 11B).
However, the fact that we observed an eliciting activity of flg15$^{E.coli}$ in transiently transformed *N. benthamiana* leaves, proves that Le1-15/19-24:GFP is a functional receptor able to confer the tomato specific responsiveness for flg15$^{E.coli}$.

Discussion

Species-specific differences of flagellin recognition have previously been reported for both the animal and plant flagellin perception systems (Andersen-Nissen et al., 2007; Robatzek et al., 2007b).

For this project, we chose to map regions on the LRR domains of the flagellin receptors of the brassicacea *Arabidopsis thaliana* and the solanacea *Solanum lycopersicum*. Our approach was to swap parts of the LRR domain of LeFLS2 into AtFLS2 and to test whether biological activity or affinity of the chimeric receptors for selected flg22-derivatives is changed. Our system had the advantage that we could make use of two well characterized flagellin receptors (Gomez-Gomez and Boller, 2000; Robatzek et al., 2007b).

Key to the project was the construction of various chimeric receptors between AtFLS2 and LeFLS2 by consecutively swapping LRRs of LeFLS2 into AtFLS2. A study using a similar approach has previously led to the successful identification of areas within the LRR domains of human TLR5 and its mouse-orthologue, which are responsible for the species-specific differences of flagellin perception between human and mouse (Andersen-Nissen et al., 2007).

In the first part of this work, we tested various flg22-derivatives for their eliciting activity in Arabidopsis, tomato and *N. benthamiana.* We used the ethylene bioassay for testing if a certain flg22-derivative is able to induce an increase of ethylene biosynthesis or not. Ethylene biosynthesis is a very reliable bioassay to detect onset of the plants defense machinery upon MAMP treatment. In the second part of this work, a series of chimeric receptors between AtFLS2 and LeFLS2 was constructed and expressed them transiently in *N. benthamiana* and stably in *fls2* Arabidopsis. The transformed plants were then analyzed for changes in ligand specificity. We further immunoprecipitated chimeric receptors expressed in *N. benthamiana* and performed binding studies. Based on these experiments, we highlight the binding characteristics of AtFLS and LeFLS2 for various flg22-derivatives and compare these characteristics with the binding characteristics of the chimeric receptors. By doing this, we identified two regions that determine species-specific features of flagellin recognition.

Arabidopsis, tomato and *N. benthamiana* each have a different pattern of flagellin perception

We tested various flg22-derivatives for the ethylene biosynthesis inducing activity in Arabidopsis, tomato and *N. benthamiana*. All three plants had a different, specific pattern of peptides which are recognized by the plant or not. All three

plants were clearly elicited by flg22 and flg15 at a concentration of 1µM (Fig. 1B and 4A). However, several flg22-derivatives are differentially recognized by these three plants. Interestingly, we could detect clear differences in the perception of different flg22-derivatives also between the closely related *N. benthamiana* and tomato.

Especially the elicitation patterns of the pairs flg22$^{E.coli}$ / flg15$^{E.coli}$ and PaRm22 / PaRm15 are noteworthy. While flg22$^{E.coli}$ is in all three plants a strong elicitor of ethylene biosynthesis, flg15$^{E.coli}$ only elicits an increase of ethylene biosynthesis in tomato plants, indicating that both *N. benthamiana* and Arabidopsis are not elicited by this 15meric peptide at a concentration of 1µM. Common to both the 22mer and the 15mer peptide is the feature that they share the C-terminal alteration LQ instead of QA at position 19 and 20 of the flg22 sequence (Fig. 1A). However, obviously this C-terminal alteration is of negligible importance for the eliciting activity when the N-terminal seven amino acids are present, as demonstrated by the clear elicitation of Arabidopsis and *N. benthamiana* when flg22$^{E.coli}$ was applied. Apparently, when the seven N-terminal amino acids are truncated, the C-terminal alteration becomes more of an issue when the peptide is interacting with the *N. benthamiana* and Arabidopsis flagellin recognition systems. It might be that the C-terminus is not only a important for activation, as it was proposed before (Meindl et al., 2000), but also has a function in stabilizing the interaction, at least for Arabidopsis and *N. benthamiana*.
This hypothesis is backed up by the observation that flg15, the N-terminally truncated derivative of flg22, still elicits a significant increase in ethylene production at the same concentration (Fig 1B). It can be supposed that *N. benthamiana* and Arabidopsis both lack the LeFLS2 typical feature(s) on their flagellin receptors that provides the tomato flagellin receptor with the ability to interact equally efficiently with both N-terminally truncated flg22-derivatives and full length flg22. Flg15$^{E.coli}$ is therefore a suitable tool to pinpoint regions of LeFLS2-LRR that are responsible for the tomato typical “insignificance” of the N-terminal seven amino acids of flg22 in contrast to Arabidopsis and *N. benthamiana* (Meindl et al., 2000; Bauer et al., 2001b).

In contrast to flg22$^{E.coli}$, the PaRm-type peptides have a C-terminal alteration that seems to be exclusively recognized by the tomato and the *N. benthamiana* flagellin perception systems. No ethylene accumulation was detected when both PaRm22 and PaRm15 were applied to Arabidopsis up to a concentration of 10µM.
Flg22 and PaRm22 differ from each other in the C-terminal residues 17, 18 and 19 (Fig. 1A). It has been shown in earlier experiments by Georg Felix and

coworkers that flg22-derivatives altered or truncated in the C-terminal region often act as antagonists for flg22 elicited defense responses (Meindl et al., 2000; Bauer et al., 2001b; Chinchilla et al., 2006). In this work, it could be demonstrated that both AtFLS2 and LeFLS2 can, however with different affinities, bind PaRm22 but only LeFLS2 gets activated upon perception. Accordingly, it can be concluded that the altered C-terminal region of PaRm22 alone is responsible for its inability to activate AtFLS2. However, PaRm22 still seems to incorporate the necessary properties to both bind with high affinity to LeFLS2 and activate it.

PaRm15, at a concentration of 1µM, exclusively elicited tomato to increase ethylene biosynthesis. Therefore, PaRm22 and PaRm15 are peptides which are suitable to pinpoint region(s) on the LeFLS2-LRR that are involved for the interaction with the C-terminal, and therefore activation guiding, residues of LeFLS2.

To summarize the features of the two peptide pairs flg22 $^{E.coli}$/flg15 $^{E.coli}$ and PaRm22/PaRm15, it appears that the **GQAIA** C-terminus of flg22$^{E.coli}$ still does have the ability to interact and activate both AtFLS2 and LeFLS2, but is probably, at least for AtFLS2, less efficient than the genuine, C-terminal part of flg22. However, for AtFLS2, the remaining residues of this peptide have to perfectly fulfill all the requirements that are needed by AtFLS2 for maximum affinity, indispensably including the N-terminal seven residues.
In contrast, the **YWSIA** C-terminus of PaRm22, even when the rest of the epitope is perfectly identical to the flg22 sequence, is not anymore able to activate AtFLS2. However, LeFLS2 activation is not influenced by this change of the C-terminus.

We further tested the flg22-derivatives flg22-Δ2 and flg22-ΔA17. Flg22-ΔA17 has been described to have a biological activity as a competitive antagonist of flagellin perception in tomato and as an agonist in Arabidopsis when tested in cell culture medium alkalinization assays (Chinchilla et al., 2006). Conversely, flg22-Δ2 has been shown to act as a competitive antagonist of flagellin perception in Arabidopsis and as an agonist in tomato when tested in cell culture medium alkalinization assays. Confirming these data, we did not see an increase of ethylene biosynthesis when we treated tomato leaves with flg22-ΔA17 and Arabidopsis leaves with flg22-Δ2, but a clear increase in ethylene biosynthesis could be measured when we applied these peptides in a reciprocal manner. However, surprisingly, we could observe that both peptides have a biological activity as agonists for the induction of ethylene biosynthesis in *N. benthamiana*. This finding emphasizes that the *N. benthamiana* flagellin perception system

shares molecular features of both tomato and Arabidopsis flagellin perception systems.

Taken together, our results show that *N. benthamiana* flagellin perception has an own pattern or specificity, which is different from the patterns of Arabidopsis and tomato. Obviously, *N. benthamiana* shares the need for N-terminal seven residues of flg22 with the Arabidopsis flagellin perception system but has a recognition of the C-terminal amino acids that resembles more the tomato type.
In the future, it will be interesting to analyze the amino acid sequences of LeFLS2 and NbFLS2 (that was identified in course of this work and will be discussed later). Because both sequences share a very high homology of 89% identical amino acids in the LRR domain, LeFLS2 and NbFLS2 are probably good candidates for targeted single site mutagenesis experiments within the regions of specificity that we identified for LeFLS2 and that will be discussed in the following.

The region of LeLRRs 7-10 is important for the higher affinity of LeFLS2 for flg22 and N-terminally truncated flg22-derivatives

We constructed a series of chimeric receptors of AtFLS2 with consecutively increasing numbers of LeLRRs replacing the AtLRRs, beginning at the LRR N-terminal domain. We used agrobacterium-mediated transient expression of these chimeric receptors in *N. benthamiana* and tested the peptides flg15$^{E.coli}$ and PaRm15 that were previously identified to elicit a defense response only in tomato but not in Arabidopsis or *N. benthamiana*. We first identified the region of LeLRR1-10 to be key LRRs of LeFLS2, responsible for mediating a change of specificity by conferring recognition of flg15$^{E.coli}$ on chimeric receptors that incorporate the tomato LRR region between amino acids 236-337 (LeLRRs 7-10). To perform comparative binding studies, we immunoprecipitated selected chimeric receptors that were expressed in *N. benthamiana*. We performed competitive binding assays on the IPs and found that the LeLRR 1-10, additionally to conferring the biological activity (ethylene induction), also massively altered binding characteristics of chimeric receptors that contain the LeLRRs 7-10 for flg22 and other derivatives.

Receptors containing the LeLRRs 7-10 showed a significantly higher affinity for flg22 and further tested flg22-derivatives. However, according to our data, it cannot be concluded with certainty that the LeLRRs 7-10 are alone responsible for the observed effects or if the LeLRRs 1-6 are needed in addition. Our data are supported a previous study by Dunning and coworkers, in which the β-sheet

region between AtFLS2-LRRs 9-15 was identified as being important for flg22 binding and onset of the seedling growth inhibition response upon flg22 treatment (Dunning et al., 2007). Our data show that this region is also important for flagellin perception of LeFLS2 and additionally, besides narrowing down the target region, allocates this region as the region that is responsible for the higher affinity of LeFLS2 for flg22 and its N-terminally truncated variant flg15 when compared to AtFLS2. We next wanted to know if this region is sufficient enough to mediate interaction with the flg22 core-epitope flg15-Δ7, that has been shown to bind to LeFLS2 but has no affinity to AtFLS2.

Flg15-Δ7 has been shown to be shortest flg22-derivative perceived by tomato (Meindl et al., 2000). Flg15-Δ7 itself exhibits no elicitor potential, but acts as a competitive antagonist for flg22 perception when concomitantly applied to tomato cell cultures. We tested IP preparations of the chimeric receptors, and again, we could see a clear shift towards tomato type of binding when we tested the chimeric receptor Le1-10:GFP, but not with LeLRR1-6 alone. This result shows that LeFLS2 already has a much higher affinity for the flg22-core sequence, independent of additional, putatively interaction-stabilizing effects of the flg22- N- and C-terminal amino acids. However, it remains to be elucidated if this region alone is able to interact with flg22 or, more probably, if it does so in cooperation with other residues on the LRR-domain.

The LRRs 7-10 cover a highly conserved β-sheet area shared between LeFLS2 and AtFLS2

When comparing the AtFLS2 and LeFLS2 LRR domains for conserved sites, an area of above average conservation (Fig. 12) can be detected within the predicted β-sheet region of LRRs 9-12. The LeFLS2 area exchanged in the chimeric receptor Le1-10:GFP ends just before the β-strand amino acid sequence of LRR 11, which β-strand sequence is in both AtFLS2 and LeFLS2 100% identical. We can therefore say that we have exchanged the bulk part of this conserved β-sheet region between AtFLS and LeFLS2. It seems that this conserved β-sheet area between LRRs 9-12 is highly important for flagellin perception in both AtFLS2 and LeFLS2. Since the β-sheet of LRR domains is often involved in protein-protein interaction it is a possible explanation that the differences between AtFLS2 and LeFLS2 in the LRRs 7-10 β-strand sequences are responsible for the detected change of specificity. However, at this point, no definite conclusion can be drawn whether it is a few different residues in the conserved β-sheet region that confer LeFLS2 specificity of if the less conserved areas, esp. the markedly below average conserved island consisting of residues

between L_2 and L_4 of the LRRs six and eight (which, interestingly, partly also extends into the β-strand region) contain the determinative residues for the differences of AtFLS2 and LeFLS2 in flagellin perception or both.

Our data allow the conclusion that the contributions of the N- and C-terminal residues of the flg22 peptide to LeFLS2 binding are much less important than for AtFLS2. Also, the determinative residues required for the detected high affinity of LeFLS2 for the flg22-core sequence flg15-Δ7 are located between the amino acids 236-337 of LeLRRs 7-10. Whether the exact residues are located within the conserved β-sheet area or not remains uncertain at this stage. It is however very likely that within the LRRs 7-10, the β-sheet region is of major importance for flagellin perception due to the detected high conservation of this region. Further studies, which are performed in the collaborating laboratory of Andrew Bent, University of Wisconsin, Madison, use site directed mutagenesis of single residues within LRRs 7-10 to study this question. Especially mutations of the amino acids in the β-sheet region of these LRRs will provide further insights to the question if the β-sheet region is indeed the key to the tomato specific LeFLS2-flg22 interaction or not.

The region of LeLRRs 19-24 is involved in the recognition of the C-terminus of the tomato specific peptides

When testing the chimeric receptors in *N. benthamiana* with the two tomato specific peptides flg15$^{E.coli}$ and PaRm15, we could observe an interesting divergence. While a gain of affinity flg15$^{E.coli}$ was observed in the chimeric receptor Le1-10:GFP and subsequently in all chimeric receptors that incorporate the LeFLS2-LRRs 7-10, responsiveness to PaRm15 could be detected only in the chimeric receptors Le1-24:GFP and Le1-28:GFP, respectively. In addition, when testing chimeric receptors that incorporate only the region of LeLRRs 19-24 and LeLRRs 11-24, respectively, no responsiveness to PaRm15 could be detected (Fig. 4D).

The results using agrotransformed *N. benthamiana* showed that perception of the tomato specific flg22-derivative PaRm15 depends on both the previously identified region of LeLRR 1-10 and additionally on an area around LeLRRs 19-24. The LRRs 19-24 are, like the area of LRRs 9-12, part of an area that shows an above average conservation of the β-sheet region (Fig 12).

However, we wanted to further characterize the role of the LeLRRs 19-24 and their importance for tomato specific recognition of the PaRm-type and other flg22-derivatived peptides. Because the endogenous *N. benthamiana* flagellin perception system is able to recognize the PaRm22 peptide as well as flg22-Δ2, we could not test perception of these peptides by our chimeric receptors in the quick and convenient *N. benthamiana* system. Therefore, we expressed the chimeric flagellin receptors in *fls2 Arabidopsis thaliana*.

Expression of chimeric receptors in *Arabidopsis thaliana* depends on the presence of the LRR-N-terminal domain and the first LRR of AtFLS2

We originally sought to express all the chimeric receptors in *fls2* Arabidopsis plants. However, we were not able to detect protein expression or reproducible biological activity of chimeric receptors transformed in *Arabidopsis thaliana*. We have previously shown that LeFLS2 is not expressed in *Arabidopsis thaliana*. However, transcription of DNA to mRNA can be detected (Robatzek et al., 2007b). It was hypothesized that translational or posttranslational quality control mechanisms like ERAD of *Arabidopsis thaliana* inhibit protein production of LeFLS2 (Robatzek et al., 2007b). We were not able to detect protein expression or biological activity of the chimeric receptor Le1-6:GFP when stably transformed in *Arabidopsis thaliana*. Therefore it can be concluded, that the cause of this phenomenon is already present within the LRR N-terminal domain and the first six N-terminal LRRs of LeFLS2. However, we could detect protein expression of the chimeric receptors Le19-24:GFP and Le11-24:GFP in transgenic *fls2 Arabidopsis thaliana* (Fig 10A). To further investigate this, we constructed an additional variant of the chimeric receptor Le1-24:GFP. The construct, termed Le1-24b:GFP, incorporated besides the signal peptide the complete LRR N-terminal domain and the first LRR of AtFLS2. After transforming *fls2 Arabidopsis thaliana* with Le1-24b:GFP, we indeed were able to detect protein expression on western blot (Fig 10A). This indicates that the LRR-N-terminal domain is important for the detected restriction of LeFLS2 expression of Arabidopsis thaliana. The LRR N-terminal domain has been shown to form a tight cap-structure that protects the hydrophobic core of the LRR-solenoid. Also, the LRR-N-terminal domain has been described to be highly important for both functioning and structure of eLRR containing proteins (van der Hoorn et al., 2005). It might therefore be that this domain is sensitive to a vigorous control mechanism to ensure proper folding and subsequent functionality of the protein. Perhaps the LeFLS2 LRR-N-terminal cap does not pass the Arabidopsis control mechanism and the emerging protein is recognized as misfolded and thus immediately

degraded. However, it remains an open question why only Arabidopsis shows this highly selective behavior, whereas AtFLS2 can be expressed without problems in tomato cells (Chinchilla et al., 2006) and N. benthamiana plants (Robatzek et al., 2007b).

Biological activity of the PaRm22 peptide can be detected in Arabidopsis when LeLRRs 19-24 are swapped into AtFLS2

Besides this setback, we successfully transformed the chimeric receptors Le19-24:GFP, Le11-24:GFP and Le1-24b:GFP into *fls2 Arabidopsis thaliana* plants and used the homozygous T3 progeny for further experiments. Protein expression could be detected by western blot and ethylene biosynthesis was clearly increased after treatment with flg22 at a concentration of 1µM, thus showing that the chimeric receptors were functional in Arabidopsis (Fig. 10B).

Our results show that LeFLS2 amino acids between position 525 to 670 (LeLRR 19-24) alone are sufficient to confer a change of specificity when swapped into AtFLS2. A clear increase in ethylene biosynthesis was detected when Le19-24:GFP transformed *A. thaliana* were treated with the PaRm22 peptide. This result allows the conclusion that the region between LeLRRs 19-24 is involved in recognition of the tomato specific recognition of the C-terminus of PaRm22.

Surprisingly, we did not detect an increase in ethylene biosynthesis when we tested the tomato specific peptide flg22-Δ2 on these plants. This implies that the molecular mechanisms, which direct the perception of these two tomato specific peptides, are not uniquely based on the same mechanism(s). Interestingly, ethylene biosynthesis was clearly increased upon flg22-Δ2 treatment in the Le11-24:GFP and Le1-24b:GFP transgenic Arabidopsis lines tested. This indicates that certain molecular features on the LeLRRs 11-19 are needed to convert flg22-Δ2 into an agonistic ligand peptide. However, whether for the recognition of flg22-Δ2 as an agonistic ligand the presence of the LeLRRs 19-24 is necessary in addition to the LeLRRs 11-18 remains to be found out.

We also detected increased ethylene biosynthesis in Le1-24b:GFP, but not Le19-24:GFP and Le11-24:GFP, transformed Arabidopsis when treated with the peptide PaRm15. This result confirmed the previous result detected in transiently transformed *N. benthamiana*.
An intriguing observation was that all transgenic Arabidopsis responded with an increase of ethylene biosynthesis upon treatment with the Arabidopsis-specific peptide flg22-ΔA17. Especially surprising was the fact that even the Le1-

24b:GFP, transformed Arabidopsis, although representing 85% of the LeLRR, perceived the flg22-ΔA17 as an agonistic ligand, just like wild type Arabidopsis.

It can be concluded that the region between Le19-24 is involved in tomato specific recognition of the C-terminus of PaRm22. Several hypothetical mechanistic models can be drawn about the molecular function of this region. It may, for example, function a molecular switch that induces the receptor to change between the inactive “sensor” state into the active signaling stage upon recognition of an agonistic ligand. However, what exactly occurs right after receptor activation is not clear. Concerning the molecular mechanism of receptor activation, it is widely thought that a conformational change happens after ligand binding. Such a conformational change then enables the FLS2-ligand complex to recruit BAK1 (and/or other signaling adapter molecules) and start signaling. Although such a mechanism is a favorite model, it has never been shown that indeed a conformational change of FLS2 is happening after binding of the ligand.

Concering the affinity of these chimeric receptors for flg22-derivatives, we observed that, despite the clear shift in biological activity of certain peptides observed in transgenic Arabidopsis plants, the tomato typical high affinity is only very little reflected when the binding characteristics of PaRm22 and flg22-Δ2 to IPs of *N. benthamiana* expressed Le11-24:GFP and Le19-24:GFP were tested. Clearly, AtFLS2:GFP and Le1-28:GFP showed great difference in affinity for the two tested peptides. Although, the two chimeric receptors Le11-14:GFP and Le19-24:GFP also showed a higher affinity for both the PaRm22 and flg22-Δ2 peptides, it was by far not to the extent that was observed for Le1-28:GFP. Based on these results, we hypothesize that the C-terminal part of flg22 and flg22-derivatives does not carry elements which are major determinants of affinity, but that it is nevertheless somehow involved in the binding process, maybe via stabilizing of the binding of flg22 N-terminal and central residues. This result strengthens the finding that, at least for LeFLS2, the N-terminal ten LRRs determine mainly the affinity between the receptor and its ligand.

Concluding remarks

In this study we identified two regions on the LeFLS2-LRR which confer independently of each other typical elements of tomato specificity when swapped into AtFLS2. We provide the new finding that at least two spatially separated areas on the LeFLS2-LRR domain are responsible for the two major differences

between tomato-specific perception compared to Arabidopsis-specific recognition, binding and receptor activation. Therefore, these areas must be involved in receptor-ligand interaction. Interestingly both identified LRR-regions compromise at least in parts areas that have an above average conservation of the putative β-sheet located on the concave face of the LRR domains of AtFLS2 and LeFLS2. The data show that the flagellin receptors AtFLS2 and LeFLS2 differ in terms of the required “address”. The N-terminal part of flg22 consists of the seven amino acids QRLSTGS. These residues are of great importance to establish efficient binding with AtFLS2, but not with LeFLS2. The presented data show that the residues which are responsible for the higher affinity of LeFLS2 for flg22 are located within the first ten LRRs of LeFLS2. For efficient binding to LeFLS2, the C- and N-terminal residues of flg22 are not important, as shown in binding assays using the flg15-Δ7 peptide. This finding implies that this region probably contains the LeFLS2 main binding site for this highly conserved core of flg22. Consequently, these first ten LRR might constitute the part of LeFLS2 that is responsible for the initial interaction of flagellin with LeFLS2. The presented data also provide evidence that the region between LeLRR 19-24 harbors molecular elements which are involved in the tomato-specific recognition of the C-terminal part of tomato specific flg22-derivatives. It has been shown that the C-terminal part of flg-peptides is important for mediating receptor-activation. Therefore, it can be assumed that the LeLRRs 19-24 are involved in the mechanism through which LeFLS is induced to change from the inactive “sensor” state to the active signaling state and thereby recruits BAK1 as a signaling-adapter molecule.

A

QRLS TGSR INSA KDDA AGLQ IA	**flg22**
R INSA KDDA AGLQ IA	**flg15**
ERLS SGLR INSA KDDA AGQA IA	**flg22 *E.coli***
R INSA KDDA AGQA IA	**flg15 *E.coli***
QRLS SGLR INSA KDDA AYWS IA	**PaRm22**
R INSA KDDA AYWS IA	**PaRm15**
QRLS TGSR INSA KDDA AGLQ - -	**flg22-Δ2**
QRLS TGSR INSA KDDA -GLQ IA	**flg22-ΔA17**

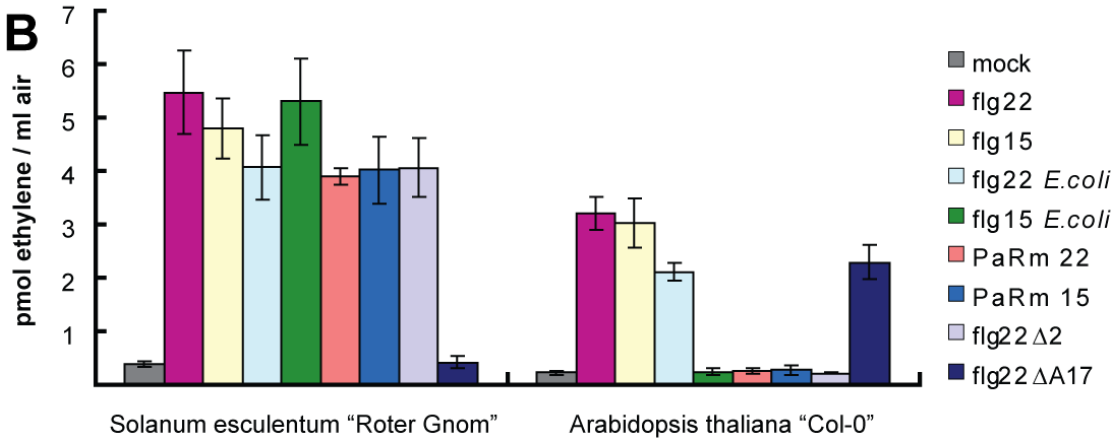

Figure 1:

Flg22-derivatives used in this study and the species-specific elicitation pattern in *Arabidopsis thaliana* and *Solanunm esculentum.*
Fig 1A: Flg22 and derivatives of flg22 used in this study. Residues differing from the flg22 sequence are marked in red. **Fig 1B:** Induction of ethylene biosynthesis upon treatment of leave strips with 1µM of each peptide. Except flg22-ΔA17, all flg22-derivatives induce an increase in ethylene biosynthesis in tomato leave tissue. *Arabidopsis thaliana* showed an increase in ethylene biosynthesis upon treatment with flg22, flg15, flg22$^{E.col}$ and flg22-ΔA17. (Error bars represent standard deviation of n = min. 6 / The experiment was repeated at least 3 times with biological replicates with similar output)

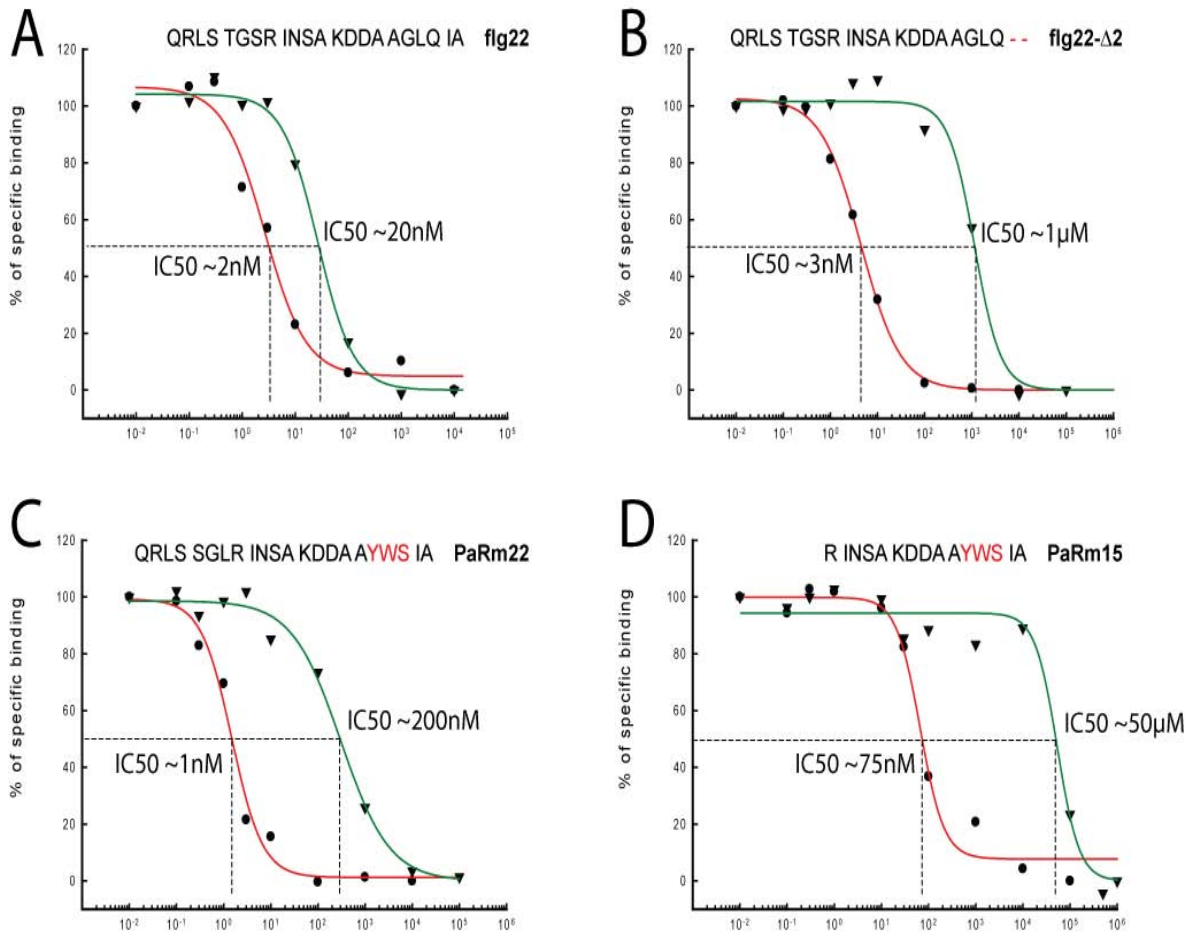

Figure 2:

Competition of ^{125}I-Tyr-flg22 and flg22 and different flg22 derivatives on wildtype Arabidopsis thaliana (Col-0) and Solanum esculentum (Roter Gnom) crude plant extracts.

Binding assays were performed with crude plant extracts of *Arabidopsis thaliana* ecotype Col-0 (triangles) and *Solanum lycopersicum var. Roter Gnom* (circles) with ^{125}I-Tyr-flg22 and various concentrations of the unlabeled peptides flg22, flg22-Δ2, PaRm22 and Parm15. Results were obtained with two independent repetitions of individually prepared crude tissue extracts and are presented as the percentage of specific binding. Dashed lines indicate the IC_{50} values observed after 30min. of incubation of the samples on ice.

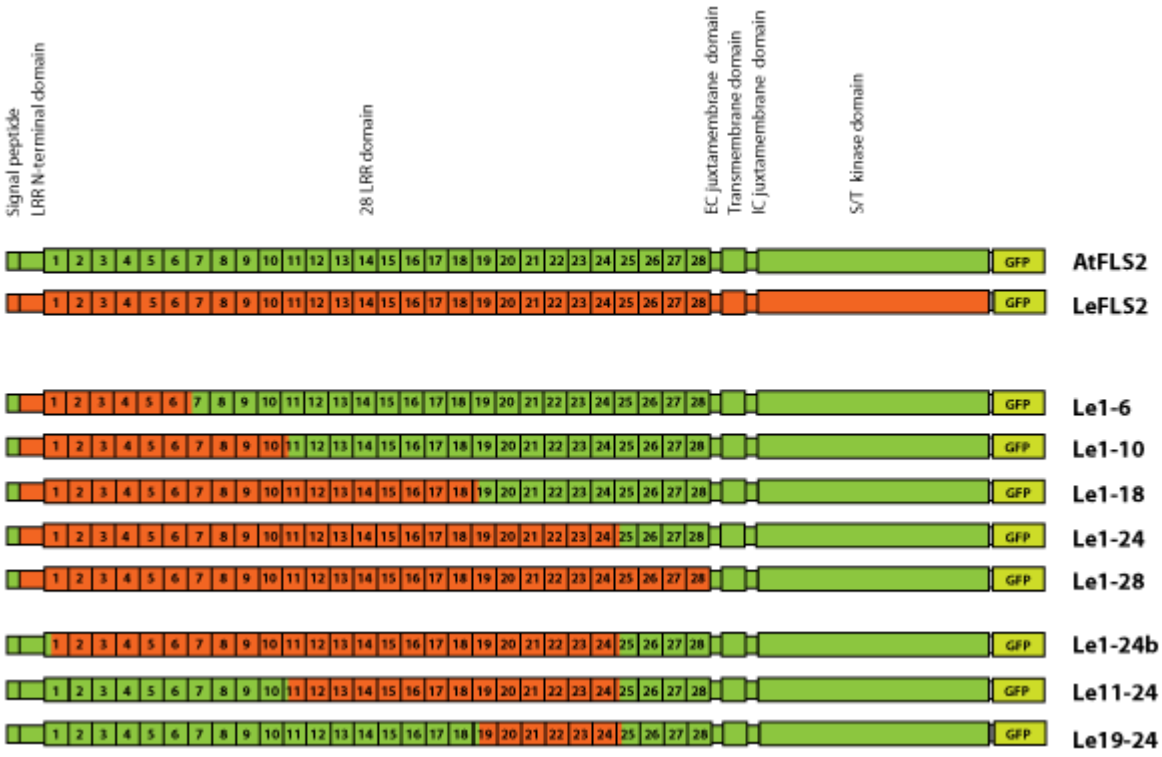

Figure 3:

Schematic representation of At/LeFLS2 chimeric receptors.
A schematic representation of the constructed chimeric receptors used in this study. For exact junction points of amino acids refer to materials and methods as well to Appendix I. The scheme is drawn to scale.

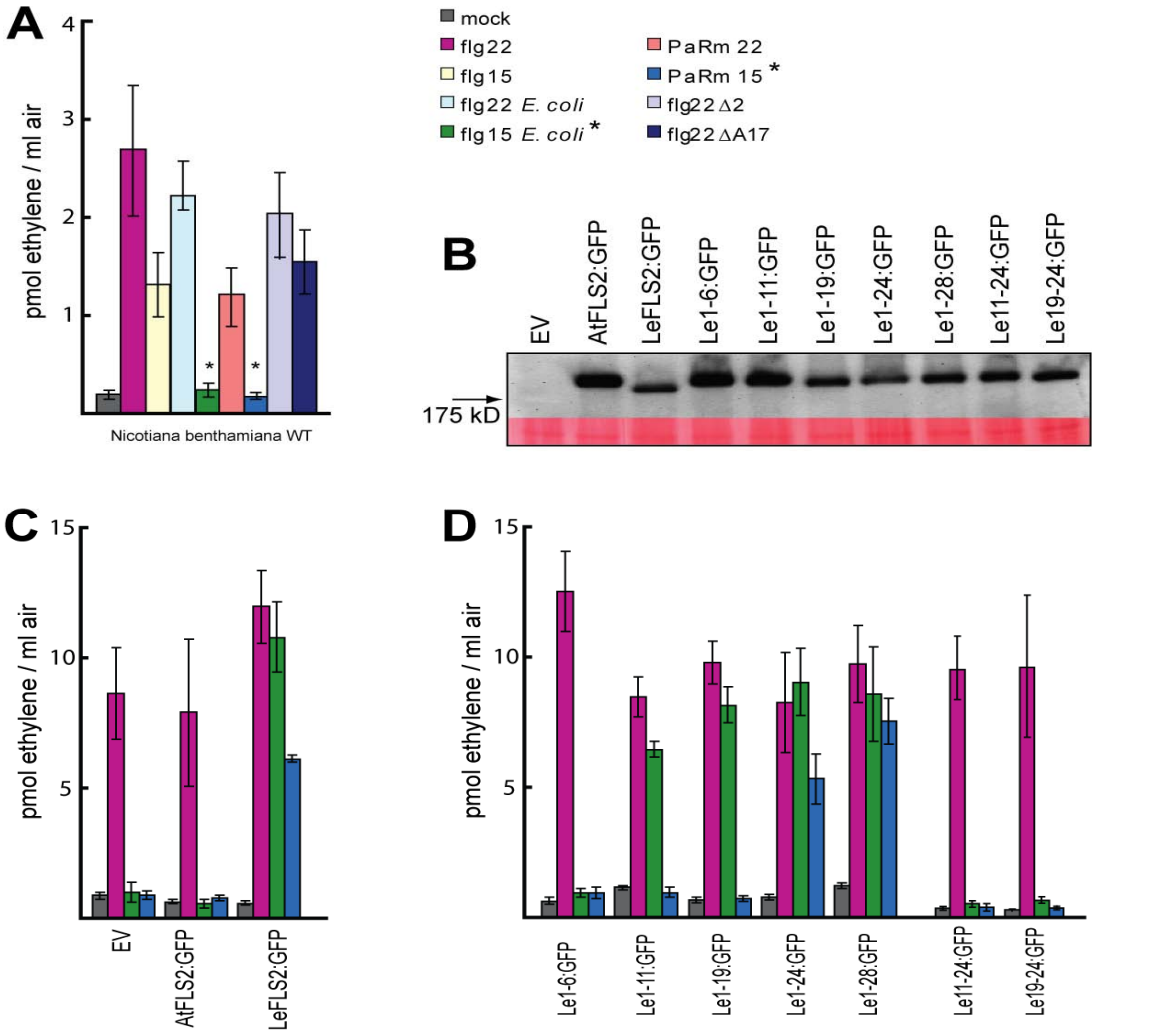

Figure 4:

Flg22 and flg22-derivatives perception of wildtype *N. benthamiana* and *N. benthamiana* transiently transformed with chimeric flagellin receptors.
Fig. 4A: Ethylene biosynthesis increase of 1µM flg22-derivative treated *N. benthamiana.* **Fig. 4B:** Expression of chimeric receptors in *N. benthamiana.* The blot was revealed with antiGFP antibodies. Equal loading is shown by the ponceau-stain. **Fig 4C:** Ethylene production of AtFLS2:GFP and LeFLS2:GFP agrobacterium-mediated, transiently transformed *N. benthamiana* upon treatment with flg22, flg15$^{E.coli}$ and PaRm15. A pSIRK:GUS construct used as empty vector control. Only LeFLS2 showed an increased ethylene accumulation upon treatment with the tomato-specific peptides flg15$^{E.coli}$ and PaRm15 **Fig 4D:** Ethylene production of Le1-6:GFP, Le1-11:GFP, Le1-19:GFP, Le1-24:GFP, Le1-28:GFP, Le11-24:GFP and Le19-24:GFP agrobacterium-mediated, transiently transformed *N. benthamiana*. Error bars represent standard deviation of n = 5. The experiment was repeated three times with biological replicates (individual transformations of *N. benthamiana*) with similar results.

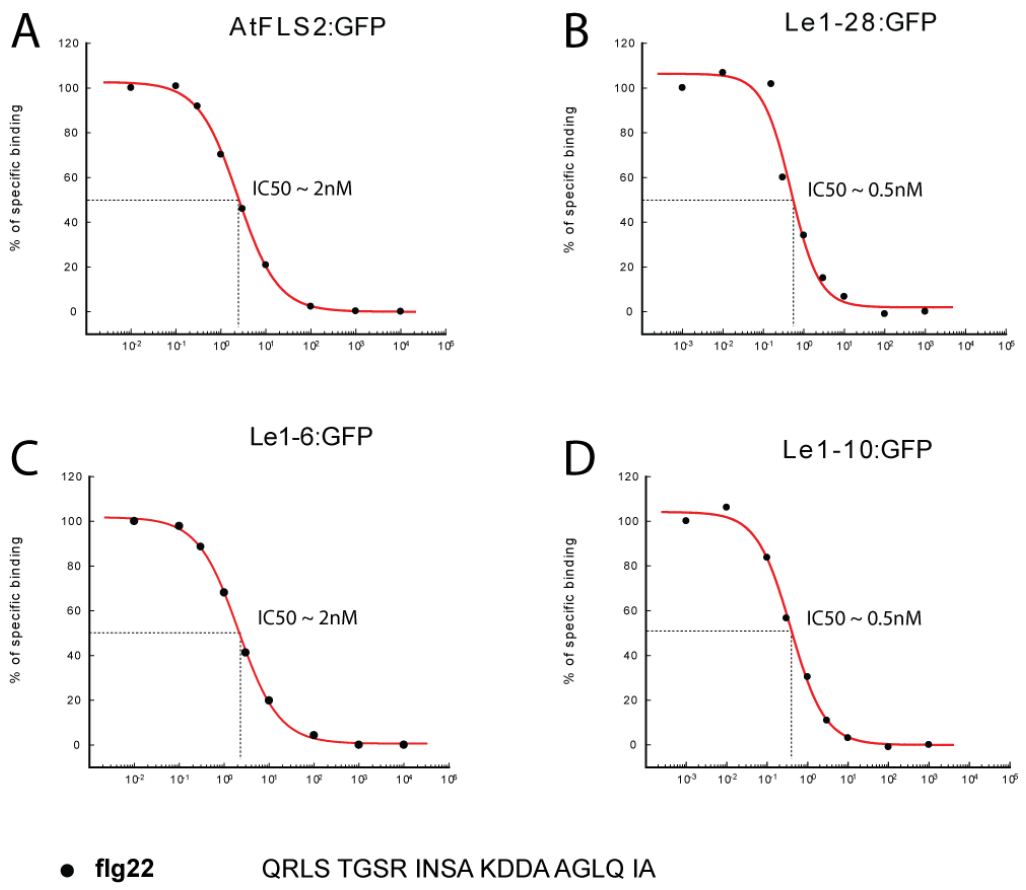

Figure 5:

Binding of ^{125}I-Tyr-flg22 and flg22 to immunoprecipitated AtFLS2:GFP, Le1-28:GFP, Le1-6:GFP and Le1-10:GFP expressed in *N. benthamiana*.
Fig. 5A and B: The full LRR domain of LeFLS2 in the chimeric receptor Le1-28:GFP has an about 4 fold higher affinity for flg22 than AtFLS2:GFP. **Fig. 5C and D:** Chimeric receptors containing only the first six LRRs of LeFLS2 show a competition of ^{125}I-Tyr-flg22 and flg22 similar to AtFLS2. The chimeric flagellin receptor Le1-10:GFP, incorporating the N-terminal ten LRRs of LeFLS2, shows a similarly enhanced affinity for flg22 as Le1-28:GFP, the chimeric receptor incorporating the full LRR domain of LeFLS2.
Results were obtained with three independent repetitions of individually prepared immunoprecipitations using biological replicates (individual transformations of *N. benthamiana*) and are presented as the percentage of specific binding. Similar results were obtained in all independent experiments. Dashed lines indicate the IC_{50} values observed after 30min of incubation of the samples on ice.

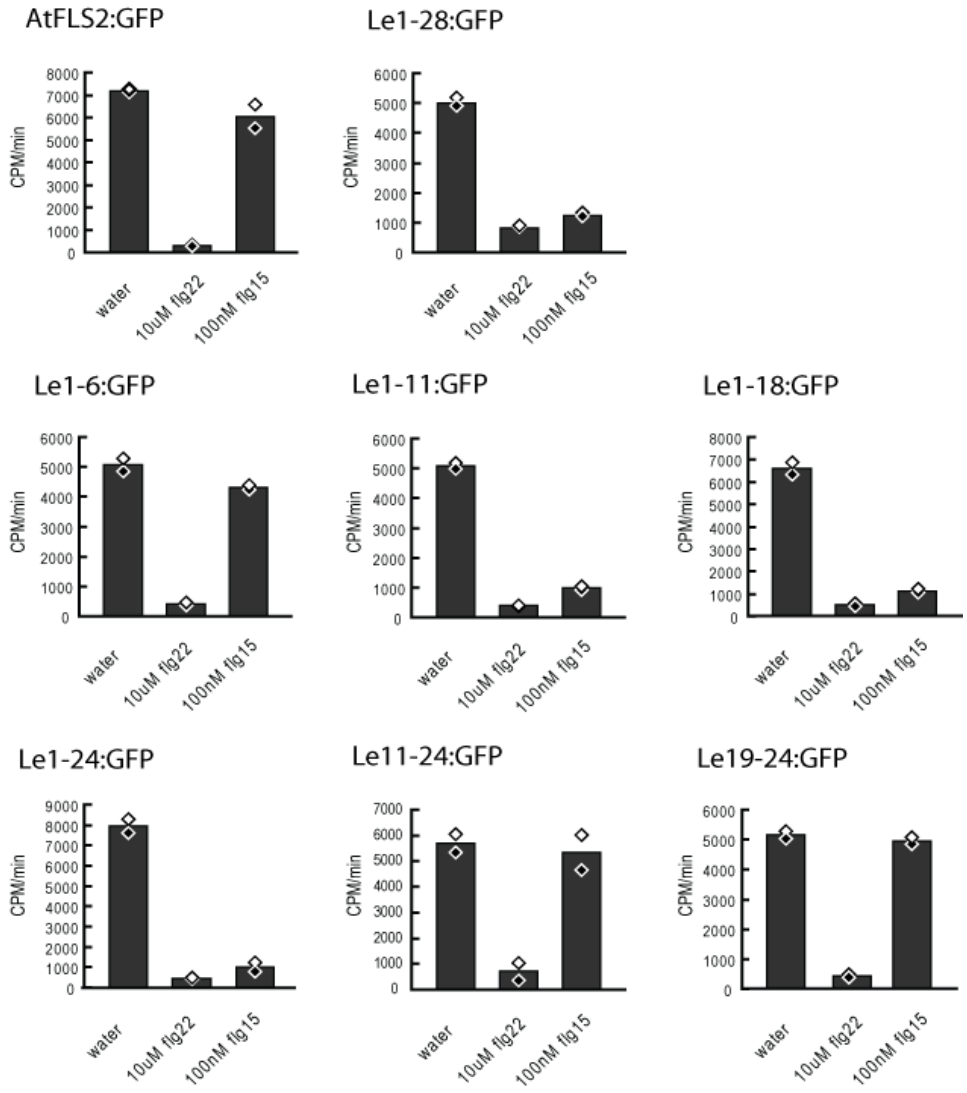

Figure 6:

Binding of ^{125}I-Tyr-flg22 and 10µM flg22 and 100nM flg15 to immunoprecipitated AtFLS2:GFP, Le1-28:GFP and Le1-6:GFP, Le1-10:GFP, Le1-18:GFP, Le1-24:GFP and Le11-24:GFP and Le19-24:GFP

In all cases 10µM unlabeled flg22 was enough to out-compete binding of ^{125}I-Tyr-flg22. For the chimeric receptors Le1-6:GFP, Le11-24:GFP and Le19-24:GFP and AtFLS2:GFP, flg15 at a concentration of 100nM was not enough to out-compete binding of ^{125}I-Tyr-flg22. For the chimeric receptors, Le1-10:GFP, Le1-19:GFP and Le1-24:GFP and Le1-28:GFP 100nM flg15 was enough to out-compete binding of ^{125}I-Tyr-flg22, showing a higher affinity of these chimeric receptors for this N-terminally truncated flg22-derivative.

Results were obtained with two independent repetitions of individually prepared immunoprecipitations using biological replicates (individual transformations of *N. benthamiana*) and are presented as the mean total CPU counts after γ-counting. Each diamond represents a single measuring point.

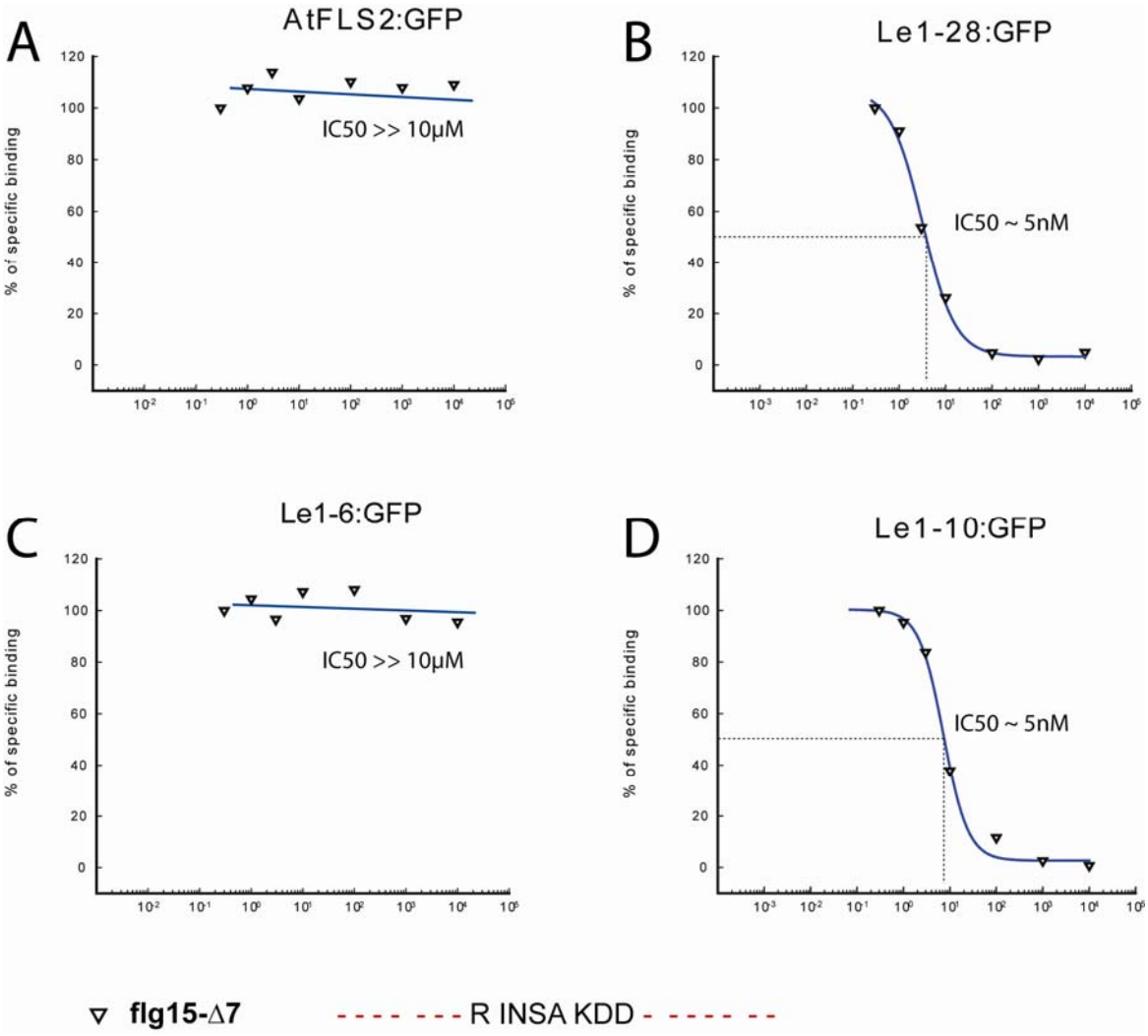

Figure 7:

Binding of ^{125}I-Tyr-flg22 and flg15-Δ7 to immunoprecipitated AtFLS2:GFP, Le1-28:GFP, Le1-6:GFP and Le1-10:GFP expressed in *N. benthamiana*

Fig. 7A/C: ^{125}I-Tyr-flg22 binding is not competed by flg15-Δ7 up to the concentration of 10µM when testing AtFLS2:GFP and the chimeric flagellin receptor Le1-6:GFP. **Fig. 7B/D:** ^{125}I-Tyr-flg22 binding is competed by flg15-Δ7 with an IC_{50} of 5nM in both chimeric receptors Le1-28:GFP and Le1-10:GFP.
Binding competition ^{125}I-Tyr-flg22 and flg15-Δ7 was confirmed in at least two independent sets of experiments. Dashed lines indicate the IC_{50} values observed after 15min of incubation of the samples on ice.

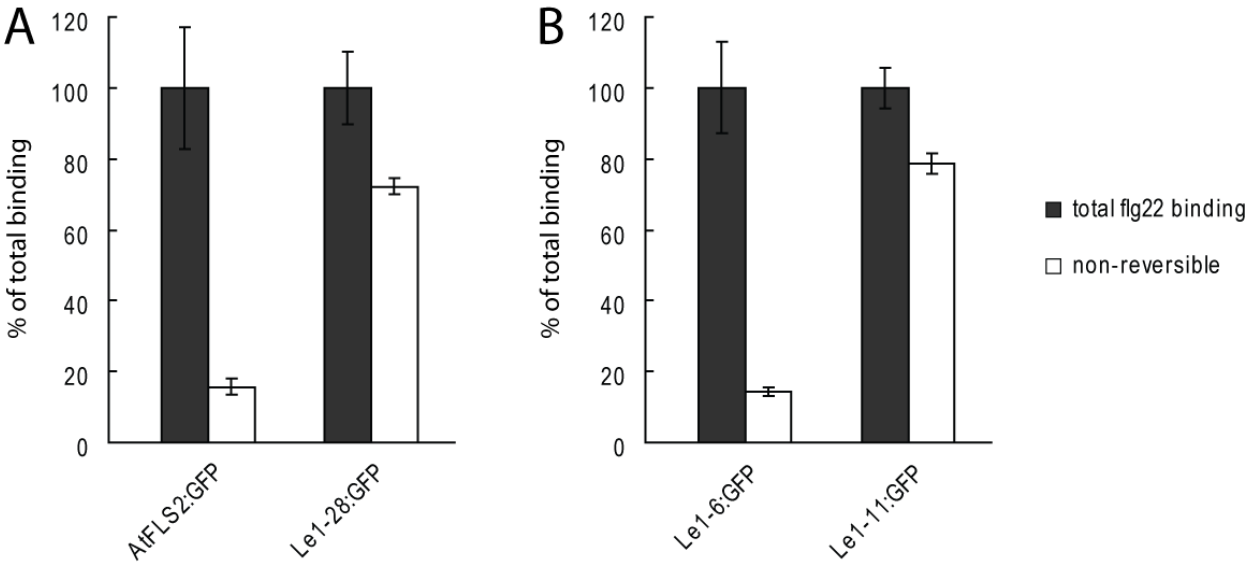

Figure 8:

Reversibility of flg22 binding to IPs of AtFLS2:GFP, Le1-28:GFP, Le1-6:GFP and Le1-10:GFP expressed in *N. benthamiana*

Fig 8A: The ability to retain bound flg22 on the receptor is for AtFLS2:GFP with approx. 20% much lower than for the chimeric receptors Le1-28:GFP with approx. 75%. **Fig. 8B:** The chimeric receptor Le1-6:GFP shows a similar sceme as AtFLS2:GFP while Le1-11:GFP has the same high retention of bound flg22 as Le1-28:GFP, the chimeric receptor containing the full LRR domain of LeFLS2.

Total binding of ^{125}I-Tyr-flg22 was measured after 30min incubation on ice. Binding-buffer containing 10µM non-radioactive labeled flg22 was added to the samples and the samples were incubated for 1h. at 4°C. After washing, the remaining amount of ^{125}I-Tyr-flg22 bound to the receptors was measured. The resulting difference between the CPM value of the total binding and the remaining amount of radioactivity is interpreted as the reversibility of flg22 binding to the receptor. The amount of total flg22 binding was set as 100% for adequate comparison.

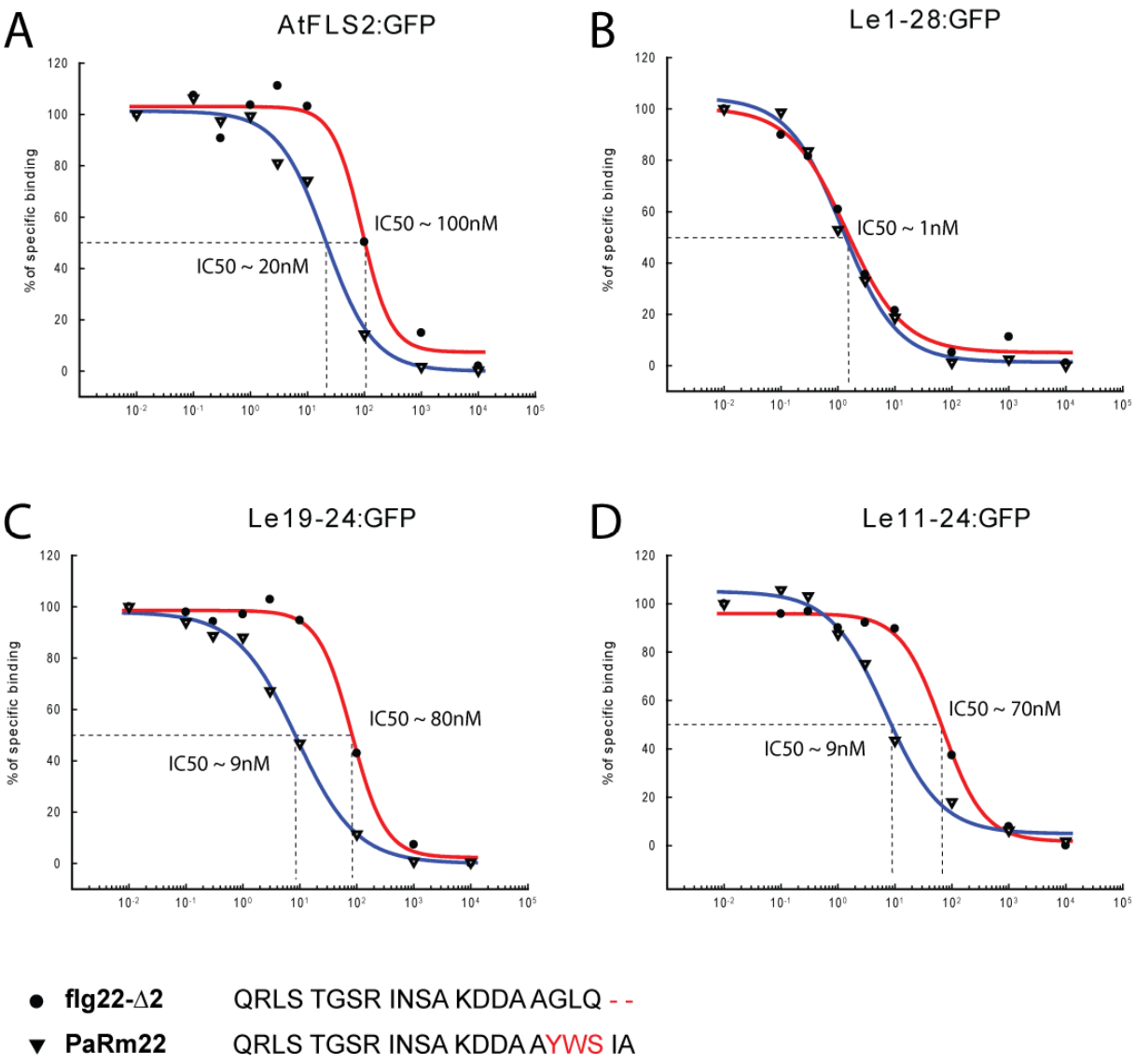

Figure 9:

Binding of ^{125}I-Tyr-flg22 and PaRm22 (triangles) and flg22-Δ2 (circles) to immunoprecipitated AtFLS2:GFP, Le1-28:GFP, Le1-6:GFP and Le1-10:GFP expressed in *N. benthamiana*.

Fig. 9A/B: AtFLS2:GFP shows different affinities for the flg22-derivatives PaRm22 and flg22-Δ2 while the chimeric receptor Le1-28:GFP, incorporating the full LRR domain of LeFLS2 does not and binds both peptides with a similar efficiency. **Fig 9C/D:** The two chimeric receptors Le19-24:GFP and Le11-24:GFP still show clear differentiation between the two peptides, however the affinity of both receptors for both peptides is enhanced. This indicates a role for the LeLRRs 19 to 24 in shifting specificity towards LeFLS2 typical recognition of the altered C-termini of these flg22-derivatives.

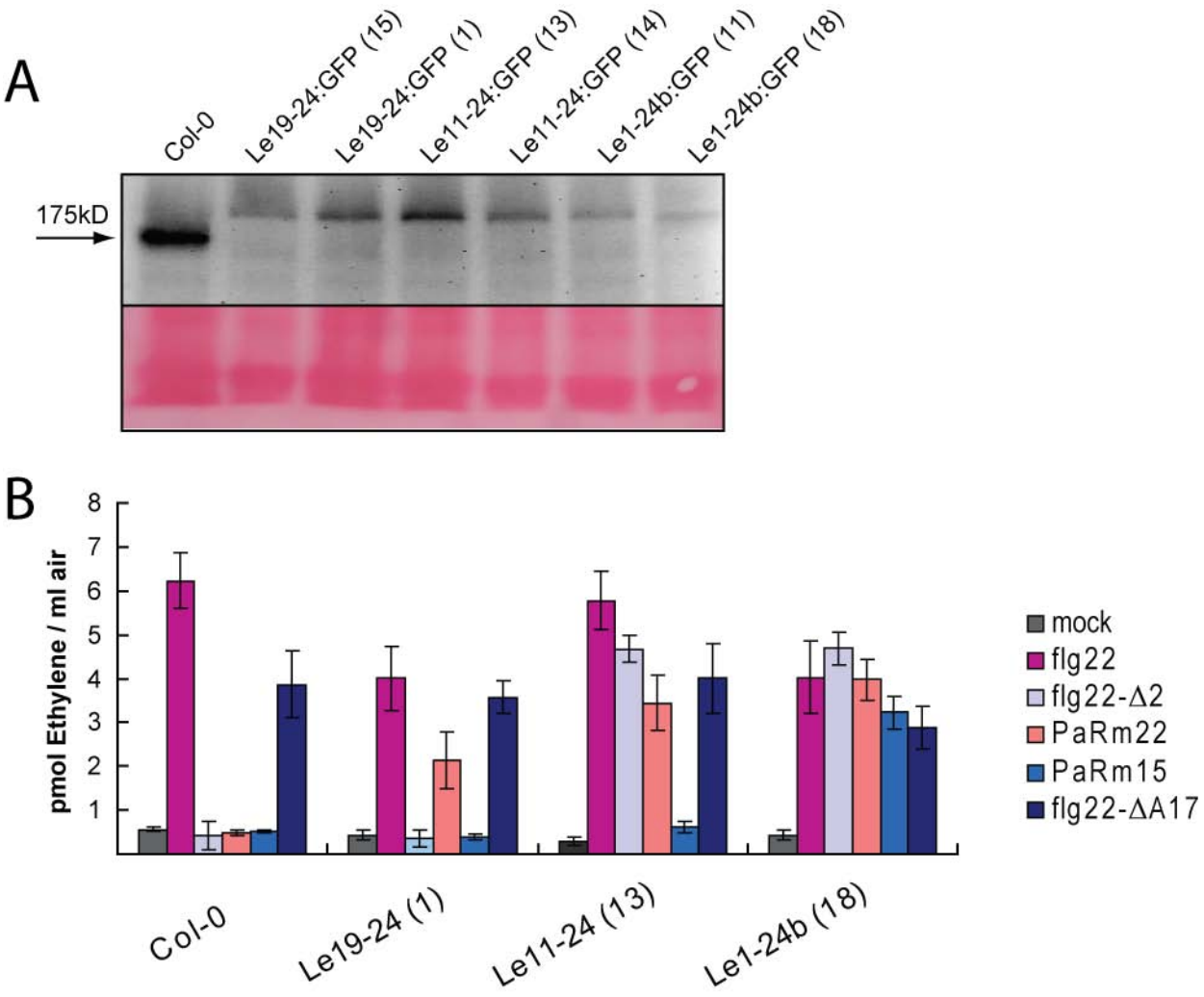

Figure 10:

Expression and biological activity of chimeric receptors stably transformed in *fls2-Arabidopsis thaliana*

Fig 10A: Expression of chimeric receptors Le19-24, Le11-24 and Le1-24b in *fls2* Arabidopsis thaliana (homozygous T3 lines). Western blot was revealed with anti-FLS2 antibodies. Equal loading is shown by the ponceau-stain. **Fig. 10B:** Ethylene response of transformed Arabidopsis to different flg22 derivatives each at a concentration of 1µM. (Error bars represent the standard deviation of n=8 individual samples)

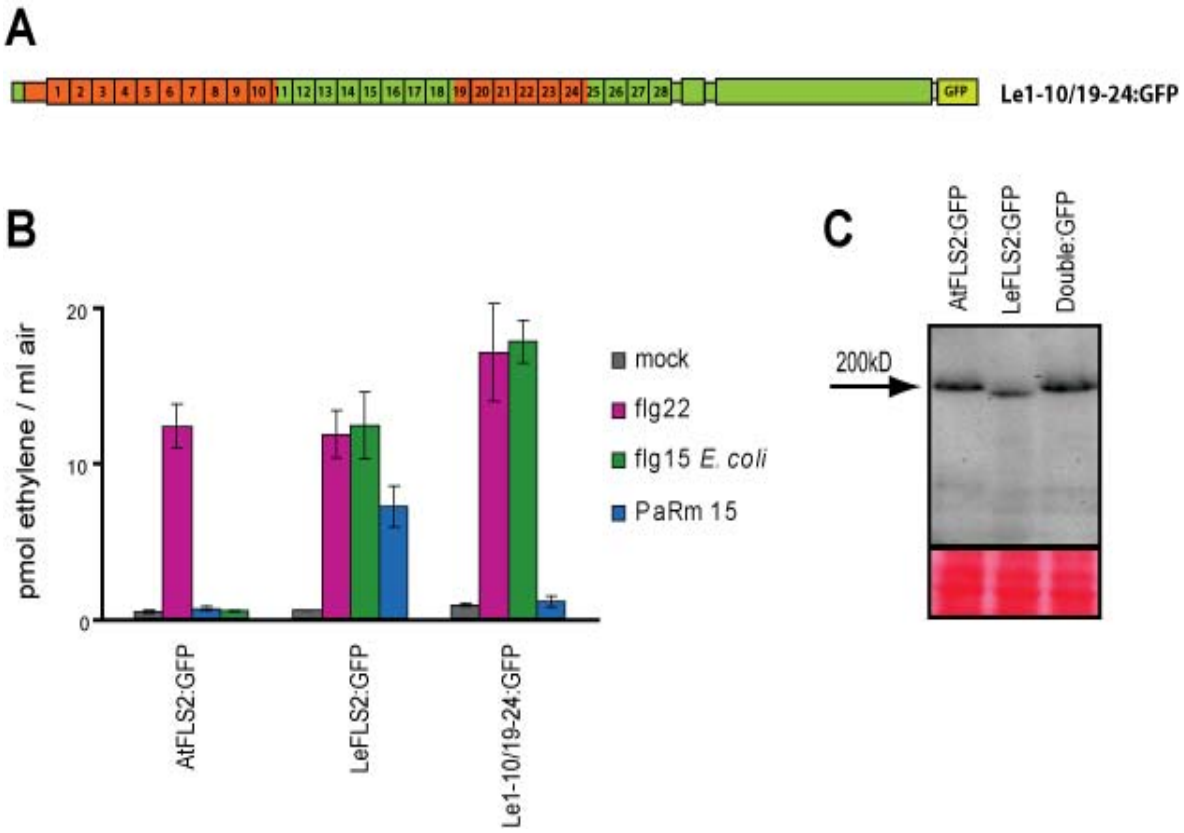

Figure 11:

Expression and biological activity of the chimeric receptor Le1-10/19-24:GFP.

Fig. 11A: Schematic depiction of the chimeric receptor Le1-10/19-24:GFP. Le1-15/19-24:GFP contains LeLRRs 1-10 and LeLRRs 19-24. For exact junction points of amino acids refer to materials and methods as well to Appendix I. The scheme is drawn to scale. **Fig 11B:** Ethylene response of agrobacterium mediated transiently transformed *N. benthamiana.* Each 1µM of peptide was used. (Error bars represent the standard deviation of n=8 individual samples) **Fig 9C:** Expression of AtFLS2, LeFLS2 and Double in *N. benthamiana*. Revealed with anti-GFP antibodies. Equal loading is shown by the ponceau-stain.

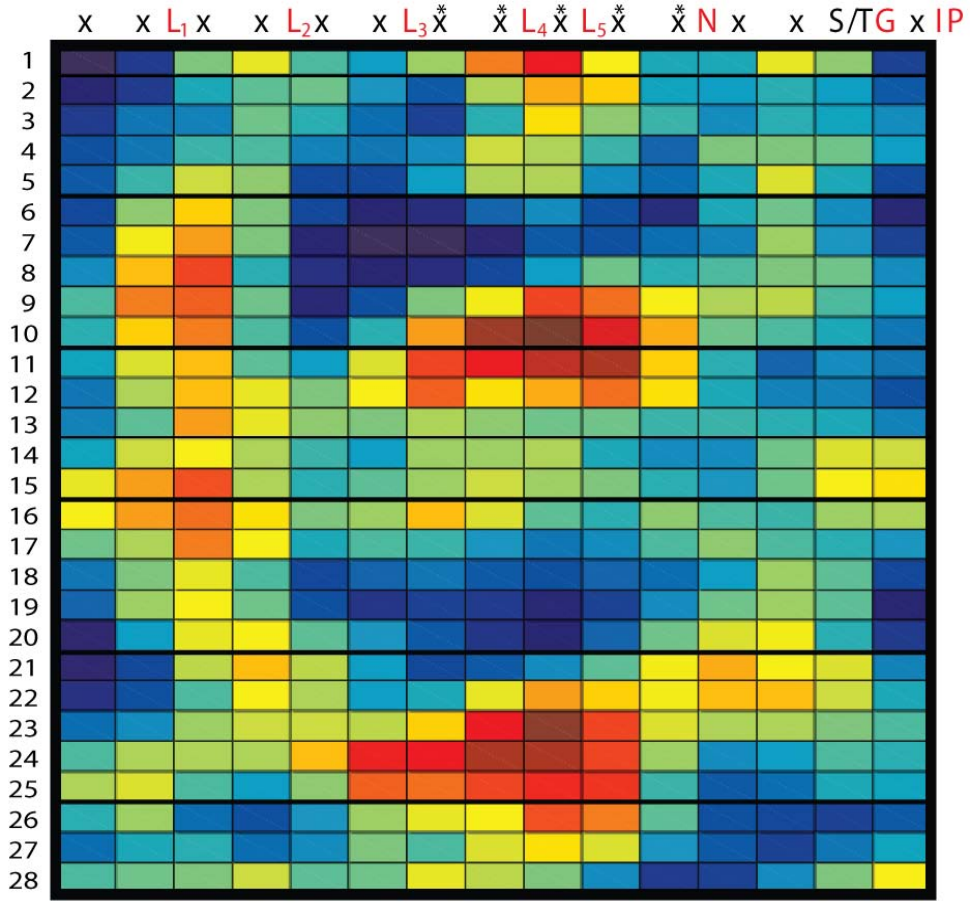

Figure 12:

AtFLS2 and LeFLS2 LRR domain conservation map.
Red indicates regions that are highly conserved between AtFLS2 and LeFLS2 while blue colors indicate lower conservation. Conserved residues belonging to the LRR-consensus sequence (red capitals) were eliminated from the map. The putative solvent exposed residues in the concave β-sheet are marked with asterisks. Two areas of high conservation can be found in the predicted β-sheet at the L_4 and L_5 positions in the LRRs 9-12 and LRRs 22-26. (Map generated in collaboration with L. Helft and A. Bent, USA)

A chimeric receptor between AtFLS2 and LeFLS2 activates the signaling pathway in the absence of flg22.

Abstract

Stable transformation of *fls2* Arabidopsis plants with a chimeric flagellin receptor produced by swapping the tomato LRRs 15 to 24 into AtFLS2 (Le15-24:GFP) resulted in an unexpected behavior of the primary transformants. Seedlings transformed with this construct suffered from a severe growth inhibition phenotype. The observed growth inhibition effect was initially comparable to the growth inhibiting effect published for wildtype Arabidopsis seedlings treated with the elicitors flg22 or elf18 (Gomez-Gomez et al., 1999; Kunze et al., 2004). However usually about two weeks after germination, the Le15-24:GFP transformed T1 seedlings died. We wanted to investigate whether the observed growth inhibition / seedling death phenotype can be correlated with the presence of the chimeric receptor Le15-24. Therefore, an inducible variant of this construct under the control of an estradiol inducible promoter was constructed and transformed into *fls2* Arabidopsis (Col-0). The progeny of such stably Le15-24^{est} transformed plants was followed up to the T3 generation. In growth inhibition assays with or without the addition of estradiol and flg22 or elf26 we found that the observed growth inhibition phenotype indeed correlated to the estradiol-induced expression of the Le15-24 protein. Furthermore, we were able to measure a clear increase in ethylene biosynthesis within 12h after induction of Le15-24 expression with estradiol. However no production of ROS within a time period of 12h could be detected. In addition to the characterization plants transformed with the inducible form of Le15-24, the AtFLS2-promoter driven construct was transformed into wildtype Arabidopsis (Col-0) plants as well a *bak1* Arabidopsis (Col-0) line. We found that wildtype Arabidopsis are equally affected by growth inhibition as *fls2* Arabidopsis upon transformation with Le15-24:GFP. In contrast, and highly interestingly, no seedling growth inhibition was observed in the progeny of stably Le15-24:GFP transformed *bak1* Arabidopsis (Col-0). As a conclusion of these findings, we hypothesize that the Le15-24 chimeric receptor functions as a constitutively active FLS2 allele. It is important to note that signaling can only be triggered via the artificial extracellular LRR domain, since the complete intracellular receptor part, e.g. transmembrane-juxtamembrane- and kinase-domain is not affected from the LRR domain

swapping. Additionally, the the presence of BAK1 was observed to be important for the phenotype. This might indicate that Le15-24 could be an FLS2 allele that recruits BAK1 via the artificial LRR-domain in the absence of its ligand, and thereby initiates signaling.

Additionally, variants of the Le15-24 chimeric receptor were constructed. A putative kinase-dead variant and two whole domain swap constructs where the transmembrane-, intracellular juxtamembrane- and kinase domain of Le15-24 were replaced by the respective domains of LRR-RLKs EFR or BRI1. While no impaired growth was detected when stably transforming the constructs Le15-24^{KD} or Le15-24^{BRI1k} into *fls2* Arabidopsis, parts of the Le15-24^{EFRk} transformed plants showed a Le15-24 similar, although much weaker, ligand independent seedling growth inhibition phenotype.

Introduction

Activation of FLS2 signaling is initiated when the extracellular LRR domain perceives bacterial flagellin or the synthetic flagellin derived peptide flg22 (Felix et al., 1999). It has been proposed that activation of FLS2 follows the address-message concept (Meindl et al., 2000). Thereby, the N-terminal part of the flg22-peptide binds in a first step to the receptor and the C-terminal part subsequently activates the receptor (Meindl et al., 2000). This hypothesis was corroborated by the finding presented in the previous part of this work that indeed spatially different areas on the LRR domain LeFLS2 are responsible for ligand-binding and perception of the receptor-activating C-terminus of flg22-derivatives. However, little is known about what occurs immediately after binding/activation of the ligand to its receptor. Recent studies identified the receptor kinase BAK1/SERK3 as a partner of ligand-binding leucine-rich repeat receptor kinases (Chinchilla et al., 2009). In particular, the brassinosteroid receptor BRI1 and the FLS2 have been shown to be dependent on the presence of BAK1 for activation of signaling (Li et al., 2002; Chinchilla et al., 2007; Heese et al., 2007). In this respect, BAK1 has been shown to positively regulate receptor functions via receptor heterodimerization, followed by receptor phosphorylation (Chinchilla et al., 2007; Schulze et al., 2010). However, it is still unknown what triggers the very initial interaction between BAK1 and the FLS2-ligand complex.

Some cases have demonstrated that ligand binding leads to a conformational change of the target receptor (Ghanouni et al., 2001; Frego and Davidson, 2006; Thomas et al., 2008). Such a conformational change allows the receptor to change from an inactive “sensor” state to an active signaling state and enables it to dimerize, recruit coreceptors and/or other downstream signaling partner molecules and thus initiates signaling. Well characterized in terms of the molecular basics of receptor activation are the G-Protein Coupled Receptors (GPCRs). GPCRs exist as a superfamily of integral membrane protein receptors that contain seven transmembrane α-helical regions, which bind to a wide range of ligands in both the animal and the plant kingdom (Tuteja, 2009). Upon activation by a ligand, GPCR have been shown to undergo conformational changes and then activate G proteins by promoting the exchange of GDP/GTP associated with the Gα subunit (Gether et al., 2002).

For the GCRPs, it has been proposed that constraining intramolecular interactions have been conserved during evolution to maintain the receptor preferentially in an inactive “sensor” conformation in the absence of agonist.

These inactivating constraints are released following agonist binding as the major part of the receptor activation mechanism (Gether et al., 2002). For the GCRPs this has been supported by the observation that constitutively activated β_2-adrenergic receptor and histamine H_2-receptor mutants are characterized by a notable structural instability and an enhanced conformational flexibility (Gether et al., 1997; Rasmussen et al., 1999; Alewijnse et al., 2000). The data presented in these studies imply that the introduced mutational changes have disrupted important stabilizing intramolecular interactions within the structure, allowing the receptors to more readily convert between the inactive “sensor” state to the active signaling state.

In the previous part of this work we demonstrated that chimeric receptors between the flagellin receptor of *Arabidopsis thaliana* and *Lycopersicon esculentum* are functional, able to bind flg22 and derivatives, and thereby confer species-typical specificities. In course of this project, we stably transformed chimeric flagellin receptors between tomato and Arabidopsis into *fls2* Arabidopsis (Col-0) and tested these receptors for functionality. We found that these receptors are functional and able to confer tomato typical patterns of flg22 and flg-derivative recognition depending on the number and position of the swapped LeFL2-LRRs. However, although AtFLS2 and LeFLS2 have most probably a very similar tertiary structure, they share only 55% amino acid identity in the LRR domain (Robatzek et al., 2007b). Therefore, the production of such chimeric receptors inevitably contained the “danger” of introducing modifications to the structure of the artificial LRR array. Such modifications might cause effects besides the changes in ligand specificity which we originally aimed to investigate. In the worst case, for example, the receptor can become completely non-functional. However, considering findings such as made with the above mentioned GCPRs, the extracellular domain of such a chimeric FLS2 allele could also be altered in a way that forces it to spontaneously adopt a conformation that is related to the conformation when the native receptor has bound its ligand. As a part of the above mentioned project, the chimeric receptor construct Le15-24:GFP was transformed into *fls2* Arabidopsis (Col-0). We could observe typical signs that would be expected for a constitutively active FLS allele. Thereby, it might expose sites or structures which are in the native form only accessible after the ligand has bound. These sites might be used as interaction domains for the recruitment of important signaling partner molecules and thereby render the receptor constitutively active.

Results

Structure of the Le15-24 chimeric receptor

The Le15-24 chimeric receptor was part of the series of chimeric receptor constructs with the aim to narrow down the region that is responsible for the tomato specific differences in recognition of C-terminally altered flg22-derivatives (see: Results I). Like the other constructs of this series, the GFP tagged version Le15-24:GFP is driven by the native AtFLS2 promoter (1000 bp upstream ATG).

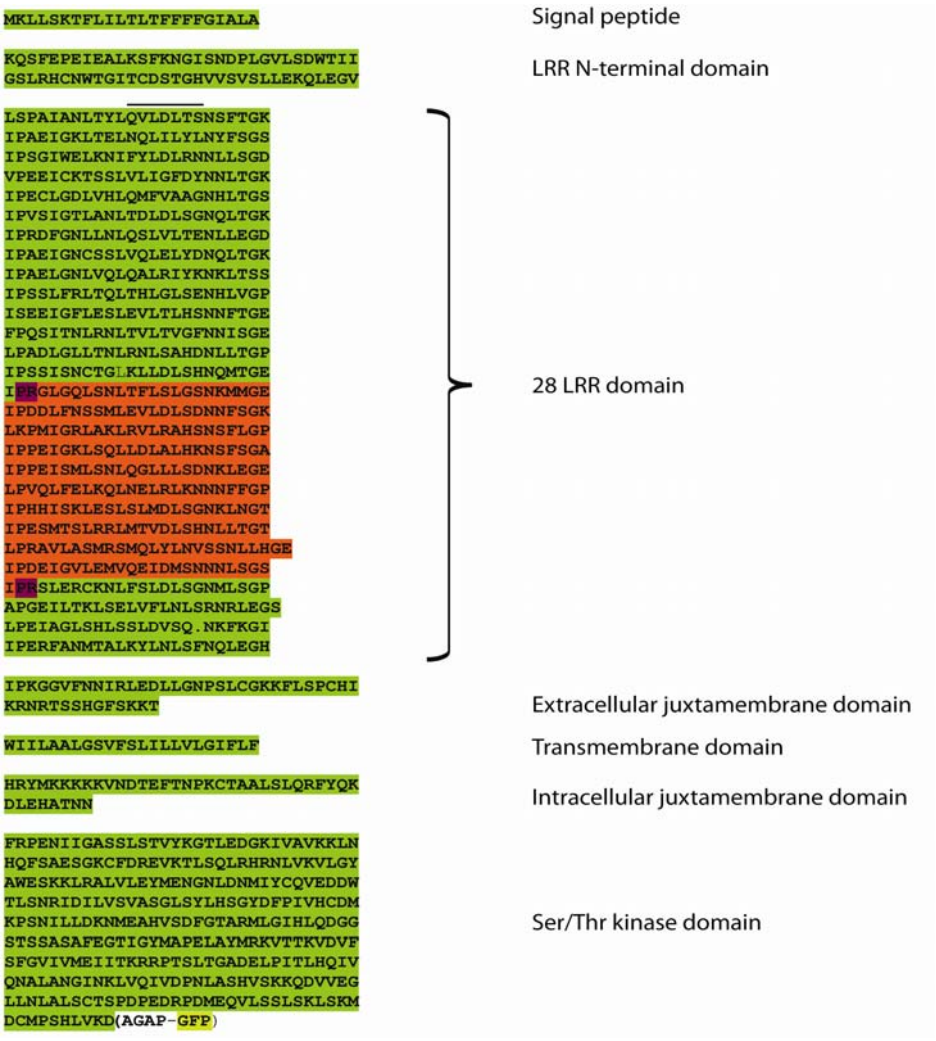

Figure 1: Structure of the Le15-24 chimeric receptor.
^{425}P and ^{425}R within LRR15 of AtFLS2 served as the N-terminal entry and fusion point to replace AtLRRs 15-24 with the corresponding LRRs from LeFLS2. Arabidopsis and tomato sequence parts are depicted in green and red, respectively. The fusion points are indicated in pink. The line atop the LRR domain represents the position of the putative β-sheet.

For the Le15-24 construct, the region of LeFLS2 between G^{427} and R^{670} was introduced into the corresponding area of AtFLS2. When analyzing the amino acid sequence of LRR 15-24 of both AtFLS2 and LeFLS2, we could detect neither in the β-sheet nor the surrounding LRRs of LRR15 an unusually high amino acid differentiation- or conservation pattern. This stands in contrast to the β-sheet of LRR24 and the directly adjacent LRRs which are conserved above average among AtFLS2 and LeFLS2 (Results I, Fig.12).

However, when comparing each the 15th LRR of LeFLS2 and AtFLS2, a peculiar, single irregularity within the usually rigid LRR consensus motive is obvious. AtFLS2, compared to LeFLS2, is lacking an amino acid between M^{431} and N^{432}:

AtFLS2 ^{424}IPRGFGRM-NL**TFISIGR**NHFTGE^{446}
LeFLS2 ^{426}IPNGLGQLSNL**TFLSLGS**NKMMGE^{449}

However, it is not known what, if any, consequences this feature contributes to the overall structure of AtFLS2 compared to the structure of LeFLS2. Nonetheless, it remains an intriguing irregularity that may or may not be important for the structural integrity of AtFLS2. If altered, this may disturb the interplay of LRR15 with its adjacent LRRs or other structural features critical for AtFLS2.

Impaired T1 seedling growth of *fls2 Arabidopsis thaliana* (Col-0) stably transformed with Le15-24:GFP

We stably transformed the construct Le15-24:GFP by floral dip into *fls2* Arabidopsis plants (Clough and Bent, 1998). In contrast to other stably transformed constructs however, we noticed that the seedlings which were positively selected for the integration of the transgene into the genome were severely impaired in growth. After germination, these seedlings did not expand their leaves and within two weeks developed heavy necrosis and finally died (Fig. 2). Transformation with Le15-24:GFP was repeated twice with the same outcome: Most of the positively selected T1 seedlings, after germination, showed this severe growth inhibition phenotype and died within the first two weeks. Although the severeness of the observed growth inhibition phenotype varied between individual primary transformants, we hypothesized that introduction of Le15-24:GFP played a role in the development of the observed phenotype.

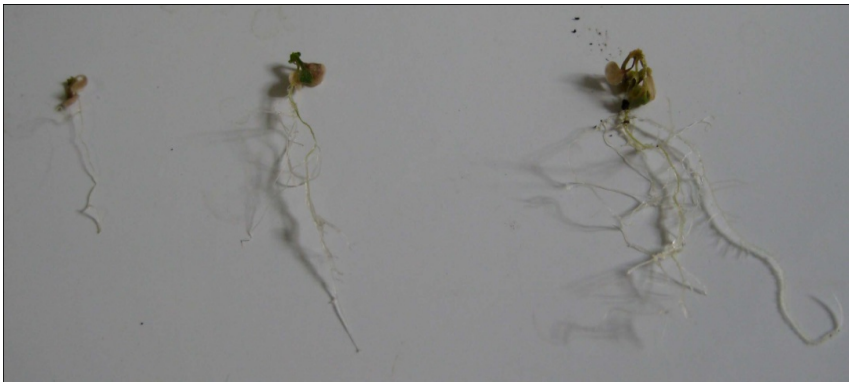

Figure 2: Examples of T1 *fls2* Arabidopsis Le15-24:GFP-transformaned seedlings suffering from different intensities of a severe growth inhibition phenotype.
Arabidopsis plants that were stably transformed with **Le15-24:GFP** by floral dip showed a strong growth inhibition phenotype. The phenotype varied in intensity, however, all plants died within the first three weeks after germination. Plants not dying shortly after the development of the first pair of real leaves, developed typical, "broccoli"-shaped, very dense arrangements of tiny leaves before the leaves, too, became necrotic and the seedlings died.

Especially intriguing, growth inhibition is a typical response of seedlings when treated with flg22. The observation of a spontaneous, ligand independent growth inhibition phenotype with an altered FLS2 allele involved, might be an indicator that LeFLS15-24 is a constitutively active FLS2 allele. We therefore decided to perform further experiments to investigate this interesting possibility in more detail.

Arabidopsis (Col-0) are also impaired in growth after transformation with Le15-24:GFP but *bak1*-deficient Arabidopsis (Col-0) are not

In parallel to the transformation of *fls2*-mutant Arabidopsis plants, the Le15-24:GFP construct was transformed into wildtype Arabidopsis (Col-0) as well as a *bak1*-deficient deficient line. For most of the progeny of transformed wildtype Arabidopsis (Col-0), we observed the same growth inhibition phenotype as for the *fls2*-mutant plants (Fig. 3). For a few individuals, however, we observed that the development of a phenotype was delayed. These plants seemed to develop normally within the first weeks, not showing signs of impaired growth. Nevertheless, after an initial "health-period" freshly developing leaves started to have a curly shape (Fig. 3, picture down right). However, these cases might represent transformation artifacts as the transgene may have integrated in regions of the genome that allowed expression of Le15-24:GFP only in a later stage of development.

Figure 3: Le15-24:GFP transformed T1 wildtype Arabidopsis (Col-0).
Examples of T1 wildtype Arabidopsis Le15-24:GFP-transformaned seedlings suffering from different intensities of growth inhibition. Interestingly, some plants that were initially seemingly healthy (during the first two to three weeks) started to develop an irregular growth phenotype by suddenly starting to develop small and curly leaves (picture below right).

As BAK1 has been recently shown to be involved in flagellin signaling, Le15-24:GFP was transformed into a *bak1 Arabidopsis thaliana* (Col-0) line (GABI-Kat # 213D09; generously provided by S. Robatzek). After transformation of the plants by floral dip, we screened the resulting T1 progeny. Interestingly, none of the positively selected transformants showed any sign of growth inhibition during the entire life cycle (Fig. 4). The T1 progeny was further propagated and brought to the T3 generation without showing signs of impaired growth over all generations. These seeds might serve as an interesting tool for further experiments.

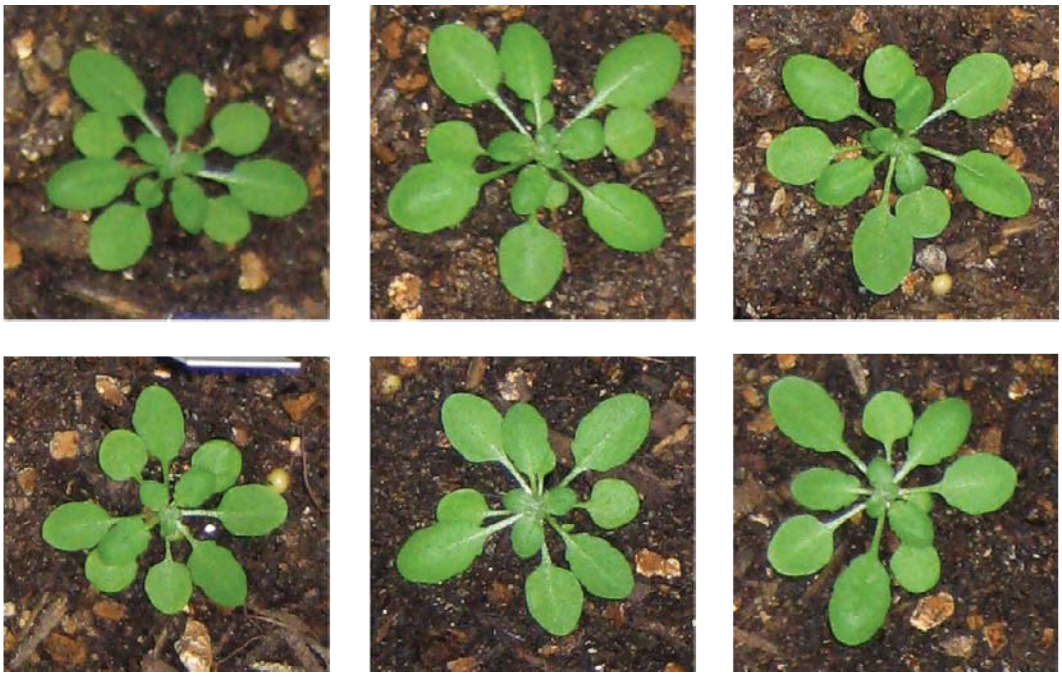

Figure 4: Le15-24:GFP transformed T1 *bak1*$^{-}$ Arabidopsis (Col-0).
Examples of T1 *bak1*$^{-}$ Arabidopsis Le15-24:GFP-transformed seedlings do not suffer from the growth inhibition phenotype observed when Le15-24:GFP was stably transformed into *fls2* Arabidopsis and wildtype Arabidopsis (Col-0).

Generation and characterization of an estradiol-inducible Le15-24 Arabidopsis line

The intensity of the observed phenotype, leading to a premature death of the transformed seedlings, was a problem for further experiments. In order to accomplish further studies, a different system of expression had to be chosen. Expression of Le15-24 controlled by an inducible promoter system instead of the native AtFLS2-promoter, as used for the Le15-24:GFP construct was selected to overcome the issue of premature seedling death.

Construction of an estradiol-inducible Le15-24 binary vector construct

The Le15-24 sequence was introduced via the gateway^{TM} cloning technique into the pMDC7 vector. pMDC7 is gateway^{TM} compatible vector consisting of a PER8 backbone (SpectR in bacteria and HygromycinR in plants) and an estradiol-inducible lexA-46 35S promoter system. The generation of lines where the expression of Le15-24 could be tightly controlled allowed us perform further bioassays like growth inhibition assays, the measurement ethylene production or the production of reactive oxygen species (ROS).

Biological characterization of an estradiol-inducible, homozygous Le15-24^{est} line

We transformed the estradiol-inducible vector-construct into fls2^{-} Arabidopsis (Col-0). After germination of the primary transformants, we noticed that the previously observed phenotype of does not occur when these transformants were germinated on MS-plates. The T1 seeds were brought to flower and T2-sister families were independently tested for responsiveness when treated with 1-10µM of the inducing agent estradiol. Indeed, we observed a strong growth inhibition when these plants were treated with estradiol indicating that the observed estradiol-induced growth inhibition phenotype must be due to the expression of the inserted transgene. By calculating the ratios of inhibited vs. non-inhibited seedlings and comparing them to the corresponding antibiotic selection pattern we confirmed the segregation pattern for single insertions, thereby further corroborating the previous results that indeed expression of a single insertion of Le15-24 is the causing agent of the observed growth inhibition. The transformants were brought to flower and the resulting T3 progeny was tested for homozygous Le15-24^{est} lines.

Growth inhibition assay with or without addition of estradiol and/or flg22 or elf26

Seedling growth inhibition has been shown to be a typical response induced by subnanomolar amounts of flg22 or elf18 (REF). Wildtype Arabidopsis (Col-0) and homozygous T3 Le15-24^{est} seedlings were germinated on MS-plates and subsequently tested in a standard 10-day growth inhibition assay. The seedlings were treated with or without 10µM estradiol as well as 1µM of the peptides flg22 and elf26 (Fig. 5, 6, 7)

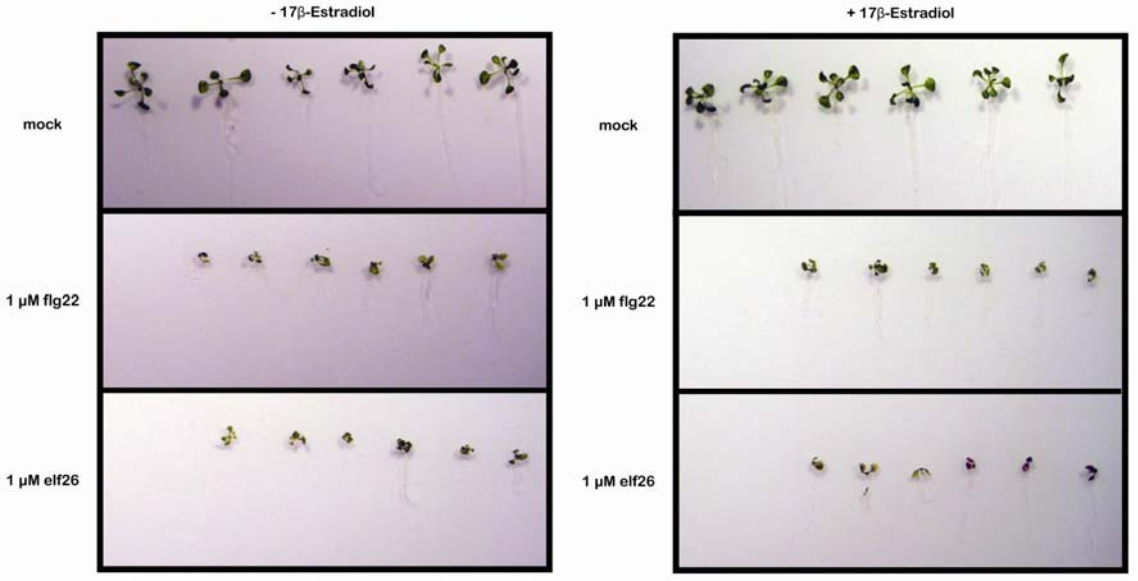

Figure 5: Wildtype *Arabidopsis thaliana* (Col-0) treated or not with estradiol and flg22 or elf26.
Seedlings were germinated on MS-plates and hereafter transferred for 10 days into liquid MS-medium containing or not 10µM estradiol alone (mock) and 1µM of flg22 or elf26. WT Arabidopsis seedlings are is not inhibited in seedling growth by the addition of 10µM estradiol compared to non-estradiol treated seedlings. As expected, the seedlings were strongly inhibited in growth when 1µM of flg22 or 1µM elf26 was added to the liquid medium. (Picture shows representatives of n=12 for each treatment; the experiment was done twice with the same output)

Wildtype seedlings were not influenced in growth by the addition of 10µM estradiol (Fig. 5). As expected, the addition of 1µM flg22 or elf26 induced the typical growth inhibition. Additionally, the presence of estradiol did not interfere with the peptide induced growth inhibition.
In contrast, when the Le15-24^{est} transformed seedlings were tested, we observed that seedlings treated with 10µM estradiol clearly showed a severe inhibition in growth, while not-treated seedlings grew comparable to wildtype seedlings (Fig. 6). This indicated that indeed expression of Le15-24^{est} is required and responsible for the observed growth inhibition phenotype. Additionally, this result shows that the inducible promoter is tight enough to suppress the expression of the transgene in the absence of estradiol. When these seedlings were additionally treated with 1µM flg22 or elf26, only elf26 induced a typical growth inhibition in the non-estradiol treated samples. Because Le15-24^{est} was transformed into *fls2*-background, non-responsiveness to flg22 was to be expected and served as an additional control.

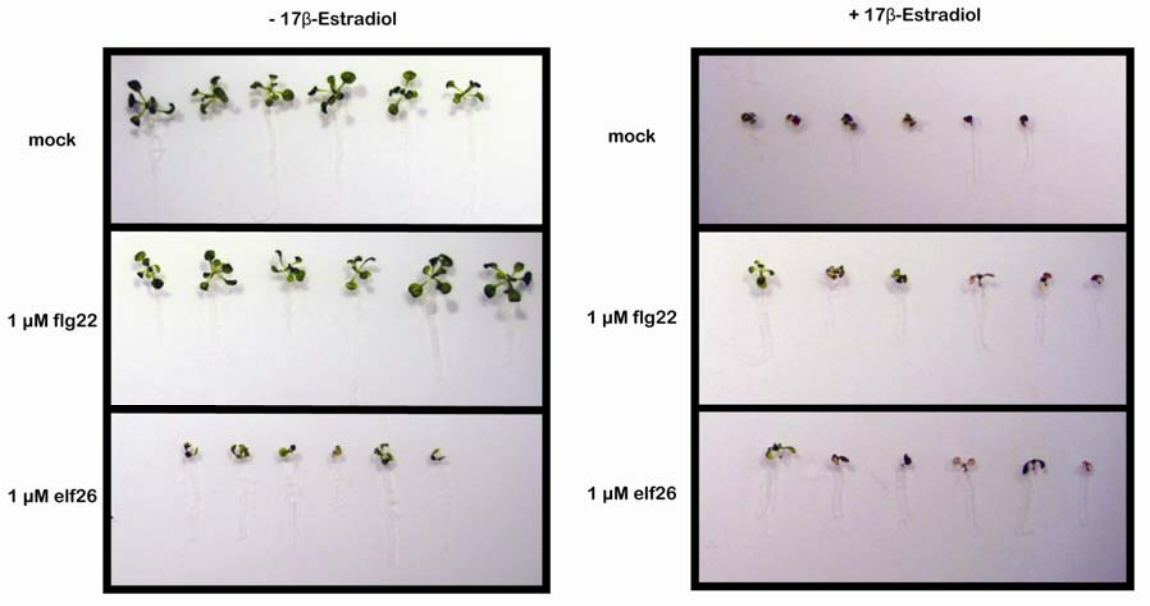

Figure 6: Homozygous T3 Le15-24^{est} transformed *fls2*$^{-}$ *Arabidopsis thaliana* (Col-0) treated or not with estradiol and flg22 or elf26.
Seedlings were germinated on MS-plates and hereafter transferred into liquid MS-medium containing or not 10µM estradiol alone (mock) and 1µM of flg22 or elf26. Seedlings treated with 10µM estradiol are strongly inhibited in growth compared to non-estradiol treated seedlings (mock). Seedlings not treated with estradiol are inhibited in growth only in the presence of elf26. By eyesight, no synergistic effects of estradiol treatment and elf26 treatment were observed. (Picture shows representatives of n=12 for each treatment; the experiment was done twice with the same output)

When analyzing the estradiol-treated samples, we observed a very strong growth inhibition phenotype similar to the one observed with the initial, AtFLS2 promoter driven construct. From visual judgment, the observed phenotype appeared to be stronger than a “normal” flg22 or elf26 induced growth inhibition on wildtype seedlings. Especially the development of early necrosis on estradiol treated Le15-24^{est} plants constitutes a difference to the flg22/elf26 induced symptoms. However, no significant difference in seedling fresh weight was detected between estradiol-induced Le15-15^{est} seedlings and flg22/elf26 treated wildtype Arabidopsis seedlings were analyzed (Fig. 7).

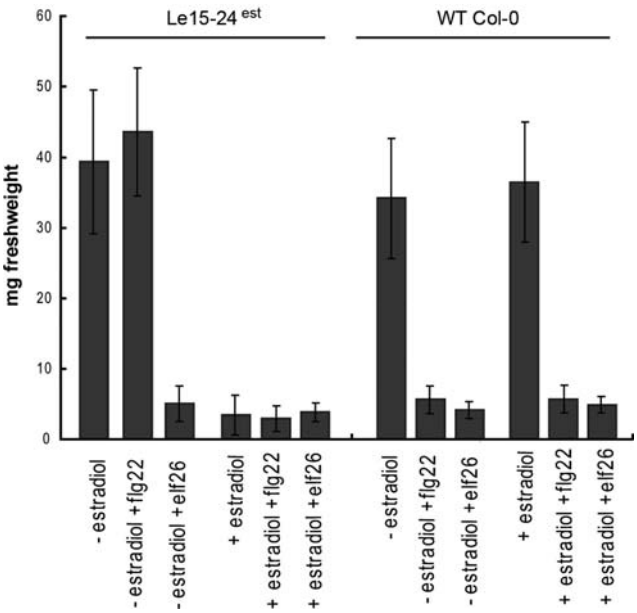

Figure 7: Analysis of seedling freshweights using homozygous T3 Le15-24^{est} transformed *fls2*$^{-}$ Arabidopsis and wildtype Arabidopsis seedlings.
T3 Le15-24^{est} transformed *fls2*$^{-}$ Arabidopsis (Col-0) and wildtype Arabidopsis (Col-0) were treated or not with 10µM estradiol and/or 1µM flg22 or elf26. Estradiol treated seedlings of homozygous T3 Le15-24^{est} were severely reduced in seedling weight while wildtype Arabidopsis (Col-0) are not affected by treatment with estradiol. Based on seedling weight no synergistic effects were observed when 10µM estradiol and 1µM elf26 were added concomitantly to Le15-24^{est} transformed seedlings. (n=12 for each treatment; the experiment was repeated twice with the same output)

Also, no synergistic effects were observed when estradiol and elf26 were added concomitantly to the seedlings, neither from visual judgment nor the statistical evaluation of seedling weight (Fig. 7).

The antagonistic flg22-derivative flg22^{SYSTEMIN} does not inhibit growth inhibition of estradiol induced Le15-24^{est}-seedlings

Flg22^{SYSTEMIN} is a potent antagonistic peptide for flg22 induced signaling in both Arabidopsis and tomato. This flg22-derivative has the sequence QRLSTGSRINSAKDDAAGGMQTD. It consists of the 16 N-terminal flg22 amino acids and a C-terminal Systemin-sequence part. In Arabidopsis cells, flg22^{SYSTEMIN} has been demonstrated to eliminate pH-shifts induced with 0.3nM flg22 when added in a concentration of 200nM (G. Felix, communication).

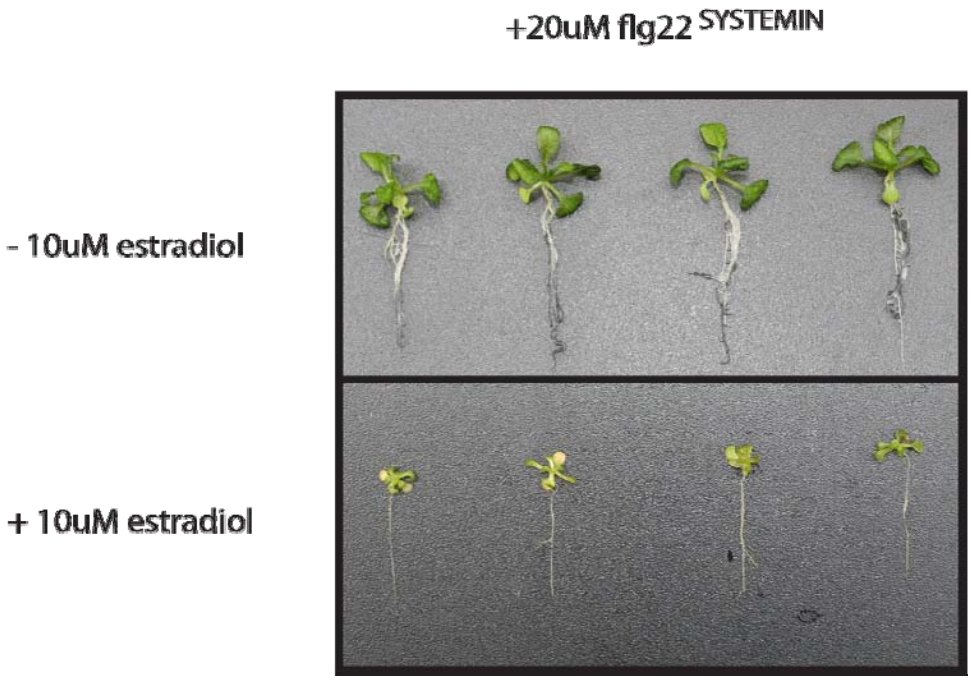

Figure 8: Growth inhibition assay using homozygous T3 Le15-24^{est} transformed *Arabidopsis thaliana* (Col-0/*fls2*$^-$).
Seedlings were germinated on MS-plates and hereafter transferred for ten days under constant light conditions into liquid MS-medium containing or not 10µM estradiol and 20µM of flg22^{SYSTEMIN}.

The aim of this experiment was to test whether an antagonistic peptide, which does not activate FLS2 but rather inhibits the docking of the agonist to the receptor, might also interfere with the observed Le15-24 induced spontaneous growth inhibition phenotype. However, we did not observe an attenuation of the previously observed growth inhibition phenotype induced by Le15-24 (Fig. 8).

Temporal dynamics of estradiol-induced expression of the Le15-24 chimeric receptor

We wanted to analyze the temporal dynamics of expression of the chimeric receptor Le15-24 in the presence of estradiol in homozygous T3 LeFLS15-24 plants. Therefore, about 2 week old seedlings were germinated on MS-plates, transferred to liquid MS-medium and after 2 weeks, 10µM estradiol was added. At time points 0, 1h, 3h, 6h,12h, and 24h random leave samples were snapfrozen in liquid nitrogen. These samples, together with similarly grown, untreated wildtype Arabidopsis (Col-0), were used for detection of AtFLS2 and Le15-24 using antiFLS2 antibodies (Fig. 9) (Chinchilla et al., 2006).

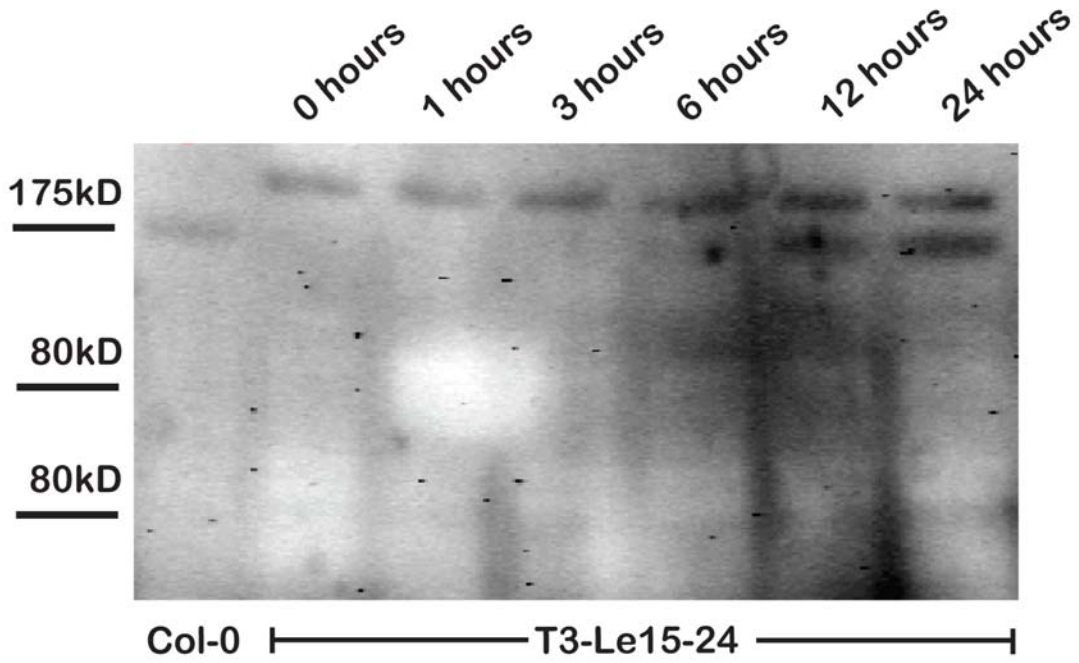

Figure 9: Time-course of expression of the Le15-24^{est} chimeric receptor in homozygous T3 Le15-24^{est} after induction with estradiol.
Western blot using wildtype Arabidopsis (Col-0) and homozygous T3 Le15-24^{est} seedlings. Anti-FLS2 antibodies were used to detect expression of AtFLS2 (WT) and Le15-24. LeFLS15-24 seedlings were treated with 10µM of estradiol and leave samples were taken at the indicated time points. First appearance of a band around 175kD, corresponding to the expected size of Le15-24 was detected faintly after 6 hours of estradiol treatment. After 12 and 24 hours of estradiol treatment, a clear band at the expected size was detected. The presence of the additional band of around 200kD in the transformed samples is most probably an artifact due to grinding which can sometimes also be detected in wildtype samples.

The earliest time point to observe a band at the expected size of Le15-24 (175kD) was 6h after the estradiol treatment. At time points 12 and 24 hours, the bands at the expected size were clearly visible. The intensity of expression between the time points 12 and 24 did not differ significantly, indicating that after 12 hours of estradiol induction expression reaches maximum levels.

Biosynthesis of ethylene is increased in estradiol treated Le15-24^{est} seedlings

Increase of ethylene production is a well characterized response when plants are challenged with pathogens or elicitors (Broekaert et al., 2006). For example, treatment of most Arabidopsis plants with flg22 or elf18 leads to a measurable increase of ethylene production that within a period of a few hours after the treatment. To investigate whether Le15-24^{est} transformed plants produce higher amounts of ethylene in the absence of elicitors than wild type Arabidopsis, 2 week old wildtype Arabidopsis (Col-0) and homozygous T3 Le15-24^{est} seedlings grown in liquid MS-medium were transferred as whole seedlings to gastight sample vials (Fig. 10) and treated with or without 10µM estradiol alone and/or 1µM of flg22 or elf 26.

Figure 10: Experimental setup to measure the ethylene biosynthesis of whole seedlings.
Whole homozygous T3 Le15-24^{est} and wildtype Arabidopsis (Col-0) seedlings were transferred into gastight vials containing liquid MS. After a recovery period of 6h, 10µM estradiol and/or 1µM flg22 or 1µM elf26 was added and the samples were closed. Ethylene concentration in the vials was measured after 12h by gas chromatography.

A clear increase in ethylene biosynthesis was detected in 10µM estradiol treated Le15-^{24est} transformed seedlings compared to likewise treated wildtype Arabidopsis (Col-0) samples after a period of 12 hours (Fig. 11). As control, untreated Le15-24^{est} seedlings, as expected, only reacted to the addition of elf26 but not flg22 with an increase of ethylene biosynthesis. Wildtype Arabidopsis (Col-0), in turn, responded to both flg22 and elf26 peptides with an increase of ethylene biosynthesis, but not to the addition of estradiol.

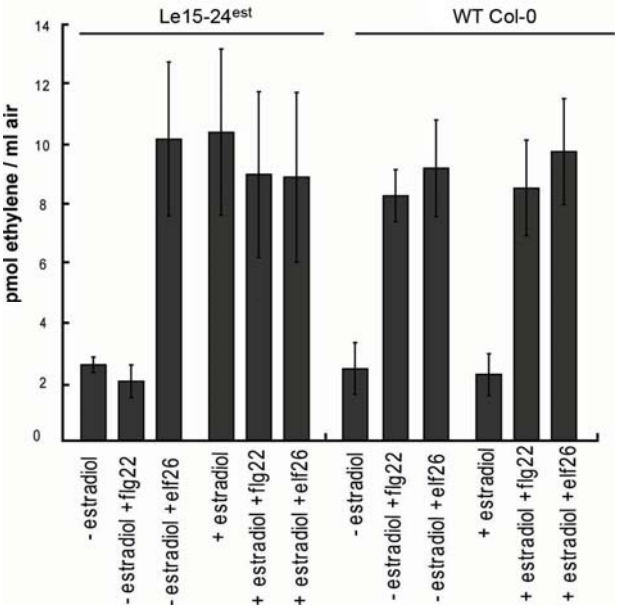

Figure 11: Increased ethylene biosynthesis of estradiol induced homozygous T3 Le15-24^{est} plants.
Statistical analysis of ethylene production. Clearly, the increase of ethylene production can be seen when estradiol is added to Le15-24^{est} transformed seedlings compared to not-estradiol treated seedlings. Estradiol does not increase ethylene production in wildtype Arabidopsis (Col-0) seedlings. Quantity of ethylene production statistically equals between estradiol treated Le15-24^{est} transformed seedlings and elicitor treated wildtype Arabidopsis (Col-0) seedlings. No quantitative synergistic effect can be observed when seedlings are concomitantly treated with estardiol and elf26. (n=10 for each treatment, the experiment was repeated 3 times with similar results)

Interestingly, the intensity of Le15-24^{est} estradiol-only treated seedlings and Le15-24^{est} estradiol + elf26 treated seedlings did not differ significantly from each other. Also, no quantitative difference in ethylene production was observed between estradiol-treated Le15-24^{est} seedlings and flg22/elf26 treated wildtype Arabidopsis (Col-0) seedlings (Fig. 11).

Production of reactive oxygen species (ROS) could not be detected with the methods used

Production of ROS is a typical quick defense response that occurs within seconds after plants are challenged with attacking pathogens (Torres, 2009). Production of ROS is considered a “quick-response”, in contrast to growth inhibition which is considered a “late-response” and the increase of ethylene biosynthesis which can be situated intermediate. Because of the quick nature of this response, the setup to test the production of ROS on a putatively constitutive active defense system is rather complex.

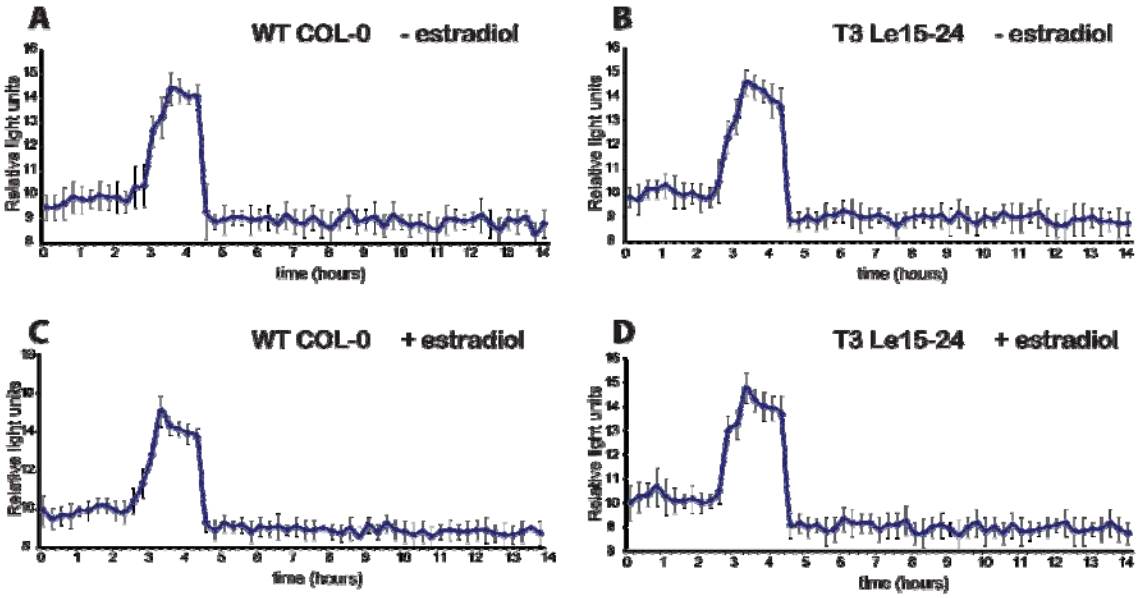

Figure 12: Oxidative burst cannot be measured with homozygous T3 Le15-24^{est} when treated with estradiol over a time-period of 12 hours.
Leave pieces of wildtype Arabidopsis (Col-0) (**A, B**) and homozygous T3 Le15-24^{est} (**C, D**) were or were not treated overnight with 10µM estradiol prior to the measurement of ROS generation in a luminescence plate reader for a time period of 12h. Spontaneous generation of ROS was detected neither in estradiol treated or untreated Le15-24^{est} transformed samples nor likewise treated wildtype Arabidopsis (Col-0).
(The spike which can be observed around 3h is an experimental artifact.)

Pieces of leaves of 4 week old homozygous T3 Le15-24^{est} and wildtype Arabidopsis (Col-0) plants were pre-incubated overnight in water containing 10µM estradiol. After the incubation, the pieces were transferred into a multiwell plate, each well containing water and a luminal/peroxidase mixture. Overnight treatment was chosen because after 12 hours the accumulation of Le15-24 appeared to reach maximum (Fig. 9). The production of ROS was measured in a

luminescence plate reader for the period of 12 hours. However, no signal could be detected during the measuring time, indicating that no production of ROS took place (Fig 12). (The spike observed after 3 hours is an artifact due to mechanical movement of the machine.)

Expression of Le15-24^{est} in *Nicotiana benthamiana*

To investigate whether the Le15-24 chimeric receptor can trigger ligand-independent increase of ethylene biosynthesis in the solanaceae *N. benthamiana*, Le15-24^{est} and AtFLS2 were transiently transformed into *N. benthamiana* by Agrobacterium pressure infiltration. Transformed leave pieces were sampled 12h, 24h and 48h after injection. Leaf pieces were kept subsequently for 6h or 12h in gastight vials containing water with or without 10µM estradiol and the amount of ethylene was measured by gas chromatography. However, we did not detect a significantly higher amount of ethylene when the infiltrated leaf pieces were harvested 12h and 24h post-infiltration and treated for both 6h and 12h with estradiol. Likewise, no increased ethylene biosynthesis was detected in samples harvested 48h post-infiltration and treated for 6h with estradiol. However, when using samples harvested 48h after infiltration and treated for 12h with estradiol, we observed repeatedly a small increase of ethylene biosynthesis of Le15-24^{est} transformed plants over AtFLS2 transformed plants (Fig. 13). This increase however, was not always significant and represents rather a trend that might be interesting to follow up.

10µM estradiol treatment / post infiltration	6h	12h
12h	-	-
24h	-	-
48h	-	-/+

Table 1: Ethylene biosynthesis of Le15-24^{est} transiently transformed *Nicotiana benthamiana*.
No increase of ethylene biosynthesis was detected with leaves sampled 12h or 24h post-infiltration when treated for both 6h and 12h with 10µM estradiol. Likewise, no increase of ethylene biosynthesis could be detected in samples harvested 48h post-infiltration and treated for 6h with estradiol. However, a trend for higher erhylene production over control samples was noticed when the samples were harvested 48h post-infiltration and treated for 12h with estradiol.

Further experiments should be performed by transformation of *N. benthamiana* with Le15-24^{est}. Additionally co-transformation of Le15-24^{est} and AtBAK1 should be performed to investigate whether AtBAK1 is specifically needed to activate ligand independent responses of the Le15-24 chimeric receptor in *N. benthamina*.

Le15-24 related constructs as tools

Additionally a series of Le15-24-related constructs were produced. The constructs Le15-24^{EFRk}, Le15-24^{BRI1k} are composed of the extracellular part of the previously described Le15-24 chimeric receptor extracellular part fused to the transmembrane, juxtamembrane and kinase domain of the EF-Tu receptor EFR and the brassinosteroid receptor BRI1. Le15-24^{KD} is a putatively kinase dead variant of Le15-24. All constructs are driven by a 2x35s promoter and were cloned into the pMDC32 gateway^{TM} compatible vector. The constructs were transformed into *fls2* Arabidopsis (Col-0) and the T1 transformants were screened for abnormal growth phenotypes.

Transformation of Le15-24^{EFRk} yields a similar, but weaker growth inhibition phenotype compared to Le15-24 when introduced into Arabidopsis

Le15-24^{EFRk} constitutes a chimeric receptor with the extracellular domain of the Le15-24 chimeric receptor and the transmembrane and intracellular juxtamembrane and kinase domain of the PRR EFR (Zipfel et al., 2006). EFR has been shown to activate the same set of responses as FLS2 upon perception of elf18. Therefore we wanted to investigate whether a chimeric Le15-25 construct containing EFR kinase domain is able to provoke a similar phenotype as Le15-24 when introduced into Arabidopsis.

Le15-24^{EFRk} was transformed into *fls2*$^{-}$ Arabidopsis (Col-0). Seedlings positively selected for integration of the transgene were transferred into soil. However, in this case, only a limited number of transformants was obtained (Fig. 13).

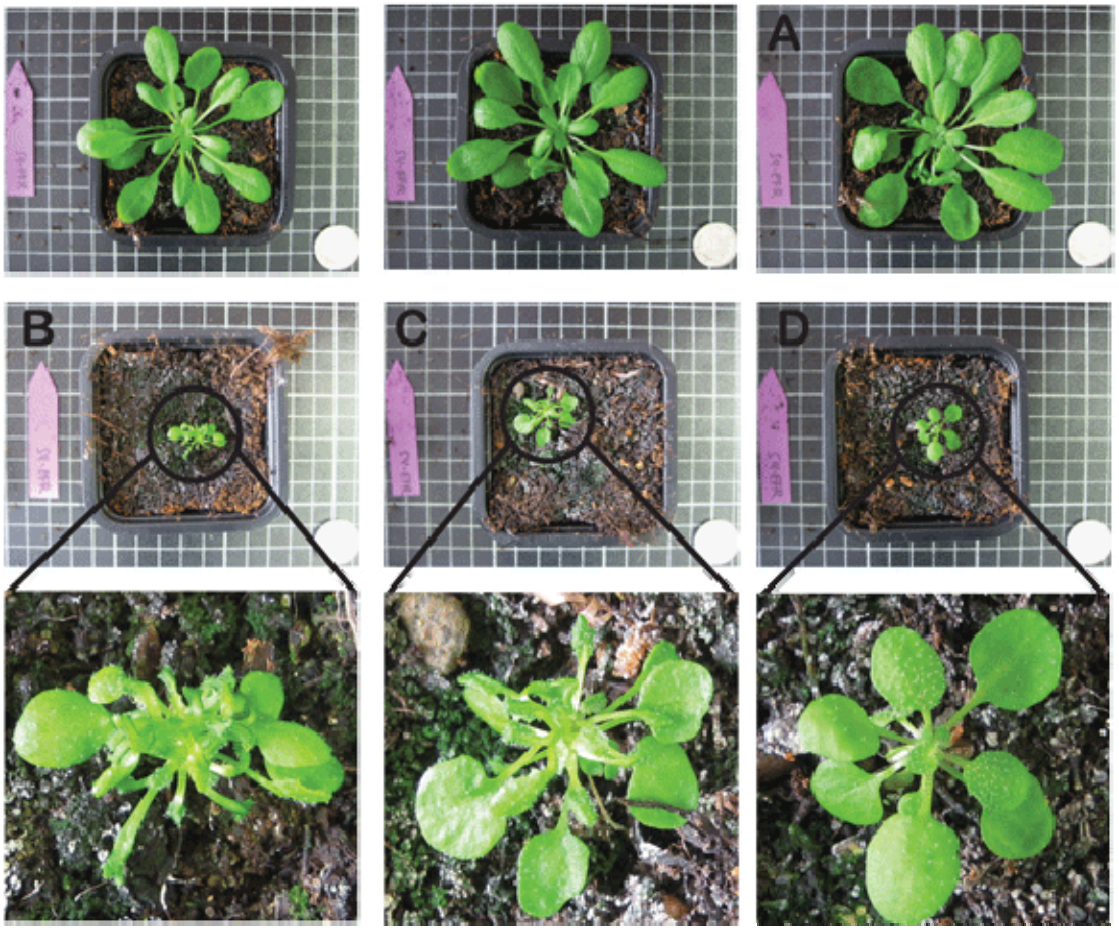

Figure 13: Four week old Le15-24^{EFRk} transformed T1 seedlings.
About 50% of the T1 transformants were inhibited in seedling growth. However, the observed phenotype was weaker than in Le15-24 transformed seedlings. Also, in contrast to Le15-24 transformed seedlings, the affected plants did not develop heavy leaf necrosis in early stages of development. **A)** represents a plant that was only affected by irregular growth at later stages of development as seen on the curly growth of newly developing leaves (Type 1). **B)** and **C)** represent plants that were affected from growth inhibition soon after germination (Type 2). **D)** represents a seedling that did not show obvious deformations in leaf development but stayed very small during the whole life cycle.

However, when we oberved that growth of about 50% of seedlings was obviously impaired. After four weeks, it was possible to clearly distinguish between plants that were strongly impaired in growth whilst others seemed to be healthy and did not show obvious signs of impaired or irregular growth. Among the plants that showed impaired growth, we could generally distinguish between two types. Type 1 was growing regularly, not showing an abnormal phenotype until week three to four. From this time on, we observed that newly developing leaves started to grow curly and the lateral expansion of the leave was inhibited (Fig. 13, A). Type 2 however was impaired in growth soon after germination. The observed growth

inhibition phenotype of type 2 seedlings Le15-24^{EFRk} transformed plants was by far not as strong as we could observe it for Le15-24 transformed plants. Leaves from such affected plants did not properly develop and lateral expansion of the leaves was nearly inhibited. Additionally curling of the leave stalk was observed (Fig. 13, B, C). The plants survived much longer than Le15-24 transformed seedlings. However, only 2 of the early affected plants could be brought to flowering stage. Like the Le15-24 transformants, most of the plants died before reaching the flowering stage. Maybe as an artifact of transformation, about 10% of the transformants did not show an obvious defective leaf phenotype, but remained in general very small during their entire lifecycle (Fig. 13, D).

Transformation of Le15-24^{BRI1k} and Le15-24^{KD} does not provoke seedling growth defects when transformed into Arabidopsis

Le15-24^{BRI1k} constitutes a receptor with the Le15-24 extracellular domain attached to the transmembrane and intracellular juxtamembrane and kinase domain of the brassinostroid receptor BRI1. Because BRI1 has been shown to be dependent on BAK1 as a co-receptor to initiate signaling, such a construct can be used to investigate whether the Le15-24 extracellular domain attached to the BRI1 kinase can act as constitutively active brassinosteroid signaling molecule. When Le15-24^{BRI1k} was transformed into *fls2* Arabidopsis we did not observe any abnormal growth of positively selected seedlings.

Le15-24^{KD} constitutes a Le15-24 chimeric receptor with a putatively inactive kinase domain. The lysine residue at position 898 of Le15-24 is replaced by an alanine. K^{898} is positioned within the kinase ATP binding site. The corresponding residue has been shown to disrupt kinase functionality in AtFLS2 (Asai et al., 2002a). This construct was used to investigate whether an active kinase is needed for the ligand independent activity of Le15-24. Indeed, when the construct was transformed into *fls2* Arabidopsis we could not detect impaired growth of the T1 progeny of the Le15-24^{KD} transformed Arabidopsis seedlings.

Discussion and conclusions

Expression of the chimeric receptor Le15-24 induces responses in Arabidopsis which accord with a constitutive active FLS2 allele

The finding that the expression of Le15-24 in the absence of ligand induces a set of responses which are typically observed when plants are challenged with flg22 was a serendipitous finding. The foremost interesting feature about Le15-24 is that the changes which were made to the receptor (swapping of the LeLRRs 15-24 into AtFLS2) are situated only in the extracellular, ligand binding domain. The complete cytoplasmic part remains completely untouched. Consequently, the observed signaling in absence of flg22 is triggered only via the chimeric LRR domain of Le15-24. Several models adopting the up-to-date knowledge about FLS2 activation and signaling will be drawn in the following to explain this finding on a molecular level.

BAK1 (SERK3) has been shown to be a partner of ligand-binding leucine-rich repeat receptor kinases, in particular BRI1 and FLS2. It has been demonstrated that among the first signaling events after flg22 binding to FLS2, BAK1 is quickly recruited into the receptor complex (Chinchilla et al., 2007). In this model, BAK1 acts as the major signaling partner for FLS2 which enables the ligand-binding receptor to transmit the signal from the outside of the cells to the inside. However, nothing is currently known what exactly happens after binding of the ligand to FLS2. It is thought to be likely that a conformational change of the ectodomain of ligand activated FLS2 allows docking processes of FLS2 adaptors (like BAK1) to form the FLS2 signaling complex. This complex-formation subsequently leads to intracellular signaling events like the phosphorylation of kinases and ultimately to the full activation of defense responses (Schulze et al., 2010).

Le15-24 is a chimeric receptor consisting of LRRs of AtFLS2 and LeFLS2. Although AtFLS2 and LeFLS2 have most probably a very similar tertiary structure, they share only 55% amino acid identity in the LRR domain (Robatzek et al., 2007b). It has been shown in the previous part of this work that swapping of the LeLRR24 does not necessarily lead to non-functional receptors. In fact, most of the receptors are functional. Therefore, the region of LeLRR15 might be a region that is particularly prone for disturbance. Dunning and coworkers have shown that the region between the β-sheet of LRRs 13,14 and 15 is particularly well conserved among the FLS2 orthologues of various brassicaceae (Dunning et al., 2007). However, when comparing AtFLS2 and LeFLS2, no particular

conservation between the respective regions can be observed (Results I; Fig 12). This indicates that the β-sheet region between LRR 13, 14 and 15 is probably particularly important for the brassicaceae derived FLS2 alleles but not for LeFLS2. Due to the tomato LRR 15 insertion, the adjacent AtFLS2 LRR14 and thereby maybe an AtFLS2 specific, critical structure may become disturbed. With respect to the constitutive active-like phenotype this receptor induces in transformed plants, it might be that the Le15-24 protein cannot adopt a functional structure and is destabilized in its inactive "sensor" conformation as it has been shown for constitutive variants of the GCPR proteins (Gether et al., 2002). Thereby the chimeric receptor might exhibit a conformation that occurs otherwise only in ligand activated FLS2. Le15-24 might, by adopting such an "active" conformation, reveal sites or structures that serve as docking stations for BAK1 or other, yet unknown, FLS2 adaptor molecules. This would enable the receptor to transmit a signal in absence of an activating ligand. This hypothesis gets strengthened by the, although preliminary, finding that *bak1*-deficient Arabidopsis are not impaired in their growth upon transformation with Le15-24 while wildtype and *fls2* Arabidopsis (Col-0) are.

However, the contribution of BAK1 to the observed, Le15-24 induced phenotypes has to be investigated in more detail before firm conclusions can be drawn. Determination of the protein levels of Le15-24 in *bak1* background transformed Arabidopsis is an essential necessity before planning further experiments. Also, co-immunoprecipitation experiments should to be performed to investigate if a spontaneous complex formation between Le15-24 and BAK1 can be detected. The estradiol inducible homozygous Le15-24 T3 lines represent suitable plant material to perform such experiments. Furthermore, the transformation of the estradiol-inducible construct into a characterized *fls2/bak1* double-mutant line might provide an additional tool for further experiments. Additionally, backcrossing experiments into wildtype Arabidopsis to test if the seedling growth inhibition phenotype reappears will be interesting to further follow up this serendipitous finding.

It has been shown that flg22 perception by FLS2 induces FLS2 endocytosis and accumulation into intracellular mobile vesicles (Robatzek et al., 2006). It has also been demonstrated that FLS2 gets ubiquitined and rapidly degraded upon flg22 perception (Gohre et al., 2008). However, the role of the complex formation of FLS2 and BAK1 for internalization and degradation remains unclear. Similarly it is unclear if the whole complex or only FLS2 gets internalized while BAK1 (and maybe other signaling molecules) are released before internalization. Additionally, it has not been shown whether the internalization process is

required for signaling or if internalization is part of a recycling process of FLS2 molecules or simply degradation of receptors which have fulfilled their duty after once being activated. The latter would stand in contrast to, for example, GCRP receptors which return to their inactive “sensor” state after activation and signaling.

In the context of this study, the fact that activated AtFLS2 is being internalized, raises the question whether a constitutively activated FLS2-allele is also internalized. If so, this would implicate a constant turnover of freshly produced constitutive active FLS2 to and from the membrane. Consequently, the fact that we deteced a considerable amount Le15-24 receptor by western blot, does not mean that these receptors are necessarily located in the plasma membrane. It might well be that most of the detected protein is in fact not located in the membrane but rather involved in various stages of the above mentioned turnover. An experiment, in which the localization of the Le15-24 receptor could be determined in more detail, might provide interesting information to answer this open question.

Expression of Le15-24 results in seedling growth inhibition and increased ethylene biosynthesis but no oxidative burst

The oxidative burst is a very quick response of the plant cell when challenged with flg22. ROS production upon pathogen challenge has been described to be mediated by plasma membrane bound NADPH oxidases (Torres et al., 2002; Torres, 2009). It has been hypothesized that ROS production upon flg22 challenge is only triggered when a sufficient amount of receptors is activated in the plasma membrane and the signaling input reaches a “critical mass” (Georg Felix, discussions). Since it has been shown that FLS2 gets internalized upon activation, the actual amount of a constitutive active FLS2-allele in the plasma membrane and therefore the signaling strength at a certain time in the plasma membrane might indeed be much lower for a constitutive active receptor than in wildtype plants, which are suddenly challenged with flg22. Supposed that Le15-24 is indeed a constitutive active FLS2 allele and that such a receptor is involved in a constant turnover to and from the membrane, Le15-24 signaling intensity at the membrane might not be able to accumulate to the critical mass which might be needed to activate ROS. It could therefore be that signaling is only sufficient to activate medium or late responses such as the indeed detected increased ethylene biosynthesis (medium response) or growth inhibition (late response). It would be interesting to obtain an inducible Le15-24 cell culture line to perform

medium alkalization experiments, another known quick response of Arabidopsis cells related to flg22 challenge.

Expression of Le15-24^{EFRk} inhibits seedling growth

It has been demonstrated that BAK1 interacts with EFR upon elf18 treatment (Schulze et al., 2010). We showed that Le15-24 transformed seedlings are likewise severely affected in growth in the absence of a ligand as wildtype Arabidopsis are when treated with flg22 or elf26. A model of the molecular mechanism for the ligand independent activity has been outlined above. Shortly, the extracellular domain of Le15-24 is proposed to be forced into conformation where it interacts with signaling activating molecules such as BAK1 in the absence of a ligand. Le15-24^{EFRk}, having the same extracellular part as Le15-24, would therefore similarly interact with the BAK1-LRR and thereby bring the BAK1 kinase and the EFR kinase in close vicinity. Consequently, these kinases would be in a position to interact with each other and thus might activate signaling. However, due to the much weaker growth inhibition of Le15-24^{EFRk} transformed plants compared to Le15-24 transformed plants, it may be speculated that BAK1 is not the preferred interaction partner of the EFR kinase. The markedly weaker growth inhibition phenotype observed in Le15-24^{EFRk} transformed plants might be an indication for this hypothesis. Another long standing hypothesis speculates that other members of the SERK/BAK family (or other small LRR-RLKs) besides BAK1, function as general signaling partners for various PRRs (and other RLKs). Each receptor is proposed to have a preference for a “main signaling partner”. Thereby, other signaling mediating molecules are proposed to, at least partially, replace the actions of the preferred signaling partner molecule. Such a “gaussian function model” of redundant signaling partner molecules could explain why the observed growth inhibition phenotype is weaker in Le15-24^{EFRk} transformed seedlings. BAK1 may not be the preferred signaling partner of EFR but is however able to substitute it when forced into place (for example by the Le15-24 LRR domain). It would be an interesting experiment to introduce Le15-24^{EFRk} into *bak1* deficient plants, other *serk/bak* mutants to screen and compare the phenotypes of the transformed plants. For future experiments, although the clearly visible phenotype is a strong indicator, expression of Le15-24^{efr} in transformed plants has to be verified by western blotting.

Le15-24^{BRI1k} does not impair seedling growth

When Le15-24^{BRI1k} was transformed into *fls2* Arabidopsis (Col-0) we did not detect abnormal growth of positively selected seedlings. This result indicated primarily that Le15-24^{BRI1k} does not feed into the same mechanisms which lead to the onset of typical flg22/elf26 related defense responses like growth inhibition and increased ethylene biosynthesis as Le15-24. It also indicates that the extracellular part of Le15-24 alone is not sufficient for inducing the growth inhibition phenotype. Growth inhibition might thus be dependent on the presence of an “immunity-type” kinase domain like the FLS2 or the EFR kinase.

However to draw firm conclusions it remains important to test whether Le15-24^{BRI1k} is expressed in transformed plants. If Le15-24^{BRI1k} is expressed in transformed plants, the next logical experiment would be to investigate whether constitutive signaling of the attached BRI1 kinase Le15-24^{BRI1k} can be detected. A possible setup for an experiment would be the use of brassinazole, a triazole-type brassinosteroid biosynthesis inhibitor (Asami et al., 2000). Transformed seedling should be grown on growth medium containing brassinazole and screened for resistance against the typical brassinazole-induced morphological changes compared to wildtype plants (Asami et al., 2000). If transformed plants indeed overcome the effects of the brassinazole induced inhibition of brassinosteroid biosynthesis, this would be a strong indication that the BRI1 kinase attached to the Le15-24 extracellular domain is in an active signaling state. Additionally, it might be interesting to test this construct also in *bak1*-deficient Arabidopsis lines.

A kinase dead variant of Le15-24 does not impair seedling growth

T1 transformants of a kinase dead variant of Le15-24 did not show impaired growth after germination**.** This result implies that an active kinase is needed for transducing the activation-signal originating from the extracellular domain of Le15-24 across the membrane to the cytoplasm. It is a strong sign that Le15-24 indeed is a constitutive active FLS2 allele. However, it still needs to be verified that Le15-24^{KD} is indeed expressed in the transformed plants. Since it has been shown that kinase dead variants of AtFLS2 are difficult to express (T. Mentzel, personal communication), protein verification should be considered a priority before planning future experiments.

The Le15-24 extracellular domain as a tool to characterize PRRs

For future thoughts, a system using a constitutive active PRR-extracellular domain, or a constitutive BAK1 interacting extracellular domain, could be used as tool to characterize other PRRs by fusing their respective kinase domain to the extracellular domain of Le15-24, then transforming these constructs into Arabidopsis and analyzing the phenotypes of the resulting positively selected seedlings. This might serve as tool to rapidly characterize PRR candidates that have a similar signaling output as FLS2 and therefore may represent yet unknown immune receptors.

Final Discussion

MAMP (PAMP) triggered plant immunity

MAMP perception and signaling received constantly growing attention during the last decade of research on plant immunity. From being an orphan research topic in the beginning, the characterization of MAMPs and MAMP receptors in plants has become an important player in plant immunity research. The evidence of the biological relevance of MAMP perception as a key pillar of plant immunity (Zipfel et al., 2004) has led to a whole new interpretation about the nature of plant immunity (Jones and Dangl, 2006). Additionally, the topic evoked the interest of animal scientists investigating the mammalian innate immune system after it became apparent that plants and animals use similar structures to perceive similar patterns (Iriti and Faoro, 2007).

Today, a constantly growing research community focuses on PTI and the molecular mechanisms of activation of PTI. PTI confers quantitative resistance to a broad range of pathogenic microbes and not only to a small subset of specialized pathogens (Boller and Felix, 2009). This makes research on PTI also interesting for approaches that are directed towards more applied goals. An R-gene mediated strategy for, for example, a crop enhancement project would involve the transfer of many different genes into each cultivar to achieve a generally enhanced resistance against a diverse range of pathogens. In contrast with only a few or even a single additional MAMP perception system(s) introgressed, it may be possible to enhance general resistance of a cultivar in a way which may lead to plants which generally require less treatment with pesticides. Indeed, such experiments have already been accomplished by transferring the recognition system for EF-Tu, which is unique to Brassicaceae into commercially important solanaceae. Exciting preliminary results have already shown that such “enhanced” plants indeed seem to be more resistant towards a broad range of bacterial pathogens (Cyril Zipfel, personal communication).

In addition, pathogens could quickly overcome resistance mediated by ETI. Because of the requirement of ETI for a particular effector-molecule, the pathogen variant would only have to lose this molecule and could evade detection. In contrast, MAMPs, by definition, are pivotal for the pathogen and thus not easily manipulated. In this context, the topic of this work, to investigate areas of FLS2 involved in recognition of the ligand and the description of the species specific specificities of flagellin perception, is a further step towards establishment of a mechanistic model of PTI.

Species specific flagellin perception

Both plants and animals recognize bacterial flagellin as a MAMP. For mammals, it has been demonstrated that mice and humans do not perceive bacterial flagellin in the exactly same way and that differences in the amino acid sequence of the respective TLR5 LRR domains are responsible for the observed difference (Andersen-Nissen et al., 2007). Comparative studies between the brassicaceae *Arabidopsis thaliana* and the solanaceae tomato showed that both species have a highly sensitive perception system for flg22 (Meindl et al., 2000; Bauer et al., 2001b; Chinchilla et al., 2006). In general, flagellin perception in plants appears to follow some common fundamental features. For example, most plants respond to treatment with flg22. However, when different flg22-derivatives were tested, many plants indeed do or do not recognize them with a distinct, species specific pattern. For example, *Populus trichocarpa, Ricinus communis, Nicotiana benthamiana, Solanum lycopersicum,* and *Arabidopsis thaliana* have been tested extensively with a variety of flg22-derived peptides and shown to respond in a species specific way to various flg22-derivatives (Meindl et al., 2000; Bauer et al., 2001b; Chinchilla et al., 2006; Robatzek et al., 2007b; Deslex, 2009). Even between the closely related solanaceae tomato and *Nicotiana benthamiana,* that share 86% identity (custalW) in the amino acid sequence of the LRR domain, clearly a species specific difference in the recognition of different flg22-derived peptides was detected (Fig. 4A and 1B).

R-genes are involved in a rapid host-pathogen co-evolution arms race process and have been demonstrated to be subjected to positive selection pressure in the LRR region and particularly the putatively solvent-exposed residues in β-sheets (Parniske et al., 1997; Ellis et al., 1999; Meyers et al., 2005). Positive selection is in line with the role of R-proteins in host-pathogen co-evolution and with the need for selection for rapid evolution of new resistance specificities to counter new or altered effector molecules. (Ellis et al., 1999)

In contrast, PRRs, by definition, recognize structures which cannot be easily masked or substituted by a pathogen. However, the fact that species specific differences in flagellin perception rather seems to be the norm than an exception, might indicate that FLS2 alleles might also be subject to diversifying pressure. The variety of species specific differences of flagellin perception might reflect a balanced selection pressure on the FLS2 locus (Mitchell-Olds et al., 2007). It will be interesting to hear about studies which investigate whether indeed a selection pressure can be identified for the FLS2 protein in general and the LRR domain in particular. Additional available FLS2 alleles will help investigating this question.

Furthermore, the identification and characterization of FLS2 orthologues provides a tool kit for refined structure-function experiments. For example, both Arabidopsis and *N.benthamiana* have been shown to be nonresponsive to the flg22 derivative flg15$^{E.coli}$. While Arabidopsis and tomato only share 55% identity in their respective LRR domain amino acid sequence, the recently cloned NbFLS2 (Wyrsch, 2010) and LeFLS2 share 86%. Using such a high conservation in combination with different patterns of flg22-derivative perception can be used in a targeted approach to identify amino acid residues which are responsible for the differences in flg22-perception, thus pinpointing specific residues involved in flagellin perception.

The tomato and Arabidopsis FLS2

The AtFLS2 and LeFLS2 LRR domains share 55% amino acid identity, the respective kinase domains 59% (Robatzek et al., 2007b). Both receptors recognize the flg22 epitope of bacterial flagellin as an elicitor. However, data about the interaction of flg22 with the receptors are scarce. Dunning and coworkers identified by alanine exchanges in nearly each of the 28 LRRs the LRRs 9-15 as contributors to flagellin responsiveness in *Arabidopsis thaliana* (Dunning et al., 2007). Supporting this finding they detected, when comparing the FLS2 sequences from over 20 different species of brassicaceae, an island of increased amino acid conservation in the β-sheet region of LRRs 9-15. Thereby, the highest conservation was found within the β-sheet region of LRRs 13, 14 and 15. Additionally, they observed a second conservation pattern within the β-sheet region of LRR 22-26 (Dunning et al., 2007; Albert et al., 2009).

When comparing the conservation between Arabidopsis and tomato FLS2 using the same method, we also found a similar conservation pattern in the β-sheet region, however, the conservation is restricted to LRR 9, 10, 11 and, weakly, LRR12 whilst the LRRs 13,14 and 15 are not conserved between AtFLS2 and LeFLS2 (Results I; Fig.12). The β-strand sequence of LRR11 is 100% identical in both AtFLS2 and LeFLS2. In this work we already detected full tomato type affinity for flg22 in the Le1-11:GFP chimeric receptor. This shows that the LeLRRs 1-10 are sufficient to form the basis of the higher affinity of LeFLS2 for flagellin derived peptides. In consequence, this result could indicate that the LRR 13, 14 and 15 have, due to the high conservation in the brassicaceae FLS2, an important function for Arabidopsis typical flagellin perception.

Assuming that for AtFLS2, as shown for LeFLS2, binding of the core residues of flg22 also takes place within the first 10 LRRs but with a lower affinity, the

AtLRRs13,14 and 15 might constitute the region which is important for stabilizing the “weak” binding of the flg22 core via interactions with the N-terminal residues of flg22.

A second conservation island detected by Dunning and coworkers is situated within the β-sheet region of LRRs 22-26. In contrast to the island detected between LRRs 9-15, our comparison of AtFLS2 and LeFLS2 results for this region in a conservation island that is nearly congruent with the location of the conservation island between the different brassicaceae FLS2. Furthermore, in an alignment of most LRR domains of identified FLS2 orthologues up to date, including besides Arabidopsis and tomato also the FLS2-LRR domains from *Nicotiana benthamiana*, *Vitis vinifera*, *Ricinus communis*, *Brachypodium distachion*, *Oryza sativa*, *Zea maize* and *Sorghum bicolor*, this region sticks out as the most highly conserved island with the location being identical to the alignment presented by Dunning and coworkers for the 20 brassicaceae FLS2-LRRs (Albert et al., 2009). The trans-order conservation for this specific island might indicate the presence of a structure which is important for all receptors. Our data implicate this region in recognition of the C-terminus of tomato specific flg22-derivates. It could thus be envisaged that irrespective of the species specific differences which this region can confer, it is implicated in a mechanism that is common to all FLS, like, for example, functioning as a molecular switch that is important for receptor activation.

The address-message concept

The address-message concept refers to compounds in which part of the molecule is required for binding (address) and part for the biological action (message). It was originally proposed by Schwyzer, who described the recognition of neuropeptide-hormones (Schwyzer and Pearse, 1980). Flagellin perception and receptor activation in plants has been proposed to occur according to the address-message concept (Meindl et al., 2000). Key to this conclusion was the finding that truncated flg22-derivatives act as competitive antagonists that specifically inhibit the agonistic activity of flg22 (Meindl et al., 2000; Chinchilla et al., 2006). Both AtFLS2 and LeFL22 are proposed to function according to the address-message concept.

The address-message concept as a mechanism for flagellin recognition has been deduced from the results of various studies using a plethora of different flagellin derived flg22-peptides (Meindl et al., 2000; Bauer et al., 2001b; Chinchilla et al., 2006) (Georg Felix, unpublished data). For both AtFLS2 and LeFLS2, it has been demonstrated that the flg22 elicitor peptide directly binds in a first step to a high-

affinity binding site on the LRR domain of FLS2 (Schulze et al., 2010) (Katharina Mueller, personal communication). In a second step the receptor gets activated via the C-terminal amino acids of flg22. Thereby, not only the presence, but also the composition of the C-terminal part of flg22-derivatives plays an important role for receptor activation on the one hand or antagonistic activity on the other hand of a given flg22-derivative (Meindl et al., 2000; Bauer et al., 2001b; Chinchilla et al., 2006). In the presented work we wanted to further investigate if binding and activation may be located to separate regions on the LRR domain of the tomato FLS2 protein. To investigate this, we not only focused on the ligands, but also included the receptor part. For this project, we had the advantage of using the well described flagellin perception systems of tomato and Arabidopsis. Our key approach was to construct chimerae between the tomato and the Arabidopsis flagellin receptors. With this approach we successfully detected determinative areas on LeFLS2 which are responsible for the observed LeFLS2 specific features like the higher affinity for flg22 and the less stringent requirements on C-terminal amino acid residues compared to AtFLS2. By combining the insights gained with the use of the flg22-derivated peptide library with the results generated with the chimeric receptors we were able to provide new insight about the interaction between (Le)FLS2 and its ligand.

The address-message nature of the flg22 peptide by consisting of a N-terminal/central part which is important for receptor binding and a C-terminal part which is important for receptor activation has been amply demonstrated and discussed. With respect to this concept, the stepwise address-message process might well include more than one interaction site of flg22 with the LRR domain. In this work we present the new finding that the address-message nature of the flg22/FLS2 interaction is indeed reflected by two spatially separated areas on the tomato FLS2 receptor which are involved in interaction with the flg22 central sequence (address) and recognition of the C-terminus (message). Interestingly, both identified LRR-regions comprise at least in parts the areas that have previously been shown by Dunning and coworker (Dunning et al., 2007) to be conserved above average among the β-sheets of AtFLS2 and LeFLS2. Our data show that the flagellin receptors AtFLS2 and LeFLS2 definitely differ in terms of the required “address”. The N-terminal part of flg22 consists of the seven amino acids QRLSTGS. These residues are of great importance to establish binding with AtFLS2, but not with LeFLS2. The minimum epitope of flg22 that can interact and establish binding with LeFLS2, but not AtFLS2, is the flg22 core motif RINSAKDDA (Fig. 1A). This motif therefore is the “address” motif of flg22 for LeFLS2 (in contrast to AtFLS2, for which the address-motif of flg22 additionally involves the N-terminal amino acids QRLSTGSRINSAKDDA). According to our

data, the LeFLS2 residues, which are responsible for interaction with the RINSAKDDA address-motif of flg22, are located within the first ten LRRs of LeFLS2. For an efficient binding to LeFLS2, the C- and N-terminal residues of flg22 are not important, as shown by binding assays using the flg15-Δ7 (RINSAKDDA) peptide. Consequently, in conformity with the address-message concept, we conclude that the LeLRRs 1-10 compose the address-acceptor site of LeFLS2 and therefore are the region where flg22 initially interacts with LeFLS2 (Fig 1BI). Also, LeLRR1-10 must contain molecular features which are important for the LeFLS2 main binding site.
Additionally, our data also provide evidence that the region between LeLRRs 19-24 harbors residues which are involved in the recognition of the C-terminal part of tomato specific flg22-derivatives. It has been shown that the C-terminus of flg22-peptides is an important factor whether a flg22-derivative is able to activate an FLS2 receptor or to act as an antagonistic peptide (Meindl et al., 2000). The C-terminus of flg22 therefore is the domain that contains the “message” and activates (or not) FLS2 receptors. Therefore, we suggest that the LeLRRs 19-24 are involved recognition of the C-terminus of flg22 and derivatives and, consequently, in a mechanism through which LeFLS2 is activated and finally probably induced to change its conformation from the inactive “sensor” state to the active signaling state (Fig. 1BII).

The tomato typical high affinity can therefore be attributed to the N-terminal ten LeLRRs while recognition of a tomato specific C-terminally altered flg22-derivative is located in the region between LeLRR19-24. This result raises the question how a relatively short peptide like flg22 can extend over this rather large distance, especially when considering that the central residues of flg22 are involved in binding. It appears that an inflexible, rigid model with a continuous horseshoe-shaped structure, as shown for ligand bound TLR3 (Liu et al., 2008), is incompatible with a conformational change which is obviously necessary to bypass the spatial distance between the two identified regions and is suspected to underlie receptor activation. Rather, one can anticipate that irregularities from the LRR consensus determine kinks or hinges in the LRR structure which enable the receptor to change conformation upon ligand perception.

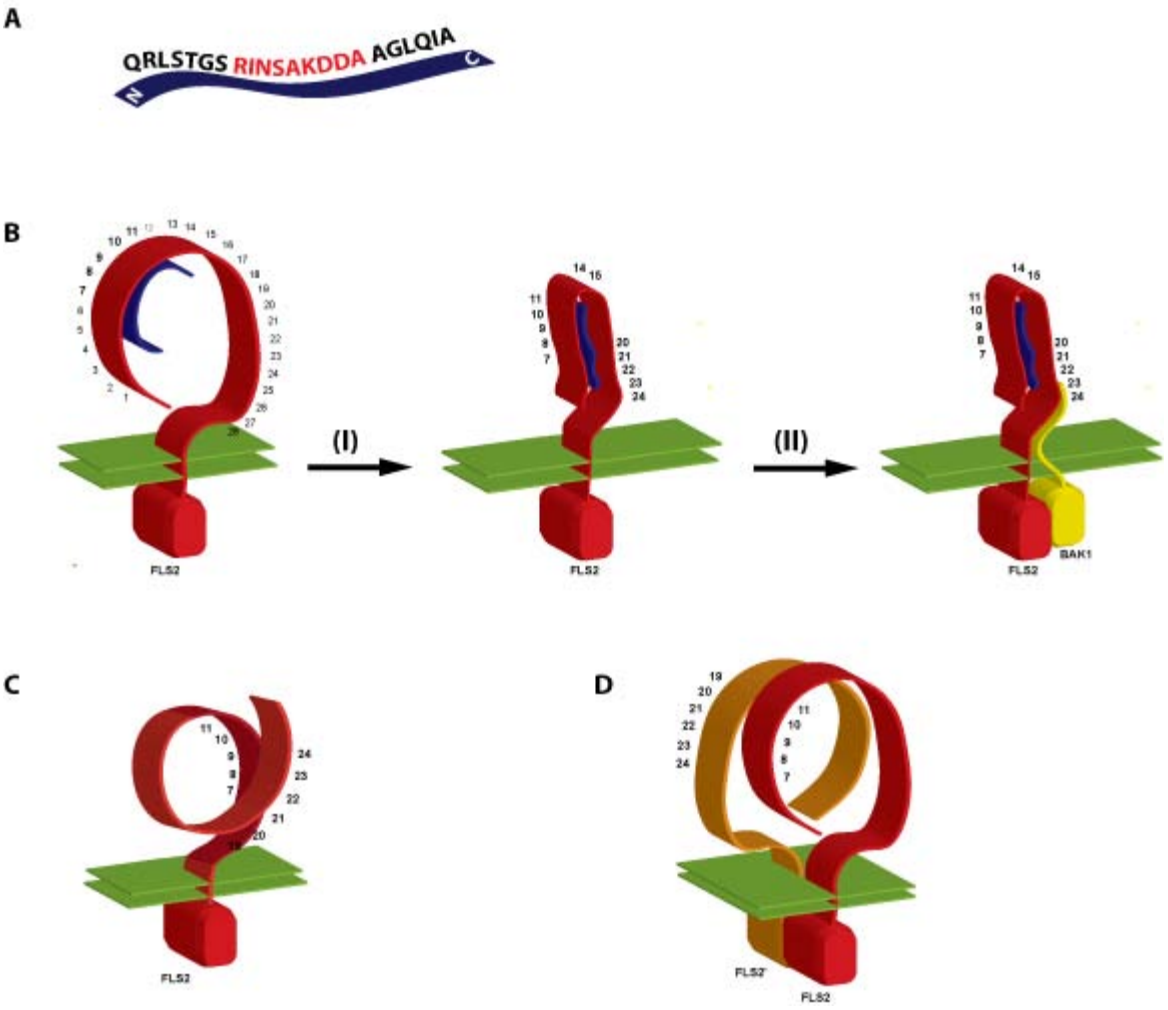

Figure 1: Models of FLS2-flg22 interaction and receptor activation.
Flg22 activates FLS2 via the address-message concept. Interaction of flg22 with FLS2 is a two-step process that leads to binding and subsequent activation of the receptor (**BI** and **BII**). For LeFLS2, the central RINSAKDDA motif (**A**; the "address") of flg22 binds to the N-terminal LRRs, involving LRRs 7-11. Connected to this process one can imagine a structural change in the ectodomain, whereby the C-terminal region of flg22 gets in contact with the region of LRR 19 to 24 which probably acts as an area which is important for receptor activation. Finally, interaction of both the address- and the message-part of flg22 allow an oligomerization process with BAK1 which finally results in the activation of signaling. Such a conformational change might be mediated via the region around LRRs 15, because a chimeric receptor between AtFLS2 and LeFLS2 with a fusion joint within LRR15 resulted in a constitutive active FLS2 allele (Results II). **C** and **D** show possible confirmations how the two identified regions of LRR 7 to 11 and 19 to 24 can be in close vicinity without the need for a rigid change in conformation.

Nonetheless, two hypothetical models can nevertheless be presented on how even in a more rigid structure a closer vicinity of the two regions can be achieved. The first model suggests a more spring-like structure for FLS2 (Fig. 1C). Since FLS2 with its 28 LRR repeats has a rather large LRR domain, it is plausible to

suspect that the N-terminal LRRs overlap the C-terminal LRRs, esp. when comparing to the known structure of TLR3 which forms an almost closed circle with only 24 LRRs. If the radius of the LRR curvature of FLS2 is only a little tighter than the TLR3 radius, the whole structure of FLS2 would much more resemble a spring than a horseshoe. Subsequently, in such a spring like conformation, the two identified regions would come in closer vicinity than in the classical, horseshoe shaped structure. A twisted superhelix structure has already been proposed for Cf9, a resistance protein containing 27 LRRs, on the basis of homology modeling (van der Hoorn et al., 2005).

The second model uses a homodimerized FLS2 complex (Fig. 1D) as a model to bypass the spatial problem. FLS2 homodimers are, however, a contentious issue. While Ali and coworkers used an in vivo bimolecular fluorescence complementation (BiFC) system and fluorescence resonance energy transfer (FRET) to show that FLS2 does not homodimerize either constitutively or in the presence of flg22 (Ali et al., 2007), Sun and coworkers claim to have detected constitutive homodimerization of FLS2 by performing co-immunoprecipitation experiments (Andrew Bent, personal communication). However, given FLS2 adopts a regular, almost circular, horseshoe-shaped structure, a FLS2 homodimer would allow a simple mechanistic model to be drawn which is able to explain how the flg22 molecule could reach both the regions of LRR7-10 and LRR19-24. By adopting a position similar to the arrangement of a ligand bound TLR3 homodimer (Liu et al., 2008), in which the two horseshoe shaped LRR domains are situated side by side and laterally inversed to each other, the LRRs 7-11 would be situated in close vicinity to the LRRs 19-24 from the other FLS2 molecule. Thereby the flg22 molecule can possibly reach both areas without the need of a significant conformational change of the receptor. In such a model, both a constitutive FLS2 dimer recognizing flg22 and FLS2 monomers which dimerize upon binding of the address can be anticipated. Consequently, dimer release or formation of dimers upon flagellin perception might be the key mechanism that leads to the recruitment of adaptor molecules like BAK1 and thus signaling.

For the future, despite the use of a variety of sophisticated methodologies to determine the structure-function relationship of FLS2/flg22, the establishment of crystals of both FLS2 and ligand-bound FLS2 is in demand. This will ultimately identify the true nature of the ligand induced course of events which lead to FLS2 activation and signaling partner recruitment.

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Frequent abbreviations

At	Arabidopsis thaliana
Avr	avirulence gene or protein
BAK1	BRI1-associated kinase (see also SERK1)
BL	brassinolide
bp	base pair
BR	brassinosteroid
BRI1	brassinosteroid insensitive 1
CC	coiled-coil
Col-0	Arabidopsis ecotype Columbia
EF-Tu	elongation factor Tu
EFR	elongation factor Tu receptor
elf	EF-Tu peptide
ETI	effector triggered immunity
ETS	effector triggered susceptibility
flg***	flagellin peptides
FLS2	flagellin sensing 2
FW	fresh weight
g	gram
GFP	green fluorescent protein
HR	hypersensitive response
IP	immunoprecipitation
kD	kilodalton
Le	Lycoersicum esculentum (Solanum exculentum)
LPS	lipolysaccharides
LRR	leucine-rich repeat
MAMP	microbe-associated molecular pattern
M	molar
MES	2-morpholinoethanesulfonic acid monohydrate
min	minute
MS	Murashige and Skoog medium
NOD/NBS	nucleotide oligomerization domain/nucleotide binding site
Nb	Nicotiana benthamiana
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PRR	pattern recognition receptor
PTI	PAMP triggered immunity
R-gene	resistance gene or protein
RLK	receptor-like kinase
RLP	receptor-like protein
ROS	reactive oxygen species
rpm	rotation per minute

s	seconds
SERK	somatic embryogenesis receptor-like kinase
SERK1	somatic embryogenesis receptor-like kinase 1 (see also BAK1)
T-DNA	transfer-DNA
TIR	Toll/Interleukin
TLR	Toll-like receptor
TRIS	tris(hydroxymethyl)aminomethan
TTSS	type III secretion system
WT	wild type

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Last, I would like to express my deep gratitude to my family and especially to Melanie Baumann for always supporting me during all these years.

Appendix

i) Protein sequences and structural aligments of the chimeric receptors between AtFLS2 and LeFLS2. AtFLS2 parts are indicated in green, LeFLS2 parts are indicated in red, respectively. EFR and BRI1 parts are underlined. xxLxLxx represents the position of the β-strand. The putative β-sheet is is indicated in bold. x indicates putative solvent exposed residues within the β-sheet.

AtFLS2:GFP

MKLLSKTFLILTLTFFFFGIALA

KQSFEPEIEALKSFKNGISNDPLGVLSDWTII
GSLRHCNWTGITCDSTGHVVSVSLLEKQLEGV

xxLxLxx

LSPAIANLTYL**QVLDLTS**NSFTGK
IPAEIGKLTEL**NQLILYL**NYFSGS
IPSGIWELKNI**FYLDLRN**NLLSGD
VPEEICKTSSL**VLIGFDY**NNLTGK
IPECLGDLVHL**QMFVAAG**NHLTGS
IPVSIGTLANL**TDLDLSG**NQLTGK
IPRDFGNLLNL**QSLVLTE**NLLEGD
IPAEIGNCSSL**VQLELYD**NQLTGK
IPAELGNLVQL**QALRIYK**NKLTSS
IPSSLFRLTQL**THLGLSE**NHLVGP
ISEEIGFLESL**EVLTLHS**NNFTGE
FPQSITNLRNL**TVLTVGF**NNISGE
LPADLGLLTNL**RNLSAHD**NLLTGP
IPSSISNCTGL**KLLDLSH**NQMTGE
IPRGFGRM-NL**TFISIGR**NHFTGE
IPDDIFNCSNL**ETLSVAD**NNLTGT
LKPLIGKLQKL**RILQVSY**NSLTGP
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IPREMSNLTLL**QGLRMYS**NDLEGP
IPEEMFDMKLL**SVLDLSN**NKFSGQ
IPALFSKLESL**TYLSLQG**NKFNGS
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IPGELLASLKN**MQLYLNF**SNNLLTGT
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IPRSLQACKNV**FTLDFSQ**NNLSGH
IPDEVFQGMDM**IISLNLS**RNSFSGE
IPQSFGNMTHL**VSLDLSS**NNLTGE
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GKANSFREDRNEDREV:AGAP:GFP

LeFLS:GFP

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NHHCNWSGIICDPSSNHVINISLIETQLKGE

xxLxLxx

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Le1-6:GFP

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Le1-10:GFP

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Le1-24b:GFP

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Le11-24:GFP

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Le19-24:GFP

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IPECLGDLVHL**QMFVAAG**NHLTGS
IPVSIGTLANL**TDLDLSG**NQLTGK
IPRDFGNLLNL**QSLVLTE**NLLEGD
IPAEIGNCSSL**VQLELYD**NQLTGK
IPAELGNLVQL**QALRIYK**NKLTSS
IPSSLFRLTQL**THLGLSE**NHLVGP
ISEEIGFLESL**EVLTLHS**NNFTGE
FPQSITNLRNL**TVLTVGF**NNISGE
LPADLGLLTNL**RNLSAHD**NLLTGP
IPSSISNCTGL**KLLDLSH**NQMTGE
IPRGFGRM-NL**TFISIGR**NHFTGE
IPDDIFNCSNL**ETLSVAD**NNLTGT
LKPLIGKLQKL**RILQVSY**NSLTGP
IPREIGNLKDL**NILYLHS**NGFTGR
IPREISMLSNL**QGLLLSD**NKLEGE
LPVQLFELKQL**NELRLKN**NNFFGP
IPHHISKLESL**SLMDLSG**NKLNGT
IPESMTSLRRL**MTVDLSH**NLLTGT
LPRAVLASMRS**MQLYLNV**SSNLLHGE
IPDEIGVLEMV**QEIDMSN**NNLSGS
IPRSLQACKNV**FTLDFSQ**NNLSGH
IPDEVFQGMDM**IISLNLS**RNSFSGE
IPQSFGNMTHL**VSLDLSS**NNLTGE
IPESLANLSTL**KHLKLAS**NNLKGH

VPESGVFKNINASDLMGNTDLCGSKKPLKPCT
IKQKSSHFSKRTR

VILIILGSAAALLLVLLLVLILT

CCKKKEKKIENSSESSLPDLDSALKLKRFEPK
ELEQATDS

FNSANIIGSSSLSTVYKGQLEDGTVIAVKVLN
LKEFSAESDKWFYTEAKTLSQLKHRNLVKILG
FAWESGKTKALVLPFMENGNLEDTIHGSAAPI
GSLLEKIDLCVHIASGIDYLHSGYGFPIVHCD
LKPANILLDSDRVAHVSDFGTARILGFREDGS
TTASTSAFEGTIGYLAPEFAYMRKVTTKADVF
SFGIIMMELMTKQRPTSLNDEDSQDMTLRQLV
EKSIGNGRKGMVRVLDMELGDSIVSLKQEEAI
EDFLKLCLFCTSSRPEDRPDMNEILTHLMKLR
GKANSFREDRNEDREV:AGAP:GFP

Le1-10/19-24:GFP

MKLLSKTFLILTLTFFFFGIALA

KQSFEVEVAALKAFKSSISDDPFSALVDWTDV
NHHCNWSGIICDPSSNHVINISLIETQLKGE

ISPFLGNLSKL**QVLDLTL**NSFTGN
IPPQLGHCTDL**VELVFYQ**NSLFGE
IPAELGNLKKL**QLIDFGN**NFLNGS
IPDSICNCTEL**LLVGFNN**NNFTGK
LPSEIGNLANL**QLFVAYT**NNLVGF
MPTSIGMLTAL**HTLDLSE**NQLSGP
IPPEIGNLSSL**GILQLHL**NSLSGK
IPSELGLCINL**FTLNMYT**NQFTGS
IPPELGNLENL**QMLRLYN**NKLNSS
IPASIFHLKSL**THLGLSQ**NELTGN
IPPQLGSLTSL**EVLTLHS**NNFTGE
FPQSITNLRNL**TVLTVGF**NNISGE
LPADLGLLTNL**RNLSAHD**NLLTGP
IPSSISNCTGL**KLLDLSH**NQMTGE
IPRGFGRM-NL**TFISIGR**NHFTGE
IPDDIFNCSNL**ETLSVAD**NNLTGT
LKPLIGKLQKL**RILQVSY**NSLTGP
IPREIGNLKDL**NILYLHS**NGFTGR
IPREISMLSNL**QGLLLSD**NKLEGE
LPVQLFELKQL**NELRLKN**NNFFGP
IPHHISKLESL**SLMDLSG**NKLNGT
IPESMTSLRRL**MTVDLSH**NLLTGT
LPRAVLASMRS**MQLYLNV**SSNLLHGE
IPDEIGVLEMV**QEIDMSN**NNLSGS
IPRSLQACKNV**FTLDFSQ**NNLSGH
IPDEVFQGMDM**IISLNLS**RNSFSGE
IPQSFGNMTHL**VSLDLSS**NNLTGE
IPESLANLSTL**KHLKLAS**NNLKGH

VPESGVFKNINASDLMGNTDLCGSKKPLKPCT
IKQKSSHFSKRTR

VILIILGSAAALLLVLLLVLILT

CCKKKEKKIENSSESSLPDLDSALKLKRFEPK
ELEQATDS

FNSANIIGSSSLSTVYKGQLEDGTVIAVKVLN
LKEFSAESDKWFYTEAKTLSQLKHRNLVKILG
FAWESGKTKALVLPFMENGNLEDTIHGSAAPI
GSLLEKIDLCVHIASGIDYLHSGYGFPIVHCD
LKPANILLDSDRVAHVSDFGTARILGFREDGS
TTASTSAFEGTIGYLAPEFAYMRKVTTKADVF
SFGIIMMELMTKQRPTSLNDEDSQDMTLRQLV
EKSIGNGRKGMVRVLDMELGDSIVSLKQEEAI
EDFLKLCLFCTSSRPEDRPDMNEILTHLMKLR
GKANSFREDRNEDREV:AGAP:GFP

Le15-24^{EFRk}:GFP

MKLLSKTFLILTLTFFFFGIALA

KQSFEPEIEALKSFKNGISNDPLGVLSDWTIIGSLRHCNWTGITCDSTGHVVSVSLLEKQLEGV

LSPAIANLTYLQVLDLTSNSFTGK
IPAEIGKLTELNQLILYLNYFSGS
IPSGIWELKNIFYLDLRNNLLSGD
VPEEICKTSSLVLIGFDYNNLTGK
IPECLGDLVHLQMFVAAGNHLTGS
IPVSIGTLANLTDLDLSGNQLTGK
IPRDFGNLLNLQSLVLTENLLEGD
IPAEIGNCSSLVQLELYDNQLTGK
IPAELGNLVQLQALRIYKNKLTSS
IPSSLFRLTQLTHLGLSENHLVGP
ISEEIGFLESLEVLTLHSNNFTGE
FPQSITNLRNLTVLTVGFNNISGE
LPADLGLLTNLRNLSAHDNLLTGP
IPSSISNCTGLKLLDLSHNQMTGE
IPRGLGQLSNLTFLSLGSNKMMGE
IPDDLFNSSMLEVLDLSDNNFSGK
LKPMIGRLAKLRVLRAHSNSFLGP
IPPEIGKLSQLLDLALHKNSFSGA
IPPEISMLSNLQGLLLSDNKLEGE
LPVQLFELKQLNELRLKNNNFFGP
IPHHISKLESLSLMDLSGNKLNGT
IPESMTSLRRLMTVDLSHNLLTGT
LPRAVLASMRSMQLYLNVSSNLLHGE
IPDEIGVLEMVQEIDMSNNNLSGS
IPRSLQACKNVFTLDFSQNNLSGH
IPDEVFQGMDMIISLNLSRNSFSGE
IPQSFGNMTHLVSLDLSSNNLTGE
IPESLANLSTLKHLKLASNNLKGH

VPESGVFKNINASDLMGNTDLCGSKKPLKPCTIKQKSSHFSKRTR

VVSGICIGIASLLLIIIVASLCW

FMKRKKKNNASDGNPSDSTTLGMFHEKVSYEELHSATSR

FSSTNLIGSGNFGNVFKGLLGPENKLVAVKVLNLLKHGATKSFMAECETFKGIRHRNLVKLITVCSSLDSEG
NDFRALVYEFMPKGSLDMWLQLEDLERVNDHSRSLTPAEKLNIAIDVASALEYLHVHCHDPVAHCDIKPSNI
LLDDDLTAHVSDFGLAQLLYKYDRESFLNQFSSAGVRGTIGYAAPGTSICKKYCLTKELSNLIVCCFFEICA
EYGMGGQPSIQGDVYSFGILLLEMFSGKKPTDESFAGDYNLHSYTKSILSGCTSSGGSNAIDEGLRLVLQVG
IKCSEEYPRDRMRTDEAVRELISIRSKFFSSKTTITESPRDAPQSSPQEWMLNTDMHTMSA:**GFP**

Le15-24^{BRI1k}:GFP

MKLLSKTFLILTLTFFFFGIALA

KQSFEPEIEALKSFKNGISNDPLGVLSDWTIIGSLRHCNWTGITCDSTGHVVSVSLLEKQLEGV

LSPAIANLTYL**QVLDLTS**NSFTGK
IPAEIGKLTEL**NQLILYL**NYFSGS
IPSGIWELKNI**FYLDLRN**NLLSGD
VPEEICKTSSL**VLIGFDY**NNLTGK
IPECLGDLVHL**QMFVAAG**NHLTGS
IPVSIGTLANL**TDLDLSG**NQLTGK
IPRDFGNLLNL**QSLVLTE**NLLEGD
IPAEIGNCSSL**VQLELYD**NQLTGK
IPAELGNLVQL**QALRIYK**NKLTSS
IPSSLFRLTQL**THLGLSE**NHLVGP
ISEEIGFLESL**EVLTLHS**NNFTGE
FPQSITNLRNL**TVLTVGF**NNISGE
LPADLGLLTNL**RNLSAHD**NLLTGP
IPSSISNCTGL**KLLDLSH**NQMTGE
IPRGLGQLSNL**TFLSLGS**NKMMGE
IPDDLFNSSML**EVLDLSD**NNFSGK
LKPMIGRLAKL**RVLRAHS**NSFLGP
IPPEIGKLSQL**LDLALHK**NSFSGA
IPPEISMLSNL**QGLLLSD**NKLEGE
LPVQLFELKQL**NELRLKN**NNFFGP
IPHHISKLESL**SLMDLSG**NKLNGT
IPESMTSLRRL**MTVDLSH**NLLTGT
LPRAVLASMRS**MQLYLNV**SSNLLHGE
IPDEIGVLEMV**QEIDMSN**NNLSGS
IPRSLQACKNV**FTLDFSQ**NNLSGH
IPDEVFQGMDM**IISLNLS**RNSFSGE
IPQSFGNMTHL**VSLDLSS**NNLTGE
IPESLANLSTL**KHLKLAS**NNLKGH

VPESGVFKNINASDLMGNTDLCGSKKPLKPCTIKQKSSHFSKRTRVPAS

LAGSVAMGLLFSFVCIFGLILVG

REMRKRRRKKEAELEMYAEGHGNSGDRTANNTNWKLTGVKEALSINLAAFEKPLRKLTFADLLQATNG

FHNDSLIGSGGFGDVYKAILKDGSAVAIKKLIHVSGQGDREFMAEMETIGKIKHRNLVPLLGYCKVGDERLL
VYEFMKYGSLEDVLHDPKKAGVKLNWSTRRKIAIGSARGLAFLHHNCSPHIIHRDMKSSNVLLDENLEARVS
DFGMARLMSAMDTHLSVSTLAGTPGYVPPEYYQSFRCSTKGDVYSYGVVLLELLTGKRPTDSPDFGDNNLVG
WVKQHAKLRISDVFDPELMKEDPALEIELLQHLKVAVACLDDRAWRRPTMVQVMAMFKEIQAGSGIDSQSTI
RSIEDGGFSTIEMVDMSIKEVPEGKLSA:**GFP**

Le15-24^{KD}

MKLLSKTFLILTLTFFFFGIALA

KQSFEPEIEALKSFKNGISNDPLGVLSDWTIIGSLRHCNWTGITCDSTGHVVSVSLLEKQLEGV

LSPAIANLTYLQVLDLTSNSFTGK
IPAEIGKLTELNQLILYLNYFSGS
IPSGIWELKNIFYLDLRNNLLSGD
VPEEICKTSSLVLIGFDYNNLTGK
IPECLGDLVHLQMFVAAGNHLTGS
IPVSIGTLANLTDLDLSGNQLTGK
IPRDFGNLLNLQSLVLTENLLEGD
IPAEIGNCSSLVQLELYDNQLTGK
IPAELGNLVQLQALRIYKNKLTSS
IPSSLFRLTQLTHLGLSENHLVGP
ISEEIGFLESLEVLTLHSNNFTGE
FPQSITNLRNLTVLTVGFNNISGE
LPADLGLLTNLRNLSAHDNLLTGP
IPSSISNCTGLKLLDLSHNQMTGE
IPRGLGQLSNLTFLSLGSNKMMGE
IPDDLFNSSMLEVLDLSDNNFSGK
LKPMIGRLAKLRVLRAHSNSFLGP
IPPEIGKLSQLLDLALHKNSFSGA
IPPEISMLSNLQGLLLSDNKLEGE
LPVQLFELKQLNELRLKNNNFFGP
IPHHISKLESLSLMDLSGNKLNGT
IPESMTSLRRLMTVDLSHNLLTGT
LPRAVLASMRSMQLYLNVSSNLLHGE
IPDEIGVLEMVQEIDMSNNNLSGS
IPRSLQACKNVFTLDFSQNNLSGH
IPDEVFQGMDMIISLNLSRNSFSGE
IPQSFGNMTHLVSLDLSSNNLTGE
IPESLANLSTL**KHLKLAS**NNLKGH

VPESGVFKNINASDLMGNTDLCGSKKPLKPCTIKQKSSHFSKRTR

VILIILGSAAALLLVLLLVLILT

CCKKKEKKIENSSESSLPDLDSALKLKRFEPKELEQATDS

FNSANIIGSSSLSTVYKGQLEDGTVIAV**K/A**VLNLKEFSAESDKWFYTEAKTLSQLKHRNLVKILGFAW
mutation in critical lysine: K898A

ESGKTKALVLPFMENGNLEDTIHGSAAPIGSLLEKIDLCVHIASGIDYLHSGYGFPIVHCDLKPANILLDSD
RVAHVSDFGTARILGFREDGSTTASTSAFEGTIGYLAPEFAYMRKVTTKADVFSFGIIMMELMTKQRPTSLN
DEDSQDMTLRQLVEKSIGNGRKGMVRVLDMELGDSIVSLKQEEAIEDFLKLCLFCTSSRPEDRPDMNEILTH
LMKLRGKANSFREDRNEDREV.

Curriculum Vitae

Name Pascal Bittel
Date of birth 18/09/1978
Nationality Swiss

EDUCATION

2005–2010 **PhD-studies in Plant Sciences, University of Basel, Switzerland**
Specialization: Molecular Plant Biology / Plant Immunology

2000–2005 **Diploma-studies in Organismic Biology, University of Basel, Switzerland**
Specialization: Plant Physiology, Cell- and Molecular Biology

1990-1998 **Humanistisches Gymnasium Basel, Switzerland**
Matura Typus B

1989-1990 **Realschule Burgstrasse, Riehen, Switzerland**

RESEARCH EXPERIENCE

2005–2009 PhD project **Innate immunity mediated by the flagellin receptor FLS2 in Arabidopsis and tomato: A molecular approach to characterize ligand binding and function, using receptor chimeras** in the lab of Prof. Dr. Thomas Boller, Uni Basel.

2004–2005 Diploma project **Chimeric receptor like kinase approach as a tool to stufy PAMP signaling in *Arabidopsis thaliana*** in the lab of Prof. Dr. Thomas Boller, Uni Basel, supervised by Dr. Silke Robatzek.

ACADEMIC EXPERIENCE

2007-2008 **Supervision of the Master students Deborah Deslex and Ines Wyrsch**

2005-2008 **Part-time teaching at the University of Basel. Practical course in Plant Physiology (4th semester students), practical course in Plant Molecular Biology (8th semester students) and tutorial course in Biology (1st semester students).**

2006–2007 **Board member of the $(PS)_2A$, the body of the PhD-students of the Zurich-Basel Plant Science Center.**

2005-2006 **OC of the 2nd Annual Plant Science Center-PhD Congress**

WORK EXPERIENCE

2009-2010 **Scientific Product Manager - Enzo Life Sciences, Inc**

2005-2009 **Research Assistant – University of Basel / Botanical Institute Basel**

2002-2005 **Eskamedia AG – Project "Erfahrungsmedizinisches Register" (EMR)**

PUBLICATIONS

Sun W., Jansen K., Bittel P., Boller T. and Bent A.F. Structure Function Analysis of Arabidopis FLS2: Gylcosylation, cysteine pairs and FLS2-FLS2 interaction. (In preparation)

Nekrasov V, Li J, Batoux M, Roux M, Chu ZH, Lacombe S, Rougon A, Bittel P, Kiss-Papp M, Chinchilla D, van Esse HP, Jorda L, Schwessinger B, Nicaise V, Thomma BP, Molina A, Jones JD, Zipfel C. Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. EMBO Journal. 2009 Nov 4;28(21):3428-38.

Bittel P, Robatzek S. Microbe-associated molecular patterns (MAMPs) probing plant immunity. Current Opinion Plant Biology. 2007 Aug;10(4):335-41

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