

Characterization of Flagellin  
Perception  
*in Arabidopsis thaliana*

**Inauguraldissertation**

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# Preface

In part, published material (Bauer et al, 2001 and Gómez-Gómez et al, 2001) was used for this manuscript. This material was dissected, rearranged and complemented with unpublished results to represent the current understanding of flagellin perception and to make additional results available for research successors.

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## (A) General Summary

Plants respond to bacterial flagellin as a part of their innate immunity. In this work we undertook two lines of approaches to identify and characterize the flagellin receptor of *Arabidopsis thaliana*, as described in Chapter I and Chapter II: In Chapter I, we characterized a high affinity, flagellin binding site at the cell surface of *A. thaliana* with specificity for flagellin-derived ligands with activity as agonist or antagonist of elicitor responses, indicating that it represents the bona fide flagellin receptor. In order to identify this binding site, we attempted its biochemical purification. We achieved an enrichment of the binding site by ligand affinity chromatography, though, we could not purify it to homogeneity. In the second line of approach (Chapter II), we tested flagellin-insensitive mutants of *A. thaliana* for impairment of flagellin binding. Five flagellin-insensitive mutants, all assigned to the previously identified receptor-candidate FLS2, a receptor-like kinase, showed strong reduction in flagellin binding. Interestingly, four of the five mutants were altered in the cytoplasmic kinase domain. In order to biochemically characterize FLS2, we engineered an epitope-tagged version of this receptor-like kinase and introduced it into *A. thaliana* and tomato. Surprisingly, co-precipitation, co-purification and co-migration of the flagellin binding site and the tagged version of FLS2 did not occur. Nevertheless, tomato cells transformed with tagged *FLS2* exhibited specificity of both flagellin binding and response that carried the characteristics of *A. thaliana*.

## (B) General Introduction

### **The knowledge about flagellin perception that formed the basis for the presented work**

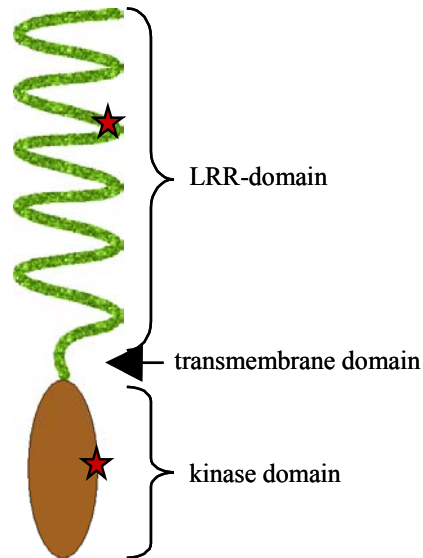
Plants have the capacity to detect and to respond to invasion by microorganisms (Somssich and Hahlbrock, 1998). In particular, Felix and colleagues discovered that bacterial flagellin, the protein subunit that composes the filament of the bacterial motility organ flagellum, is recognized in several plant species by a highly sensitive and specific perception system (Felix et al, 1999). They demonstrated that the most conserved sequence of flagellin, consisting of 22 amino acids, is sufficient to elicit full response in a number of plant species, including tomato and *Arabidopsis thaliana*. Flagellin was shown to induce defence-related responses, such as oxidative burst, ethylene production, callose deposition and medium alkalinization in cell suspension cultures, and induced genes coding for pathogenesis-related proteins (Felix et al, 1999; Gómez-Gómez et al, 1999). Changes in the sequence of synthetic flagellin peptides led to a reduction of elicitor activity or to antagonistic inhibition of the response demonstrating the specificity of this perception system (Felix et al, 1999).

Several research approaches have been initiated to elucidate the mechanism of flagellin perception and the identification of participating signalling components. Meindl and colleagues (Meindl et al, 2000) characterized a specific, high affinity flagellin binding site in tomato cells and microsomal membranes, using radioactively labelled  $^{125}\text{I}$ -Tyr-flg22 peptides. Binding was found to be nonreversible in cells and in membranes. Peptides lacking the C-terminus of flg22, which acted as competitive antagonists of elicitor action (Felix et al. 1999) also competed for binding. Thus, a two-step mechanism receptor model was proposed according to the address-message concept, in which binding of the N-terminus (address) is the first step, and activation of responses with the C terminus (message) is the second step. Chemical crosslinking specifically labelled a polypeptide of 115 kDa (Meindl et al, 2000).

In order to identify the flagellin receptor, Gómez-Gómez and colleagues identified flagellin-insensitive ecotypes and mutants of *A. thaliana*, exploiting the growth inhibition effect of flagellin peptide on seedlings (Gómez-Gómez et al, 1999; Gómez-

Gómez and Boller, 2000). Crosses of the insensitive ecotype Ws-0 with the sensitive ecotypes La-*er* and Col-0, respectively, resulted in sensitive F1 seedlings. In the F2 generation of both crosses, sensitivity segregated as a single trait with markers of chromosome 5 and a ratio of 3:1. This locus has been termed *FLS1* (FLagellin Sensing 1). Two EMS-flagellin insensitive mutations were mapped closely to the locus *FLS1* (Gómez-Gómez and Boller, 2000). Sequencing revealed point mutations in one single gene, coding for a leucine-rich-repeat receptor-like kinase, that was termed *FLS2* (FLagellin Sensing 2; Fig. 1.). Transformation of the mutants with wild type *FLS2* restored sensitivity, demonstrating that loss of sensitivity was in fact due to mutations in *FLS2*. The deduced amino acid sequence of *FLS2* has all characteristics of a receptor protein kinase. Two hydrophobic domains are present in *FLS2*, at the N terminus (amino acids 1-23) and between amino acids 815-831. These are consistent with a signal peptide that directs targeting to the membrane and a transmembrane domain (von Heijne, 1990). The putative extracellular domain (amino acids 88-745) contains 28 tandem copies of a 24-amino acid long LRR-motif. (Gómez-Gómez and Boller, 2000) In the *fls2-24* allele, a mutation was found at codon 318, changing glycine to arginine. Pairs of conservatively spaced cysteines flank the LRR domain. The presence of 23 N-glycosylation sites (N-X-S/T) indicates that *FLS2* might be a glycosylated protein. A putative leucine zipper motif (Landschulz et al, 1988) is localized in the extracellular domain at positions 460-481. The C-terminal region of *FLS2* (amino acids 870-1150) comprises a putative protein kinase catalytic domain (Hanks and Quinn, 1991), which has a consensus amino acid region characteristic for serine/threonine substrate specificity. In the *fls2-17* allele, amino acid 1064 is changed from glycine to arginine (Gómez-Gómez and Boller, 2000).

Though, *FLS2* was found to be tightly linked to *FLS1*, crossings between *fls2-24*, that carries a point mutation in the leucine-rich-repeat domain, and Ws-0, revealed a codominance effect suggesting that *fls2* mutants are not allelic to *fls1* (Gómez-Gómez and Boller, 2000). Also, transformation of Ws-0 with *FLS2* did not restore flg22-sensitivity confirming that *FLS2* is distinct from *FLS1* (Gómez-Gómez and Boller, 2000). In two further mutations, sequencing did not reveal any change in the *FLS2* sequence and therefore they were assumed to be allelic to *FLS1* (*1-2* and *1-19*: Lourdes Gómez-Gómez, personal communication).



**Fig. 1: Schematic structure of FLS2.** The red stars represent sites of identified mutations, that caused insensitivity to flagellin. *Fls2-24* exhibits a mutation in the LRR-domain, *fls2-17* in the kinase domain. The structure of FLS2 is described in the text.

## The goal of this work

The goal of this work has been to characterize flagellin binding in *A. thaliana* and to find out if the putative flagellin receptor FLS2 is the flagellin binding site.

## Literature overview of topics related to flagellin perception

### *What is the biological function of flagellin recognition?*

Induction of defense-related activities in response to bacterial flagellin was discovered by pure chance in the tomato cell line Msk8, in an attempt to characterize harpin (Felix et al, 1999). Arabidopsis similarly recognizes flagellin. However, *A. thaliana* mutant plants lacking the capability to recognize flagellin, do not show any striking phenotype. Moreover, one ecotype, Ws-0, appears to live happily without being able to recognize flagellin, albeit all seven other ecotypes tested were flagellin-sensitive (Gómez-Gómez et al, 1999). It is being discussed that flagellin plays a role in nonhost resistance. It is suggested, that plants often respond in similar ways to host and nonhost pathogens and that the difference between these two forms of resistance might reside in the solidity of

the recognition leading to resistance (Thordal-Christensen, 2003). Namely, nonhost resistance is suggested to be based on a robust surveillance with many independent recognition events, while host resistance is based on a single R-/Avr-gene recognition (Thordal-Christensen, 2003). Flagellin might represent one of several bacterial signals and lack of its recognition might be complemented by recognition of further signals. These could be EF-Tu (Gernot Kunze, Jürg Felix, personal communication) LPS (Meyer et al, 2001) and the cold-shock-protein (Felix and Boller, 2003). Hence, recognition of flagellin might not be absolutely essential, but helpful in some circumstances. Attempts to detect some difference in resistance to *P. syringae* by flagellin sensitive and flagellin insensitive *A. thaliana* plants failed (Ana Cristina Molteni, Silke Robatzek, Jürg Felix, unpublished data). However, it has been shown that *A. thaliana* plants pre- or co-treated with 1  $\mu$ M flg22 are more resistant to virulent *P. syringae* than non-treated plants. (Cyril Zipfel and Silke Robatzek, unpublished data). Also, flg22 was found to induce massive and rapid transcriptional changes in wildtype *A.thaliana* but not in the flg22-insensitive (*fls2-17*) mutant (Cyril Zipfel and Silke Robatzek, unpublished data). Many of these changes were found to belong to signaling gene families. Interestingly, many genes encoding R proteins and receptor-like kinases, which might be involved in the recognition of other elicitors or Avr proteins, are activated (Cyril Zipfel, unpublished data). Hence, flagellin might, as an early warning, enhance sensitivity to other signals deriving from potential pathogens or mutualistic symbionts. Notably, transient overexpression of truncated AtMEKK1, constitutively active AtMKK4 and AtMKK5 or WRKY29, factors involved in downstream flagellin signaling, conferred resistance of Arabidopsis leaves to infection by the bacterial pathogen *Pseudomonas syringae* or the fungal pathogen *Botrytis cinerea* (Asai et al, 2002). It has been concluded, that elicitation with flagellin leads to activation of signal elements and transcription factors that enhance resistance to bacterial and fungal pathogens (Asai et al, 2002). Substantially, *A. thaliana* plants were found to be protected against infection by *P. syringae* by previous colonization of *P. brassicacearum*, that constitute 60 % of the Brassicaceae microflora (Wafa Achouak, personal communication). This enhanced resistance might be induced by flagellin that is delivered by one of the two phases of *P. brassicacearum* into the environment in bulk masses (Wafa Achouak, personal communication).



Interestingly, recognition of flagellin is not restricted to plants, but it also induces defense responses in *Drosophila* (Lemaitre et al, 1997) and mammals (McDermott et al, 2000; Eaves-Pyles et al, 2001; Sierro et al, 2001). Recently, it has been found that the immune response of mice to purified *Salmonella* flagellin is mediated by the Toll-like receptor (TLR) 5 (Hayashi et al, 2001). Toll-like receptors recently were found to have a role in innate immunity of *Drosophila* (Lemaitre et al, 1996; Williams et al, 1997) and mammals (Medzhitov et al, 1997). Rock and colleagues highlighted the general structural features of the TLR family, namely the presence of multiple leucine-rich repeats in the ectodomain and the Toll-homology domain found in the cytoplasmic tail of all members of this protein family (Rock et al, 1998).

Janeway and Medzhitov have provided a set of definitions to formalize a description of the components of the innate immune system (Janeway and Medzhitov, 1998). They proposed calling the motifs pathogen-associated molecular patterns (PAMPs) including mannans in the yeast cell wall, formylated peptides and various bacterial cell wall components such as lipopolysaccharide (LPS), lipopeptides, peptidoglycans and teichoic acids (Janeway and Medzhitov, 1998), and now flagellin (Hayashi et al, 2001). These motifs have essential roles in the biology of the invading agents, and are therefore not subject to high mutation rates. And, as different classes of microbe (bacteria, fungi or viruses) carry different PAMPs, the immune system may be able to “classify” the invader (Janeway and Medzhitov, 1998).

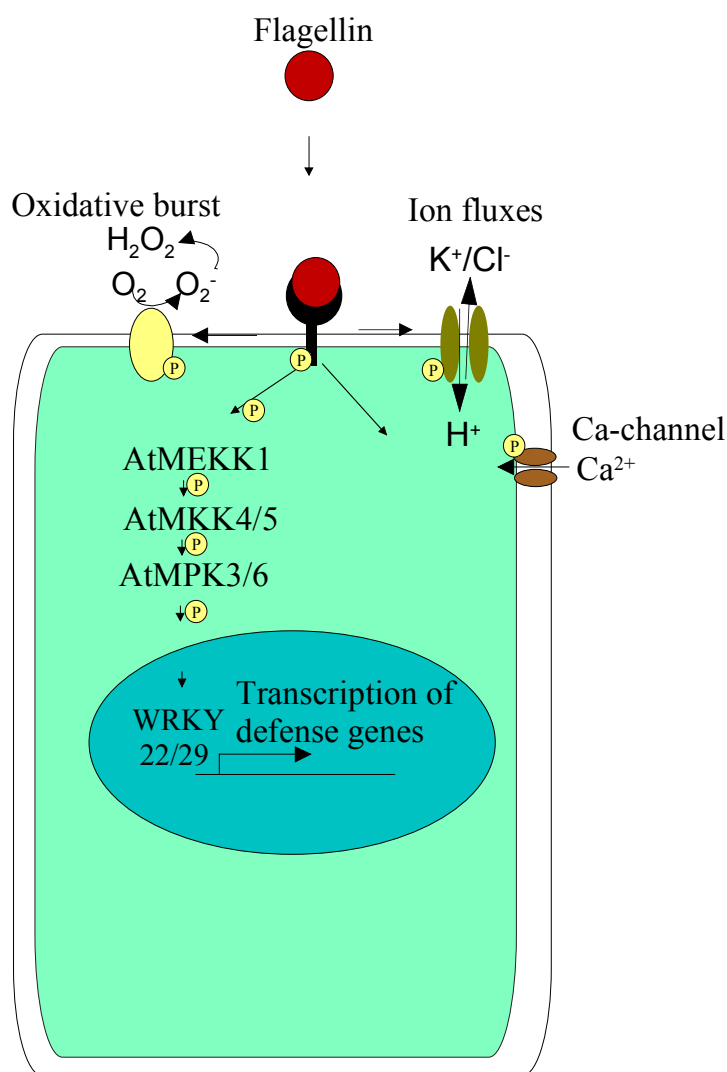
In plants, flagellin has been classified as a general elicitor (Felix, 1999). General elicitors are substances characteristic of whole groups of microorganisms, such as microbial glycopeptides, glycolipids, lipopolysaccharides, mannans and sterols (Boller, 1995; Boller and Keen, 1999; and Gómez-Gómez and Boller, 2002) and are contrasted to specific elicitors encoded by avirulence genes that induce defense reaction, including hypersensitive reaction, when the plant race or cultivar harbors the matching resistance gene (Flor, 1971). Perception of general elicitors, however, may trigger only some reactions associated with defense responses, thus providing an early warning for the presence of a foreign organism, or contribute substantially to reactions associated with the hypersensitive response (Boller and Keen, 1999).

Similarly, the role of innate immunity in animals is regarded as to distinguish different classes of pathogenic bacteria, viruses and fungi in order to provide a "quick-and-dirty" holding operation and to prod the slow-acting adaptive immunity into action (Brown, 2001). Gómez-Gómez and Boller elucidated these parallels between PAMPs and general elicitors (Gómez-Gómez and Boller, 2002) and, given the high similarity between the Toll-like-receptors and FLS2, they suggested that the plants' perception systems for characteristic non-self molecules, exemplified by the recognition system for bacterial flagellin, are highly reminiscent of animals' innate immunity response. Furthermore, they pointed out that the term "PAMP" neglects that many of the microorganisms in the environment are potentially mutualistic symbionts rather than pathogens (Gómez-Gómez and Boller, 2002). They speculated that the innate immune system in animals and plants might represent a primeval recognition system.

### *The current view of downstream flagellin signaling*

There has been much effort to identify downstream signaling elements that mediate the response to flagellin by its receptor (Overview: Fig. 2). Transient changes in the ion permeability of the plasma membrane appear to be a common early element in defense signaling (Atkinson et al, 1993; Keppler et al, 1989; Baker et al, 1993). Ion fluxes across the plasma membrane ( $H^+$  influx,  $K^+$  and  $Cl^-$  efflux) can easily be determined by pH measurements in the medium of cell suspension cultures. This feature promoted the medium alkalization assay to a standard assay in our lab. A strong and rapid induction of medium alkalization was the major characteristic that facilitated the identification of flagellin as a potent elicitor (Felix et al, 1999). At present, the function of these ion fluxes are unclear. We do not know, if they act as second messenger or have a direct function. Also, elicitor-activated increase of cytosolic  $Ca^{2+}$ -levels was observed in many examples (Zimmermann et al, 1997; Gelli et al, 1997; Blume et al 2000). In tomato cell cultures, an induction of biphasic  $Ca^{2+}$  signals has been observed after flg22 treatment but not after  $\beta$ -heptaglucan-treatment for which no elicitor activity has been described in tomato (Chantal Ebel, doctoral thesis 2000). 7 nM flg22 were found to be required for half-maximal-activity. The  $Ca^{2+}$ -chelator BAPTA (5 mM), which depletes  $Ca^{2+}$  from the extracellular medium, blocked the flg22 induced  $Ca^{2+}$ -response completely indicating that the increase in cytosolic  $Ca^{2+}$  is due to an influx of  $Ca^{2+}$  from the extracellular medium. Preincubation of tomato cells with 5 mM EGTA, another  $Ca^{2+}$ -

chelator, blocked ethylene synthesis upon flg22-elicitation completely suggesting that  $\text{Ca}^{2+}$  is an essential second messenger in flagellin signaling (Chantal Ebel, doctoral thesis, 2000).  $\text{Ca}^{2+}$  was also found to be required to activate oxidative burst in parsley (Blume et al, 2000). Oxidative burst is also a downstream response of flagellin signaling in tomato (Felix et al, 1999) and *A. thaliana* (Gómez-Gómez et al, 1999). Extracellular production of reactive oxygen species during the oxidative burst is catalyzed by a plasma membrane-located NADPH oxidase (Scheel, 2001). Also, the role of oxidative burst is not clear at present. It might be a direct defense mechanism against invaders or a signaling component, or both.



**Fig. 2: Schematic view of downstream flagellin signaling.** The model is described in the text.

Mitogen-activated protein kinases (MAPKs) have been established in a variety of plant processes as signal transduction components (reviewed by Jonak et al, 2002). Accordingly, AtMPK6, is activated by flg22, concomitantly with medium alkalization (Nühse et al, 2000). In vivo labeling demonstrated the dual phosphorylation of AtMPK6 on threonine and tyrosine residues.

Induction of medium alkalization, production of reactive oxygen species and activation of AtMPK6 all were shown to be blocked by the protein kinase inhibitor K-252a (Jürg Felix, unpublished data; Felix et al, 1999; Nühse et al, 1999) demonstrating the role of phosphorylation in the early part of flagellin signaling.

Asai et al (Asai et al, 2002) examined the role of a number of different MAPKs in flagellin signaling. A variety of MAPKs, MAPKKs, and MAPKKKs were transiently coexpressed in protoplasts with *FLS2* and the protoplasts were elicited with flg22. The results of this work place AtMPK3 and AtMPK6 downstream of the closely related MAPKKs AtMKK4 and AtMKK5, and the MAPKKK atMEKK1 downstream of *FLS2*. The targets of the MAPK pathway are suggested to be two plant specific transcription factors of the WRKY family WRKY22 and WRKY29.

Using *A. thaliana* cell-suspension cultures pulse-labelled with [<sup>33</sup>P] phosphate, Peck and colleagues (Peck et al, 2001) showed by two-dimensional gel-electrophoresis, that at least 30 proteins are differentially phosphorylated within the first 4 min after flg22 treatment. One of these 30 proteins, termed AtPhos43, was identified by mass spectrometry. Importantly, phosphorylation of AtPhos43 occurred in Col-0 and La-er Wt but not in Ws-0, *fls2-24*, *fls2-17* and *fls1-19* (Peck et al, 2001 and Scott Peck, unpublished data), demonstrating that AtPhos43 is downstream of *FLS2*. AtPhos43 was also found to be differentially phosphorylated upon chitin treatment. Eds1-2 mutant and NahG-expressing seedlings were not affected in AtPhos43 phosphorylation indicating that neither EDS1 nor SA is required for flg22-induced phosphorylation of AtPhos43. AtPhos43 is a predicted cytosolic protein with two ankyrin motifs at the C-terminus, indicating its involvement in protein-protein interactions. The role of AtPhos43 is yet unknown.

Also plasma membrane proteins become phosphorylated after flg22 elicitation. Nühse and colleagues (Nühse et al, 2003) identified a plasma membrane syntaxin that was

rapidly phosphorylated in response to flg22, in a calcium-dependent manner. Syntaxins are known to be important in membrane fusion and exocytosis.

KAPP, a kinase-associated protein phosphatase, might be a putative negative regulator of flagellin perception. Lourdes Gómez-Gómez demonstrated interaction of KAPP with the FLS2 kinase domain in a yeast two-hybrid system, and in a KAPP-overexpressing line flagellin binding and response were abolished (Gómez-Gómez et al, 2001). However, more recent results put these findings into question. Silke Robatzek recovered flagellin response (binding was not determined) in this KAPP-overexpressing line with an FLS2:myc construct driven by the native promoter (Silke Robatzek, personal communication).

In conclusion, current research has elucidated several aspects of flagellin signal transduction, though, much work has to be done yet to determine the complete signal network.

### *Further plant receptor systems in the literature*

A number of recent findings have begun to shed light on the molecular basis of signal perception in plants. Learning about other plant perception systems facilitates understanding flagellin perception.

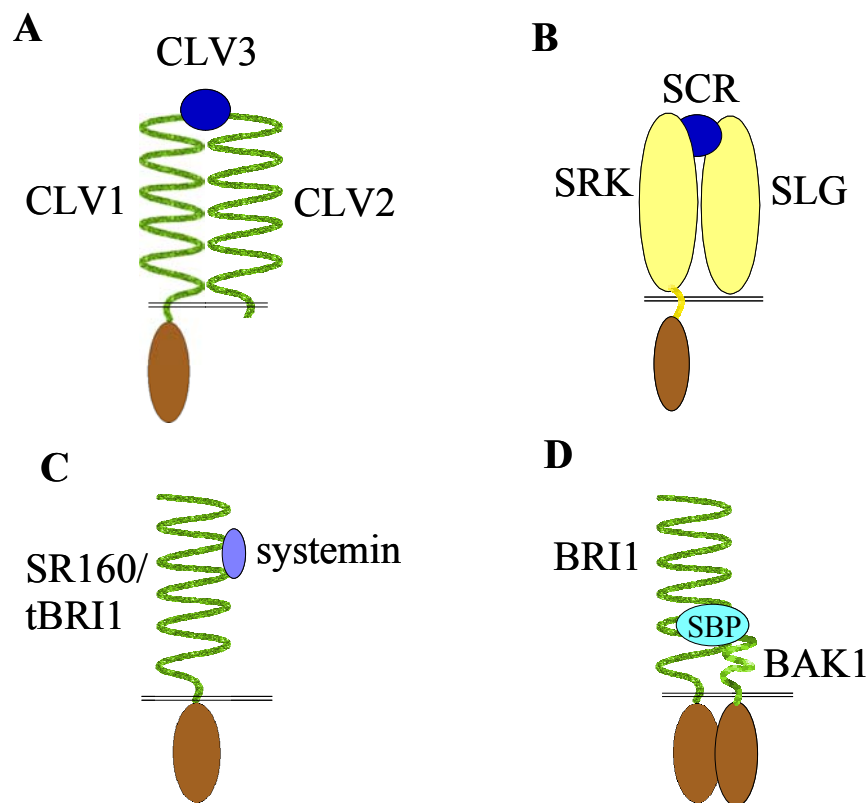
- Perception of peptide signals, involved in growth and development regulation, occurs via RLKs

The understanding of plant perception systems is most advanced in the area of peptide signal perception. Four described examples show the involvement of receptor-like kinases (RLK's), three out of them having leucine-rich-repeats in the extracellular domain, and hence, belong to the same RLK family as FLS2.

A major paradigm for plant receptors is the **CLAVATA** system that regulates the balance between cell proliferation and organ formation at the Arabidopsis shoot and flower meristems. To continuously generate new organs, the meristem maintains a population of undifferentiated cells at the center of the meristem while directing appropriately positioned progeny cells toward differentiation. The CLAVATA genes encode a receptor kinase (CLV1), a receptor-interacting protein (CLV2) and a small

secreted protein (CLV3). (Clarke 2001; Fig. 3A) Loss-of-function mutations in either gene cause progressive enlargement of the shoot apical meristem and floral meristem overgrowth (Fletscher et al, 1999). CLV1 exists in two complexes of 185 kDa and 450 kDa. The 185 kDa complex is thought to be a heterodimer of CLV1 and CLV2, interlinked covalently by disulfide bonds. It is postulated that CLV3 interacts with the CLV1-CLV2 receptor complex (of 185 kDa) to induce the formation of the active receptor complex (of 450 kDa). The ligand-receptor relationship of the CLV proteins has been confirmed by co-immunoprecipitation of CLV3 with CLV1 and co-migration of the proteins in gel filtration experiments in wildtype but not in certain CLV1 mutant lines (Trotochaud et al, 2000). However, these findings have recently been retracted (Nishihama et al, 2003). The 450 kDa complex comprises the 185 kDa complex plus a kinase-associated protein phosphatase (KAPP) and a Rho-GTPase-like protein (Rop). The kinase activity of CLV1 was shown to be required for CLV3 binding. In the 450 kDa complex, KAPP serves as a negative regulator of CLV1 function (Trotochaud et al, 1999). Interactions between the homeodomain transcription factor WUSCHEL (WUS) and CLV1 act to maintain meristem identity and size. CLV3 produced by the stem cells interact with CLV1 in a negative feedback loop to repress WUS gene expression.

Another peptide signal is **phytosulfokine** (PSK), a 5-amino acid peptide, which has sulfated tyrosin residues. Together with auxin and cytokinin, PSK induces plant cells to dedifferentiate and reenter the cell cycle at nanomolar concentrations. PSK is processed from the COOH-terminal region of ~80 amino acid precursor proteins ubiquitously expressed in the leaf, apical meristem, hypocotyl, and root of seedlings, as well as in suspension cells in culture. Evidence for the existence of high-affinity binding sites for the phytosulfokine peptide (PSK) has been provided by binding assays with radiolabeled PSKs (Matsubayashi, 1997; Matsubayashi, 1999). Recently, 120- and 150-kDa binding proteins for PSK were identified in the plasma membrane of suspension-cultured rice cells by photoaffinity labeling (Matsubayashi and Sagakami, 2000). More recently, the receptor for PSK has been biochemically purified (Matsubayashi et al, 2002). Matsubayashi and colleagues used PSK affinity chromatography followed by hydroxyapatite column chromatography. The fractions containing the binding site were identified by binding assays. These fractions still contained numerous bands when subjected to SDS-PAGE. The bands representing the binding site were identified by two



**Fig. 3: Models of well-studied receptor complexes.** Shown are features of the CLAVATA system, regulating cell proliferation at the meristem (A), and the self-incompatibility system of Brassicaceae (B), the systemin receptor (C) and the brassinosteroid receptor complex (D). The models are described in the text.

means: Firstly, in a control experiment the elution was performed with the inactive, but highly similar [2-5]PSK peptide: Only the 120- and the 150-kDa bands, that were labeled by photoaffinity, were not present in the [2-5]PSK elution. Secondly, these two bands showed the same 10 kDa shift upon digestion with PNGase F as the photoaffinity labeled bands. The 120 kDa band was tryptically digested, and peptides were identified by mass spectrometry. A cDNA representing the identified peptides was obtained from a carrot cDNA library. The role of the encoded RLK was studied by overexpression of the cDNA in sense and antisense orientation, and the results indicated that it was involved in PSK binding and signaling. The number of binding sites was substantially increased ( $B_{max}=570$  fmol/mg in the overexpressed lines and 34 fmol/mg in control lines with similar binding affinities). Interestingly, overexpression revealed the

expression of the 150 kDa protein in addition to the 120 kDa protein indicating that both proteins are encoded by a single gene, and purified antibodies against the LRR domain of the 120 kDa protein detected the 150 kDa protein as well (Matsubayashi et al, 2002).

The 18 amino acid peptide **systemin**, is known as a “hormone” mediating the response to wounding. In previous work, a high-affinity binding site was characterized in the plasma membrane of *Lycopersicon peruvianum* (Meindl et al, 1998). Systemin was found to bind with the N-terminal part to its receptor but to need the C-terminal part to activate it (Meindl et al, 1998). A 160 kDa high-affinity binding protein was photoaffinity labeled (Scheer and Ryan, 1999). Photoaffinity labeling of a bulk quantity of suspension cultured *L. peruvianum* cells was used to purify the systemin binding site under denaturing conditions. The purification revealed a LRR-RLK highly homologous to BRI1 (see next paragraph), termed SR160 (Scheer and Ryan, 2002; [Fig. 3C](#)).

- Brassinosteroid, a non-peptide plant hormone, is perceived by the same LRR-receptor-kinase as systemin

The putative **receptor BRI1 for brassinosteroid (BR)**, a plant steroid hormone involved in a wide range of developmental processes, is a typical plasma membrane associated LRR-RLK, which carries an N-terminal signal peptide and an extracellular domain of 25 imperfect leucine-rich repeats (Li and Chory, 1997). The presence of a 70-amino-acid loop-out "island" found between repeats 21 and 22 of BRI1 is characteristic of a specific family of RLKs. Such "islands" are also observed in the Cf-9 (Jones et al, 1994) CLAVATA2 (Jeong et al, 1999) and TOLL LRR receptors (Hashimoto et al., 1988), which lack a cytosolic kinase domain. At either end of the LRR region in BRI1, pairs of cysteine residues are found that, together with a putative leucine-zipper motif at the N-terminus, may facilitate the dimerization of BRI1.

To address the hypothesis that BRI1 plays a direct role in perception of BR, He et al (2000) generated a series of RLKs in which domains were swapped between BRI1 and Xa21, and these chimeric RLKs were expressed in rice cultured cells. The chimeric RLK that possessed the extracellular LRR, transmembrane, and juxtamembrane domains derived from BRI1 and the cytoplasmic kinase domain from Xa21, was able to trigger defense responses, including cell death, oxidative burst, and activation of defense-related gene expression, upon BR application. The activation of defense



responses required both the proper protein kinase activity of Xa21 and a 70-amino-acid loop-out island within the BRI1 LRR domain. These results indicated that the 70-amino-acid loop-out island is essential either for direct binding of steroid hormone or for proper folding and interaction of the LRR domains of dimerized BRI1 with some accessory factor(s) that may be required for BR binding. Using a C-terminal BRI1 translational fusion to the green fluorescent protein (GFP), BRI1 was localized to the plasma membrane (Friedrichsen and Chory, 2001). Binding studies with tritiated BL were used in plasma membrane binding assays to demonstrate specificity and high affinity of BL binding by BRI1 (Wang et al, 2001). Most importantly, BR binding activity could be co-immunoprecipitated with the BRI1 protein, indicating that BRI1 is a critical component for BR signaling. Still, since LRRs are supposed to be involved in protein-protein (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995) and not in protein-steroid interactions, the role of putative sterol binding proteins (SBPs), that are represented by several genes in the Arabidopsis genome, is discussed (Arabidopsis Genome Initiative, 2000; Li et al, 2001). These proteins might interact with BRI1 (i.e. with the 79-amino-acid loop out island). Immunoblotting of protein extracts prepared from BRI1-GFP-expressing plants with antibodies recognizing either the N-terminal region of BRI1 or GFP revealed a shift in size suggesting autophosphorylation of BRI1 in the presence of BR (Wang et al, 2001).

Recently, a second component of the BR-receptor complex, termed BAK1 (BRI1 Associated receptor Kinase 1) was identified by two different laboratories (Nam and Li, 2002 and Li et al, 2002). BAK1 encodes an LRR-RLK with a predicted signal peptide at its N terminus, followed by four leucine zippers, five LRRs, a proline-rich region, a single transmembrane domain, and a serine/threonine protein kinase domain. BRI1 and BAK1 interact in vitro and in vivo. In addition, it was shown, that BRI1 and BAK1 can phosphorylate one another, and that the autophosphorylation activity of BAK1 is stimulated by BRI1 (Li et al, 2000). Nam and Li proposed following model for the activation of a membrane BR receptor upon BR binding. BRI1 and BAK1 exist mainly as inactive monomers but can form ligand-independent heterodimers on the cell surface through interactions between their extra- and intracellular domains. The inactive monomers are in equilibrium with the active dimers. BR binding, most likely via a BR binding protein, would stabilize the BRI1/BAK1 heterodimerization, resulting in

transphosphorylation of specific serine/threonine residues of one receptor kinase by its respective partner (Nam and Li, 2002; [Fig. 3D](#)).

Amazingly, the tomato homolog of *A. thaliana* BRI1 (tBRI1) was found to be identical with SR160, the putative systemin receptor (see above). The significance of tBRI1 for brassinosteroid-signaling was confirmed by (partial) insensitivity to BL in a missense (abs1) and a nonsense (cu-3) mutant of tBRI1 (Montoya et al, 2002). Clear evidence that SR160 is a bona fida systemin receptor has recently been provided (Scheer et al, 2002): Tobacco, a plant that does not express a systemin precursor gene and does not respond to systemin, when transformed with the SR160 receptor gene, showed binding of systemin in photoaffinity-labelling experiments. Additionally, systemin induced an alkalization response in the transgenic tobacco cells similar to that found in tomato cells, but not in wildtype tobacco cells. Further, cu-3 was found to show severely reduced response to systemin (Scheer et al, 2003). These results suggest, that one single RLK might be involved in the perception of two structurally entirely different hormones that also exhibit diverse functions (defense vs. development). On the basis of these findings we may have to jettison our current notions of cellular signaling.

- Perception of several non-peptide plant hormones occurs via histidine-kinases

The **ethylene receptors and the cytokinin receptors** belong to the His kinase family with a significant sequence similarity with bacterial His kinases (reviewed by Schaller et al, 2002). Sensor His kinases typically contain a variable input domain and a conserved transmitter domain. The transmitter domain includes characteristic sequence motifs and a conserved His residue that is the site of autophosphorylation (reviewed by West and Stock, 2001)

Ethylene, one of the classic plant hormones, is perceived by a family of five membrane-localized receptors ETR1, ETR2, EIN4, ERS1, and ERS2 in Arabidopsis. ETR1 and ERS1 contain three transmembrane domains and a conserved histidine kinase domain, and have been shown to function as homodimers. ETR2, EIN4 and ERS2 have four membrane-spanning regions and a degenerate histidin kinase region. Only ETR1, ETR2, and EIN4 have receiver domains at their C termini. Ethylene binding occurs at the N-terminal transmembrane domain of the receptors, and a copper co-factor is

required for the binding. In the absence of an ethylene signal, ethylene receptors activate a Ralf-like kinase, CTR1, and CTR1 in turn negatively regulates the downstream ethylene response pathway, possibly through a MAP-kinase cascade. Binding of ethylene inactivates the receptors, resulting in deactivation of CTR1, which allows EIN2 to function as a positive regulator of the ethylene pathway (reviewed by Wang, Li, and Ecker 2002).

There are three putative receptors of cytokinin, another classical plant hormone, CRE1/AHK4, AHK2 and AHK3, that share a region of sequence similarity in the predicted extraplastic CHASE region (cyclases/histidine kinases-associated sensory extracellular) (reviewed by Hutchison and Kieber, 2002). AHK2 and AHK3 contain three predicted transmembrane domains, CRE1/AHK4 contains two. Binding of cytokinin by CRE1 (Cytokinin response 1) was confirmed by *in vitro* binding assays using membrane preparations of *Saccharomyces pombe* expressing CRE1 (Yamada et al, 2001). CRE1 binds radiolabeled cytokinin isopentyl adenine with high affinity and specificity. A single amino acid substitution in the extracellular CHASE domain destroys cytokinin binding *in vitro*, suggesting that this domain is the cytokinin binding site.

- Perception of Avr-gene products

Despite of valiant efforts by many laboratories, the recognition of pathogen-derived avirulence (Avr) effectors by plant resistance (R) proteins, which results in disease resistance, is still a mystery. The basic assumption is that R proteins behave like receptors for the effector ligands (Gene for gene hypothesis, Flor, 1942). Structural features of the R proteins support this model, as a majority of the R proteins, similarly to FLS2, have a well-conserved leucine rich repeat domain, which is believed to mediate protein-protein interactions (Kobe and Deisenhofer, 1995; Dangl and Jones, 2001). However, a direct physical interaction between LRR-containing R-proteins and corresponding Avr effectors has only been shown for the rice Pita and corresponding AvrPita of the rice blast fungus (Jia et al, 2000). Attempts with dozens of other similar R-Avr pairs have failed to identify such an interaction. One such example is the Avr9-Cf-9 interaction.

The tomato **Cf-9** protein is a disease resistance protein involved in recognizing the fungal pathogen *Cladosporium fulvum* expressing the avirulence gene *avr9* (Jones et al, 1994). All known *Cf* genes are predicted to encode extracytoplasmic, membrane-anchored leucine-rich repeats without cytoplasmic functional domain. It has been proposed that Cf-9 is the receptor for *avr9* (Jones et al, 1994), according to Flor's gene-for-gene hypothesis (Flor, 1942). Valiant efforts have been undertaken to demonstrate interaction between Cf-9 and *avr9*. However, studies performed in parallel in four different laboratories did not show a direct interaction (Luderer et al, 2001). Additionally, a high-affinity binding site for Avr9 was found in tomato lines regardless of whether they expressed the Cf-9 gene (Kooman-Gersman et al, 1996). These observations, together with the fact that the Cf-9 protein lacks any obvious domain suitable for signal transduction, suggest that additional proteins may be involved in Avr9 perception. Cf-9 has recently been shown to be present in a heteromultimeric complex of approximately 420 kDa (Rivas et al, 2002). Unlike CLV2, however, Cf-9 did not form disulfide-linked heterodimers. No ligand (i.e. Avr9)-dependent shift in the molecular mass of the Cf-9 complex was detected, and no Rops were found to be associated with Cf-9. Recent findings indicate that further proteins are involved in the perception by the Cf gene products. For example, Rcr3, a tomato gene required for Cf-2 function, encodes a protein with strong homology to secreted cysteine proteases of the papain family. Interaction between Cf-2 and Rcr3 is being tested (Krüger et al, 2002).

**Syringolides**, acyl glycosides produced in several Gram-negative bacteria, are recognized in soybean cultivars expressing the Rpg4 resistance gene (Ji et al, 1997). However, soluble high affinity binding sites for syringolide are present also in cultivars that does not express Rpg4. Hence, the binding site was not expected to be encoded by the Rpg4 gene (Ji et al, 1997). Ligand affinity purification of the syringolide binding site identified a 34-kDa protein that showed homology to thiol proteases (Ji et al, 1998).

In light of such observations, the original receptor-ligand model of Avr-R interaction was amended. The R protein has been assigned the role of guarding key virulence targets (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). This guard hypothesis proposes that Avr products interact with and modify non-R cellular factors. The R protein perceives the altered status of the virulence target and induces a rapid defense response. Such a key bacterial virulence target is the Arabidopsis **RIN4** protein, that

plays a role in basal resistance (Mackey et al, 2002). Two recent studies suggest, that the bacterial avirulence effectors **AvrB**, **AvrRpm1** and **AvrRpt2** alter this key protein that is guarded by the R proteins **RPM1** and **RPS2** (Mackey et al, 2003; Axtell and Staskawicz, 2003).

- Little is known about the molecular structure of general elicitor receptors

Perception of general elicitors is especially interesting in the context of this thesis, since flagellin was classified as a general elicitor (Felix et al, 1999). High affinity binding sites have been described for several general elicitors, and crosslinking experiments indicated the molecular weight of these putative receptors. A high affinity binding site for Pep13 has been described in parsley microsomes (Nürnberg et al, 1994), and crosslinking labeled a 91 kDa polypeptide (Nürnberg et al, 1995). Basse and colleagues characterized a high affinity binding site for the glycopeptide elicitor in membranes and cells of tomato (Basse et al, 1993). Purification of the glycopeptide binding site was attempted, but its identification could not be achieved (Fath and Boller, 1996). Also, for xylanase and elicitors, such as cryptogein, the presence of high-affinity binding sites has been described (Hanania and Avni, 1997; Wendehenne et al, 1995; Bourque et al, 1999, ) and crosslinking labeled a 66 kDa polypeptide for the xylanase binding site (Hanania and Avni, 1997). Also for chitin fragments, high-affinity binding sites were described in membrane fractions of rice and tomato cells (Shibuya et al, 1993; Baureithel et al, 1994) and crosslinking labeled a 75 kDa protein with rice membranes (Ito et al, 1997), and two bands at ~53 kDa and 83 kDa in tomato membranes (Baureithel, 1996). Purification experiments have been performed for the chitin fragment binding site, but sequence data of the purified proteins revealed no homology to any known receptors of other organisms (Baureithel, 1996). The hepta- $\beta$ -glucan binding site (Cosio et al, 1988 and 1996; Cheong et al, 1991) represents a positive exception in this group: Mithöfer and colleagues extracted a protein of the size from soybean root membranes (~75 kDa) by ligand affinity purification (Mithöfer et al, 1996), that was found in photoaffinity labeling experiments (Cosio et al, 1992). Another group performed similar purification experiments as Mithöfer and colleagues, and isolated a gene coding for the putative receptor protein (Umemoto et al, 1997). The structure of the corresponding protein was surprising, because it showed no homology

to any known receptors, and it had no membrane-spanning domains, although it was found to be membrane-associated. In summary, little is known about the molecular structure of general elicitor receptors. The single identified binding site up to date shows no homology to receptors, and the others, based on the molecular weight of the affinity labeled proteins, might be too small to represent receptor kinases.

- Which is the best paradigm for the flagellin perception?

It is difficult to judge which of the described receptor paradigms (Fig. 3) might be most appropriate for the flagellin receptor. The most extensively described receptors of peptide signals, that show high homology to FLS2, would not perfectly fit, since these signals are plant specific regulators of growth and development, and flagellin represents a non-self molecule. On the other hand, perception of peptides might resemble the perception of flagellin, since it is a polypeptide.

Perception of avirulence gene products could be an ideal paradigm, since these signals derive from pathogens and most of them are proteins. But, they also exhibit a role as virulence factors. As such, many of them interfere with the functioning of plant cells, for instance by enzyme activity or binding to target proteins. Hence, the primary binding sites might be these target proteins, and the actual receptors might perceive changes of the target proteins, according to the guard hypothesis (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). In contrast to avirulence proteins, general elicitors or PAMP's are thought to solely act as signals, without effector activity within the plant cells. Nevertheless, perception of other general elicitors is poorly understood at present, and only few of them are proteins or peptides. In summary, comparison with other perception systems can give much information for flagellin perception, but none of the described examples provides a perfect paradigm.

## (C) Material and Methods

### General Material and Methods

#### *Flagellin-derived peptides and radioiodination*

The flagellin-derived peptides were synthesized according to the consensus sequence for the most highly conserved region in the N-terminus of eubacterial flagellin (Felix et al, 1999). Flg22, Tyr-flg22, flg15, flg13, flg22-Δ2, flg15<sup>E.coli</sup>, flg15<sup>R.mel</sup>, and flg22<sup>A.tum</sup> were synthesized and purified on reversed phase HPLC by F. Fischer (Friedrich Miescher-Institute). Peptides were dissolved in H<sub>2</sub>O (stock solutions of 1 to 10 mM) and diluted in a solution containing 0.1% BSA and 0.1 M NaCl. Tyr-flg22 was iodinated using chloramine T to I-Tyr-flg22 (Meindl et al, 2000) or labeled with <sup>125</sup>I-iodine to yield 3-<sup>125</sup>I-iodotyrosine-flg22 (<sup>125</sup>I-Tyr-flg22) with a specific radioactivity of >2000 Ci/mmol by Anawa Trading SA (Wangen, Switzerland).

#### *Cell suspension cultures of A. thaliana and tomato*

Cell cultures of *A. thaliana*, originally derived from plant tissue of ecotype *Landsberg erecta*, were grown as described before (May and Leaver, 1993). Cell cultures of tomato (line Msk8) were subcultured as described before (Felix et al, 1991). The cells were subcultured in weekly intervals and used for assays 6 to 8 days after subculture, containing approximately 80 mg cells / ml (fresh weight).

#### *Plants of A. thaliana*

*A. thaliana* seeds of ecotypes La-er, Zürich and Ws-0 were obtained from J. Paszkowski (Friedrich Miescher Institute). Seeds of ecotypes Col-0, Mühlen, Estland, Cri, AUA-Rhon, No-0, Col-PRL and Kandavill were obtained from Lehle Seeds (Round Rock, TX). The seeds were grown in soil in growth chambers programmed for cycles of 12 h light of 60 μE m<sup>-2</sup>s<sup>-1</sup> (Biolux lamps; Osram, Munich, Germany) at 20°C and 12 h of dark at 16°C with 70 % relative humidity.

### *Binding buffer*

25 mM MES/ KOH pH 6,0, 3 mM MgCl<sub>2</sub>, 10 mM NaCl.

### *Cell homogenization*

Filtered cells were mixed with binding buffer and 4 mM DTT (1,4-Dithio-DL-threitol) in a ratio of 1g cells (fresh weight) to 2 ml buffer. The cells were broken in a Parr cell disruption bomb (Parr Instrument Co., Moline, IL) by incubating the cells at 1000 psi nitrogen for 30 min at 4°C by stirring. The cells were broken by sudden release to normal atmospheric pressure.

### *Preparation of microsomal membranes*

Microsomal membranes were prepared from cell suspension cultures as described before (Grosskopf et. al, 1990). The cell homogenates were sequentially centrifuged at 10,000 x g for 20 min to yield pellet 1 (P1) and at 100,000 x g for 45 min to yield pellet 2 (P2) containing microsomal membranes.

### *Binding assays with intact cells and cell fractions*

Aliquots of cells and cell fractions were incubated in binding buffer in a total volume of 100 µl with <sup>125</sup>I-Tyr-flg22 (60 fmol in standard assays; >2000 Ci / mmol) for 25 min either alone (total binding) or with 10 µM of competing flg22 (nonspecific binding). Cells or cell fractions were collected by vacuum filtration on glass fibre filters (Macherey-Nagel MN GF-2, 2.5-cm diameter, preincubated with 1% BSA, 1% bactotrypton and 1% bactopecton in binding buffer) and washed for approx. 10 s with 15 ml of ice-cold binding buffer. Radioactivity retained on the filters was determined by γ-counting. Specific binding was calculated by subtracting nonspecific binding from total binding.

### *Storage of cell preparations*

Cell preparations were stored at -80°C.



### *Protein determination*

Protein content of cell fractions was determined with Micro BCA Protein Assay Reagent Kit (Pierce)

### *SDS-Polyacrylamide Gel-electrophoresis*

SDS-Polyacrylamide gels were performed with Mini-Protean II Dual and Protean II Xi slab cells. The samples were mixed with 5x Sample buffer (0.6 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50 % (v/v) Glycerol, Trace bromophenol blue) and 2-mercaptoethanol to a final concentration of 5% (v/v). Samples were boiled at 95 °C for 5 min and loaded on an SDS-polyacrylamide gel consisting of a lower separating and an upper stacking gel with the following composition: Separating gel (6 ml for mini and 15 ml for maxi gels): 25 % (v/v) separating gel buffer (1.5 M Tris/HCl pH 6.8, 0.4 % SDS), 8-12 % (v/v) bisacrylamide, 0.075 % (w/v) ammonium persulfate and 0.05 % (v/v) TEMED (N,N,N',N'-Tetramethylethylenediamine). Ammonium persulfate and TEMED cause polymerization of the gel and were added at last. Stacking gel (3 ml for mini and 7 ml for maxi gels): 25 % stacking gel buffer (1.5 M Tris/HCl pH 8.8, 0.4 % SDS), 4.5 % (w/v) bisacrylamide, 0.06 % ammonium persulfate and 0.05 % TEMED.

### *Coomassie blue staining of SDS-Polyacrylamide gels*

Coomassie blue: Gel was incubated by gentle shaking in stain solution (0.25 % Serva Blue R in Destain solution) for 20 min to over night. For destaining gel was incubated in Destain solution (40 % ethanol, 10 % acetic acid) until the color of the gel equaled the color of the solution. Then the Destain solution was exchanged for fresh one.

### *Solubilization of membrane proteins*

Triton X-100 in binding buffer was given to microsomal fractions or P1 to a final concentration of 1%. The mixture was rotated at 4°C for one hour and ultracentrifuged at 100,000 x g for one hour. The supernatant was regarded as the solubilized fraction.

For binding studies with the solubilized fraction filters were preincubated in 3 % (w/v) polyethyleneimin.

### *Methanol-Chloroform precipitation of proteins*

Precipitation was used to concentrate proteins and remove detergents, salts and sugars for loading on SDS-PAGE. To an aliquot of protein solution, three volumes of methanol were added. After vortexing, one volume of chloroform was added. After vortexing, four volumes of water were added, the mixture was vortexed intensely and centrifuged for one min. Since the proteins accumulate in the interface between the organic phase (bottom) and the aqueous (upper) phase, the aqueous phase was removed without disturbing the interface. Then, four volumes of methanol were added. After vortexing, the solution was placed at -20°C for 10 min, and the precipitated proteins were collected by centrifugation.

### *Acetone precipitation of proteins*

Protein solutions were mixed with four volumes of acetone and incubated for 1 h at -20°C. The precipitated proteins were pelleted by centrifugation.

### *Flagellin affinity chromatography*

Purified flagellin (1-3 mg) was covalently bound to 0.4-1 ml Affi 15 gel (BioRad) according to the manufacturers description, and poured into a 1 ml column. The column was equilibrated with binding buffer containing 1% Triton X-100, then loaded with solubilized fraction, then washed with 1% Triton X-100 in binding buffer and eluted with 0.1 M flg22 in 1% Triton X-100 in binding buffer. Binding in the flow through was measured throughout the chromatography.

## **Special Material and Methods for Chapter I**

### *Measurement of alkalinization response*

Aliquots of the *A. thaliana* cell suspension were incubated in open flasks on a rotary shaker at 150 cycles per min (Gómez-Gómez et al, 1999). Extracellular pH was measured with a small combined glass pH-electrode (Metrohm, Herisau, Switzerland) and either recorded continuously using a pen recorder or measured 20 min after elicitation.

### *Protoplast preparation*

4-5 day old cell suspensions were centrifuged for 5 min at 1000 rpm. The medium was removed and 50 ml enzyme mix (1% cellulase (Onozuka R-10), 0.25% Macerozyme (R-10), 0.5 M Mannitol, 8 mM  $\text{CaCl}_2$  were stirred for one hour and filter sterilized) was added. The mixture was distributed in three petri dishes (10 cm diameter, Corning) and incubated over night in darkness. The protoplasts were filtered through a 100  $\mu\text{m}$  metal mesh and then a 63  $\mu\text{m}$  metal mesh to remove undigested cell-clumps. The flow through was pelleted at 800 rpm for 5 min. The pellet was washed twice in Mannitol- $\text{MgCl}_2$ -solution (0.4 M Mannitol, 15 mM  $\text{MgCl}_2$  and 0.1 % MES, filter sterilized) by centrifugation at 800 rpm for 5 min. The pellet was mixed with 2.5 ml Mannitol- $\text{MgCl}_2$ -solution and 5 ml Saccharose mix (0.5 M saccharose, 0.01 % MES; filter sterilized) and 1 ml Mannitol- $\text{MgCl}_2$  and then centrifuged at 800 rpm for 10 min. The pellet contained cell remnants. The protoplasts floating on the surface were collected and used for experiments.

In some experiments a mixture of protease inhibitors was added containing following chemicals with their final concentration: bestatin (40  $\mu\text{g/ml}$ , Sigma), EDTA (0.5 mg/ml), Leupeptin (0.5  $\mu\text{g/ml}$ , Sigma), Pefabloc (1 mg/ml; Boehringer Mannheim), Pepstatin A (0,7  $\mu\text{l/ml}$ , Sigma), PMSF (170  $\mu\text{g/ml}$ , Sigma), TLCK (5 mg/ml, Sigma) and EGTA (0,5 mg/ml).

### *Chemical crosslinking*

Aliquots of 100 µg microsomal protein in a total volume of 100 µl binding buffer containing 60 fmol  $^{125}\text{I}$ -Tyr-flg22 were incubated for 30 min at 4°C in the presence or absence of unlabeled peptides used as competitors. Crosslinking was initiated by addition of 5 µl 50 mM dithio-bis-(succinimidylpropionate) (Pierce) in DMSO (dimethylsulfoxide) directly to the incubation mixture. After further incubation for 30 min at room temperature the reaction was stopped by addition of 2.5 µl 1 M Tris/HCl pH 7.5. Samples were solubilized in SDS-sample buffer under non-reducing conditions (5 min, 95 °C). Proteins were separated by SDS-PAGE on gels containing 8% (w/v) acrylamide. Gels were dried, analyzed and quantified using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA).

### *Cell wall purification*

Cell homogenates were centrifuged at 2000 g for 5 min. The pellet ( $P_0$ ) was resuspended in binding buffer, further homogenized with the Polytron mixer (Kinematica AG, Littau-Luzern, Switzerland) and filtrated through Miracloth (Calbiochem). This process was repeated three times. The white fibrous material remaining in Miracloth was regarded as the cell wall.

### *Determination of cell number in cell suspensions*

To separate cells from each other, they were treated with 1% pectinase and 1% cellulase in 1 M Mannitol for 2-4 h. The separated cells were diluted 5 times and counted in a Thoma-chamber.

### *Preparation of flagellin protein*

Preparation of flagellin protein was modified after Felix et al (1999) as follows:

*Pseudomonas syringae* pv. *tabaci* was streaked on King B Agar (2% Proteose Peptone, 1% glycerin, 0.15 %  $\text{K}_2\text{HPO}_4$ , 0.15 %  $\text{MgSO}_4$ , pH 7.2, 1.5 % Agar) and incubated for 1-2 days at 27°C until single colonies were grown. One colony was

picked and inoculated into 50 ml liquid King B medium and incubated for one day at 27°C under vigorous shaking. 0.5 ml of the inoculum was added to 400 ml King B liquid medium in six 2.5 l flasks, shaking over night. The cells were tested for motility under a light-microscope. Non-motile cells quivered due to Brownian molecular movement. Motile cells swam quickly through the microscopic field, in different directions.

**Purification of flagellin:** Cells were harvested by centrifugation at 10,000 x g for 20 min, and resuspended in 100 ml 20 mM Tris pH 7.0. Flagella were sheared off from cells in a waring blender twice for 30 min. Cells were pelleted by centrifugation for 10,000 x g for 30 min, and the supernatant was further used. Remaining cells were pelleted by a second centrifugation round. The supernatant was ultracentrifuged at 100,000 x g for 30 min to pellet flagella. The pellet was resuspended in 20 ml ddH<sub>2</sub>O and ultracentrifuged again. The new pellet was resuspended in 1 ml ddH<sub>2</sub>O, the pH was adjusted to 2.5 with HCl and boiled for 10 min to dissociate flagella to flagellin molecules. The solution was ultracentrifuged at 100,000 g for 1 hour to pellet contaminants. The pH of the supernatant containing flagellin was adjusted back to 7 with NaOH. We avoided adding buffers in order to test the preparation for elicitor activity in the alkalization assay.

The flagellin preparation was tested for activity by medium alkalization assays, and for quantity and purity by SDS-PAGE. Flagellin of *Pseudomonas syringae* pv. *tabaci* migrates at 30 kDa.

### *Con A affinity chromatography*

Con A agarose beads (Sigma) were packed into a 0.5 ml column (Micro Bio-Spin Chromatography Column, BioRad) and equilibrated with binding buffer containing 1% Triton X-100 and 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub>. The column was loaded with solubilized fraction, then washed with 1% Triton X-100 in binding buffer and eluted with 1 M methyl- $\alpha$ -D-mannopyranoside in 1% Triton X-100 in binding buffer. Binding in the flow through was measured throughout the chromatography.

### *Binding assays with ConA-beads-bound binding sites.*

Con A-agarose beads were added to solubilized binding sites containing 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  and 1 mM  $\text{MnCl}_2$  and incubated by rotation for 30 min at 4°C. Then the beads were sedimented by centrifugation, washed with binding buffer and used for binding assays.

### *Colloidal Coomassie blue staining of SDS-Polyacrylamide gels*

The SDS-polyacrylamide gel was incubated for 3h to overnight in 500 ml Fix-solution (50 % Ethanol, 2 % Phosphoric acid (85 % solution)) by shaking, and washed three times for 10 min in ddH<sub>2</sub>O. Then, the gel was preincubated for 1 hour in 500 ml stain solution, that consisted of 17 %  $(\text{NH}_4)_2\text{SO}_4$ , 3 % phosphoric acid (85 % solution) and 34 % methanol. Methanol was added at last, after the other chemicals were solved. Subsequently, 330 mg Coomassie Blue G-250 (Serva Blue G # 35050) was solved in an aliquot of stain solution and added to the stain solution with the gel. The gel was incubated in the coomassie blue stain by gentle shaking for 1-3 days. For destaining, the gel was washed for several times in ddH<sub>2</sub>O.

### *Crosslinking of solubilized binding sites*

Ten ml solubilized fraction with approx. 10.000 cpm / 100  $\mu\text{l}$  specific binding activity was incubated for 30 min at 4°C with 0.5 nM  $^{125}\text{I}$ -Tyr-flg22 in the presence or absence of 10  $\mu\text{M}$  flg22. 12 mg DSP in DMSO was added and the mixture was incubated for further 30 min at room temperature. The crosslinking reaction was stopped by addition of 1 M Tris pH 7.5 to a final concentration of 20 mM. The binding sites were precipitated with Con A-sepharose beads by incubating the solution with Con A beads for 1 h at room temperature. Then, the Con A beads were washed four times with 1 ml 1% Triton X-100 in binding buffer. For loading on a 7 % SDS-gel, the beads were boiled in sample buffer, centrifuged, and about 1/10 of the supernatant was applied.

## Special Material and Methods for Chapter II

### *Preparation of plant homogenates*

Individual *A. thaliana* plants, weighing 0.1-0.5 g fresh weight, were homogenized in 1-5 ml ice-cold binding buffer (10 ml of buffer/g tissue) with a Polytron mixer (Kinematica AG, Littau-Luzern, Switzerland). Big fragments of tissues were removed by passing the homogenate through one layer of Miracloth (Calbiochem). (Section II.3.1.) Or, later on (Section II.3.2.) 0.1 g leaf tissue was collected in an 1.5 ml reaction tube and seven glass beads with 1.7-2.0 mm diameter were added. The samples were frozen in liquid nitrogen and shaken with Silamat SS Vivamed for 15 s at the maximum speed. Afterwards, the samples were frozen in liquid nitrogen and shaken again. Alternatively, Quiagen Mixer Mill was used with two 3 mm Tungsten Carbide Beads (Quiagen) with non-frozen samples. These samples were used for immunoblots, binding assays and RNA-extraction.

### *Binding Assays with plant homogenates*

Binding assays with plant homogenates in section were performed according to binding assays with cells, cell homogenates and microsomes (see General Material and Methods). For equilibrium binding assays, aliquots of plant homogenates containing 500 µg of protein were incubated with radioligand and competing unlabeled flg22 as described for chapter I. After incubation of 20 min on ice, free label was separated from label bound to P1 by centrifugation (10,000 x g for 5 min).

### *Ethylene biosynthesis in *A. thaliana* leaf pieces*

Leaves of *A. thaliana* plants were cut in 1- to 3-mm slices (~30 mg fresh weight per assay) and floated overnight on H<sub>2</sub>O. The leaf slices were transferred to 6-ml glass tubes containing 1 ml of H<sub>2</sub>O. After addition of elicitor preparations to be tested, vials were closed with rubber septa and placed horizontally on an orbital shaker (100 rpm). Ethylene accumulating in the free-air space was measured by gas chromatography after 2 h of incubation.

### *Oxidative burst*

As described for ethylene synthesis, leaves of *A.thaliana* plants were cut in 1 to 3 mm slices (~30 mg fresh weight per assay) and floated overnight on H<sub>2</sub>O. The leaf slices were transferred to assay tubes containing 0.1 ml of H<sub>2</sub>O supplied with 20 µM luminol and 1 µg of horseradish peroxidase (Fluka). Luminescence was measured in a LKB 1250 luminometer (LKB Wallac, Turku, Finland) for 20 min after the addition of the test solution.

### *Transformation of fls2 mutants with wild type FLS2*

Transformation with the vector pBBFLS2 was performed by Lourdes Gómez-Gómez as described previously (Gómez-Gómez and Boller, 2000).

### *Constructs*

Triple-myc was provided by Suzanna Rivas (lab of Jonathan Jones, Sainsbury) in the pBSK vector to be cut out with the restriction sites EcoRI-HindIII. The *FLS2* gene construct was received from Lourdes Gómez-Gómez in the plasmid pBB FLS2 that represents pCAMBIA 3300 containing the 12 kb Bam HI-fragment of MPL12.8.

The plasmid pCAMBIA 2300 P+T was kindly provided by Scott Peck. It contained the cauliflower mosaic virus 35S Promoter and terminator with the following polycloning site: KpnI, SmaI, BamHI, XbaI, SalI, PstI.

### *Engineering of FLS2:myc*

*FLS2* was amplified via PCR with pBBFLS2 as a template, using the Expand High Fidelity PCR-System (Boehringer) with following primers:

Forward primer: 5' GTTAGGTACCGGATCCGTCTAAACCATGAAGTTACTCTC<sup>3'</sup>.

This primer contains a BamHI and a KpnI restriction site. Reverse primer:

5' TTGTTACCCATATGCACCTCTCGATCCTCGTTACGATCTTCTCG<sup>3'</sup>. This

primer contains an NdeI restriction site and no stop-codon. The PCR-program was as



follows: 1: 94 °C 2 min, 2: 94 °C 15 s, 3: 55 °C 30 s, 4: 72 °C 3 min, 5: 30 times to 2, 6: 72 °C 5 min.

The PCR product was run in a 0.5 % agarose gel. The band between 3000 und 4000 kb was cut out, purified and ligated in pGEM from (Promega) that contains overhanging A-ends. The ligation product was transformed into JM109 competent cells. Minipreps (Qiagen) of white colonies were digested with NdeI-KpnI. The NdeI-KpnI-fragment was cut out of the gel and purified (Quiagen Gel Extraction Kit).

Triple-myc was amplified via PCR with pBSK-myc plasmid, provided by S. Rivas, with Pwo DNA polymerase (Boehringer) with the following primers:

Forward primer: 5' ATGCATATGGGTGAACAAAAGTTG<sup>3'</sup>. This primer contains the NdeI restriction site. Reverse primer:

5' ATGGTCGACCTATCCGTTCAAGTCTTCTTCT<sup>3'</sup>. This primer contains the stop codon TAG and the Sall restriction site. The PCR-Program was as follows: 1: 94 °C 2 min, 2: 94 °C 15 s, 3: 53 °C 30 s, 4: 72 °C 44 s, 5: 30 times to 2, 6: 72 °C 5 min.

The PCR-product was ligated into EcoRV-cut (blunt ended) pBSK. The ligation product was transformed into DH5 $\alpha$  competent cells. Minipreps were digested with Sall. Due to blunt end ligation, triple myc was inserted in two different orientations. Since pBSK had an intrinsic Sall restriction site in the poly-cloning-site, cutting with Sall was used to identify clones with the correct orientation: Triple-myc inserts in the correct orientation were cut out, inserts in the wrong orientation were not. Plasmids with the correctly oriented insert were digested with KpnI and NdeI, using restriction sites of pBSK, run in an agarose gel and purified.

The two KpnI-NdeI fragments *FLS2* and triple-myc-pBSK were ligated and the product was transformed into XL10-Gold ultracompetent cells (Stratagene) to produce *FLS2*:myc-pBSK. The correctness of the fragment was verified by sequencing.

*FLS2*:myc-pBSK and pCAMBIA 2300 P&T were digested with BamHI and Sall. After gel purification of *FLS2*:myc and the cut pCAMBIA 2300 P&T, the two fragments were ligated and cloned into XL10-Gold ultracompetent cells.

### *Transformation of cell suspensions with 35S:FLS2:myc pCAMBIA 2300 by particle bombardment*

4-5 day old cell suspensions were vacuum filtered on sterile filter paper with 5.5 cm diameter. The filters with cells were laid on mannose agar plates (cell suspension medium, 0.7 % agarose, 0.5 M mannose) and incubated for several hours. The plasmids were used to coat gold particles as follows: Nine µl plasmid FLS2:myc pCAMBIA 2300 P&T containing 10µg plasmid was mixed with 5 µl gold particles (=microcarriers, 100 µg/l), 50 µl 2.5 M CaCl<sub>2</sub>, 20 µl spermidine free base and vortexed for 3 min. 100 µl cold 95 % ethanol was added and the mix vortexed. 200 µl 95 % ethanol was added again and the mix was shaken without vortexing. The particles were precipitated at -20°C for 30 min and centrifuged for 10 min at 15,000 rpm and 4°C. The supernatant was decanted. The pellet was mixed with 60 µl 95 % ethanol and sonicated to break clumps of gold particles. The particle bombardment was performed with the Biolistic Particle Delivery System (Model PDS-1000/HE, Biorad). 650 Psi rupture disks were used. The rupture disks, meshes and macrocarriers were sterilized in 70 % ethanol. For each bombardment 10 µl plasmid-gold-particle-ethanol mixture was distributed on the surface of a macrocarrier and air-dried. The bombardment was performed according to the manufacturers description. The bombarded cells were treated as follows: for the first day the plates were wrapped in aluminium foil. The second day the foil was removed and the filters carrying the cells were transferred on agar plates without mannose. The third day the filters were transferred on agar plates containing kanamycin (cell suspension medium, 0.7 % agar and 50 µg / ml kanamycin). On the kanamycin plates, nonresistant cells died showing white color (*A. thaliana*) or brownish color (tomato), and resistant cells grew producing calli with the original color. The calli were transferred on fresh kanamycin plates. To establish liquid cell suspension cultures, the calli were transferred to 50 ml flasks containing 20 ml liquid kanamycin medium (50 µg /ml), then after one week 2 ml were transferred in a 250 ml flask containing 80 ml kanamycin medium.

*Transformation of A. thaliana plants with 35S:FLS2:myc pCAMBIA 2300 by Agrobacterium tumefaciens*

For transformation, the "flower-dip" method was used. *Agrobacterium tumefaciens* GV3101 (provided by the group of Barbara Hohn) that is resistant against rifampicin and gentamycin, was grown and transformed with the FLS2:myc plasmid as follows: One clone was inoculated in YEB liquid medium (5 g/l Gibco beef extract, 1 g/l Bacto yeast extract, 1 g/l Bacto peptone, 5 g/l sucrose pH 7.4, autoclaved and supplemented with filter sterilized 10 mM MgSO<sub>4</sub> and 100 µg/ml rifampicin) and incubated by shaking over night at 4°C in 50 ml. The cells were harvested by centrifugation for 10 min at 5000 rpm and 4°C and resuspended in 20 ml 10 % glycerin. Fifty µl aliquots were frozen in liquid nitrogen and kept at -80°C for usage. For transformation, 1 µl plasmid DNA containing 1.2 µg 35S:FLS2:myc pCAMBIA 2300 was added to 50 µl bacteria. Electroporation was performed in pre-cooled cuvettes with a gap of 2 mm at 2.5 kV, 200 Ω and 25 µF. 600 µl NZY+ medium (10g NZ amine (casein hydrolysate), 5g yeast extract, 5g NaCl pH 7.5 with NaOH, after autoclaving addition of 12.5 ml 1M MgCl<sub>2</sub> and 12.5 ml 1 M MgSO<sub>4</sub> and 10 ml 2 M filter-sterilized glucose) was added to the electroporated bacteria, and the culture was shaken for 30 min at 37°C and for 1 h at 28°C. 50 µl and 5 µl were plated on YEB agar plates (YEB medium, 15 g/l agar, 10mM MgSO<sub>4</sub>, 50 µg/ml kanamycin and 100 µg/ml rifampicin). Colonies were tested via PCR if they contained FLS2:myc.

Colonies containing FLS2:myc were grown for "flower-dip" transformation: One colony was inoculated into 25 ml YEB liquid medium supplied with MgSO<sub>4</sub>, Rifampicin and Kanamycin. The culture was shaken over night at 28°C. Four times 12.5 ml of this inoculum was added to 800 ml YEB liquid medium supplied with MgSO<sub>4</sub>, Rifampicin and Kanamycin, and the cultures were shaken for 24 h at 28°C. The cells were harvested by centrifugation for 30 min at 5000 rpm and room temperature and resuspended in infiltration medium (for 2 l: 4.4 g MS salts, 6.4 g Gamborg's B5 basal medium with minimal organics, 100 g sucrose, 1.0 g MES pH 5.7 with KOH and 2µl of 10 mg/ml benzyladenin. 0.05 % Extravon was added after autoclaving). The OD was adjusted to a value of 2.

The *A. thaliana* plants (Ws-0 Wt, La-*er* Wt, La-*er fls2-24*, *fls2-17*, *fls11-2* and *fls11-19*) to be transformed were grown for 2 month at 8h light daily. The flowers were removed 3 days prior to transformation to enhance the development of new flowers. Fully developed flowers showing petals (white) were removed prior to transformation, since they were already too old.

The plants were dipped headlong into the bacterial suspension for 3 s to cover most of the flowers. The plants were covered with a plastic bag for one day for recovering and left growing for seed maturation.

The seeds of the transformed plants were collected and surface sterilized as follows: Two hundred mg seeds (~100,000 seeds) were incubated with 20% sodium hypochlorite and 0.05 % Tween-20 for 10 min. The liquid was removed and the seeds were washed three times in sterile water and dried on the sterile bench.

For germination, the sterilized seeds were mixed with 8 ml 0.7 % liquid agar and distributed on two 13.5 cm diameter agar plates (0.8 % phytoagar, 0.44 % MSMO (Murashige and Skoog Minimal Organics including Nitsch vitamins, Duchefa cat. no. M0256) and 100 µg/ml kanamycin). To control germination rate, an aliquot was distributed on agar plates without kanamycin. The plates were incubated for two weeks at 12h daily light.

All seeds on control plates germinated. On kanamycin plates only 0-9 seedlings out of >2000 survived. The rest of the seeds started to germinate but yielded seedlings with white leaves, which died subsequently. All surviving seedlings and a couple of control plants were planted in soil.

### *Anti-myc antibodies*

The antibodies used recognize the 10 amino acid epitope of the human protooncogene c-myc with the sequence EQKLISEEDL (Evan et al, 1985). We used mouse monoclonal 9E10 isotype IgG1 (FMI-produced, Sigma or Upstate), mouse monoclonal 9E10 alkaline phosphatase conjugate (Sigma) and rabbit polyclonal antibodies (Upstate).

### *Immunoblots*

The SDS-polyacrylamide gel was blotted on Millipore Immobilon-P Transfer membrane by Millipore MilliBlot-SDE System according to the manufacturers instructions. Then the blots were rinsed twice in PBS and “blocked” in 3 % BSA in PBS (0.8 % NaCl, 0.02 % KCl, 0.144 % Na<sub>2</sub>HPO<sub>4</sub>, 0.024 % KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) for 20-60 min. Then the blots were rinsed twice and washed once for 15 min and three times for 5 min in PBS. The blots (6 cm x 8 cm) were wrapped in nylon foil and 10 µl of the monoclonal anti-myc antibody or 1 µl of the polyclonal anti-myc antibody was added in 10 ml 3 % BSA in PBS. The blots were incubated on a revolving wheel over night at 4°C and washed again as described above. Then 1 µl of the secondary antibody (anti mouse IgG-alkaline phosphatase conjugate for monoclonal anti-myc or anti rabbit IgG-alkaline phosphatase conjugate for polyclonal anti-myc, Sigma) was added in 10 ml 3% BSA in PBS. The blots were incubated on the wheel for 1 h at room temperature. Then, they were washed as described above and incubated for 5 min in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris pH 9.5). The blots were put in a new nylon foil. 20 µl 25 mM CSPD (Roche) was given to 2 ml alkaline phosphatase buffer. The solution was given to the blots and distributed equally on the surface and then discarded. The blots were layed onto X-ray films (Kodak Scientific Imaging Film X-omat AR) in X-ray-cassettes, exhibited for 5-25 min, and the films were developed by AGFA Curix 60.

### *Estimation of molecular weights in immunoblots*

We routinely used prestained molecular weight markers from Invitrogen (BenchMark™ Pre-stained Protein Ladder) to approximate the molecular weight of blotted crossreacting bands. For precise molecular weight determination we used an unstained marker (Fermentas Protein Ladder, 10-200 kDa). In this case, the marker was cut from the blot before blocking and stained with coomassie blue. The stained piece of blot with the markers was fitted to the developed film. The pre-stained protein markers showed higher molecular weights then the unstained marker.

### *Immunoprecipitation*

Aliquots of Triton X-100 solubilized extracts of transgenic Msk8 (Tom2) or *A. thaliana* (At6 or At8) cell cultures were incubated with monoclonal or polyclonal anti-myc antibodies at 4°C over night. Protein G-sepharose for monoclonal or protein A-sepharose for polyclonal anti-myc antibodies (Amersham Pharmacia Biotech AB), as indicated in the figure legend, was added and incubated for several hours at 4°C. The beads were separated from the supernatant by spinning and washed three times in binding buffer with 1% Triton X-100.

### *ConA-sepharose precipitation*

Triton X-100 solubilized fractions of transgenic FLS2:myc plants or cell cultures were incubated with ConA-sepharose 4B (Sigma) and 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub> for 1 hour at 4°C. The beads were pelleted by spinning and washed in 1% Triton X-100 in binding buffer.

### *Deglycosylation*

Enzymatic deglycosylation with Oxford GlycoSystems Peptide-N-Glycosidase-F was done according to the manufacturers protocol. Briefly, 30 µl of crude extract of FLS2:myc transgenic *A. thaliana* cell culture were boiled for 5 min in Tris/HCl pH 7.5, 1 mM EDTA, 0,5 % SDS and 5 % β-mercaptoethanol. After cooling to room temperature, 10 % octylglycoside was added (final concentration), and the sample was incubated over night at 37°C, dried in speedvac and resolved in SDS-sample buffer.

Chemical deglycosylation with trimethylfluorosulfonic acid (TFMS) was performed according to the protocol of GlycoFree Deglycosylation Kit. Briefly, 500 µl crude extract of FLS2:myc transgenic Msk8 cell culture (Tom2) were desalted by spinning down and washing the pellet with ddH<sub>2</sub>O three times. Subsequently, the sample was lyophilized and transferred to 1 ml glass reaction vessels with teflon cover and cooled down in dry-ice-ethanol-bath. Sixty µl 100 % toluene and 300 µl TFMS (Sigma) were mixed, and 50 µl of this mixture were slowly added to the sample with a Hamilton-syringe. The sample was frozen for 5 min at -20°C, then shaken, frozen for 5 min and

shaken for two more times, then frozen for 4 hours. Then, the teflon cover was removed, and the sample was cooled in dry-ice-ethanol-bath and neutralized with 150  $\mu$ l of 60 % pyridine, 20 % methanol and 20 % ddH<sub>2</sub>O and 400  $\mu$ l 0.5 % ammonium-hydrogencarbonate. The sample was centrifuged. The pellet was washed three times in ddH<sub>2</sub>O and resolved in SDS sample buffer.

### *RT-PCR*

An aliquot of 0.1 g (FW) cell suspension culture or leaf material was homogenized with a Retsch Mixer Mill (Quiagen). RNA was extracted with RNeasy Plant Mini Kit (Quiagen). The yield was assessed via spectrophotometer ( $c [\mu\text{g/ml}] = \text{OD}_{260} \times \text{dilution factor} \times 40$ ) and viewing aliquots on a agarose gel. The RNA was treated with DNase (DNA-free, Ambion), and 2  $\mu$ g of RNA was used for cDNA synthesis (SuperScript<sup>TM</sup>, Invitrogen). Semi-quantitative PCR was performed with primers of a 420 kDa fragment of the C-terminal end of FLS2 (500 kDa DNA fragment with intron) and ended after 28 cycles. For controlling equal loading we amplified actin-fragments with primers provided by Cyril Zipfel in the same PCR. For a more accurate comparison of the level of RNA transcript we diluted cDNA-samples of *FLS2:myc* transgenic cells twice, 4-fold and 8-fold and compared them to the undiluted cDNA-sample of the control cell line.

(D<sub>I</sub>) Chapter I: Characterization and partial  
purification of the flagellin binding site in  
*Arabidopsis thaliana*



### I.1. Summary

*A. thaliana* has a sensitive and specific perception system for the most conserved domain of bacterial flagellin (Felix et al, 1999; Gómez-Gómez et al, 1999). Binding of flagellin and flagellin-derived peptides by different fractions of *A. thaliana* extracts was investigated to characterize the first step of the perception. Intact cells, showed specific binding at low salt concentrations and with a pH-maximum between 5 and 6. When cells were homogenized and fractionated by differential centrifugation, both the first pellet (P1), obtained by centrifugation at 10,000 g for 20 min, as well as the second pellet (P2, microsomes) obtained by ultracentrifugation at 100,000 g for 45 min, showed binding with very similar characteristics. Binding exhibited saturability and a high affinity of 1-3 nM. The number of binding sites was assessed at  $2-6 \times 10^4$  sites per cell. 70-95% of the binding sites was associated with the pellet P1 and only 5-30% with microsomes. In terms of specific activity (binding per mg protein), the microsomal fraction showed a 3- to 5-fold higher binding activity than P1.

The binding site exhibited specificity for flagellin-derived peptides with biological activity as agonists or antagonists of the elicitor responses. Cells showed irreversibility of binding, that partly disappeared during fractionation, to give rise to approximately 60 % reversibility in the microsomal fraction. Chemical crosslinking specifically labeled a polypeptide with an apparent molecular weight of ~120 kDa.

In order to obtain information on the molecular structure of the binding site, its biochemical purification was attempted. In order to solubilize the binding site, cell fractions were treated with 1 % Triton X-100. Thereby, we obtained maximally 5% of the binding site in solubilized form, the rest remained insoluble in the pellet. Solubilized binding sites were subjected to Mono Q anion exchange chromatography. The binding site from the ion exchange column was eluted in two broad peaks. Therefore, this method was not considered further for purification. Furthermore, affinity chromatography was tested for its suitability for purification. Concanavalin A, a lectin binding the carbohydrate moiety of glycoproteins, bound the binding site tightly, indicating that the binding site is a glycoprotein. Because elution from Concanavalin A column was inefficient, it was not used for purification. As next, we tested ligand affinity chromatography. As a prerequisite for

this method, solubilized binding sites were found to exhibit full reversibility. Flagellin protein was found, in contrast to flg22 peptide, well suitable for ligand affinity chromatography. The binding site was retained on the column and eluted with high pH or salt concentrations, or 0.1 M of flg22 peptide. Presence of the binding site in the eluted fraction could be determined by pulling it down with Concanavalin A-agarose beads and removing the elution reagent by washing. However, this fraction still contained a high number of contaminating proteins. Nevertheless, partial purification and 12-fold concentration of the binding site was achieved.

The major results of this Chapter have been published in Bauer et al, 2001.

## I.2. Introduction

Biochemical studies on ligand binding sites are a classical approach for receptor characterization. Binding characteristics of these proteins, such as saturability, high affinity, and reversibility of ligand binding, together with a direct correlation between the binding affinities and the elicitor activities provide strong evidence for the roles of these proteins as physiological receptors. Such binding studies have not only been performed for the flagellin binding site in tomato (described in the General Introduction) but also for a number of other different elicitors. The knowledge of high affinity binding sites for general elicitors is based on the work performed with the classical paradigm of a general elicitor, the hepta- $\beta$ -glucan binding site (Cosio et al., 1988; Cheong et al., 1991). High affinity binding sites have also been characterized for chitin fragments (Shibuya et al, 1993; Baureithel et al, 1994), the oligopeptide elicitor Pep13 (Nürnberg et al, 1994) and many other molecules involved in plant-pathogen interactions. Accordingly, such biochemical binding studies were performed to characterize the flagellin binding site in *A. thaliana*. Additionally, characterization of pH- and salt-dependence of the binding site was performed to identify the optimal experimental conditions. Localization of the binding site and assessment of the molecular weight via chemical crosslinking further elucidated the properties of the putative flagellin receptor.

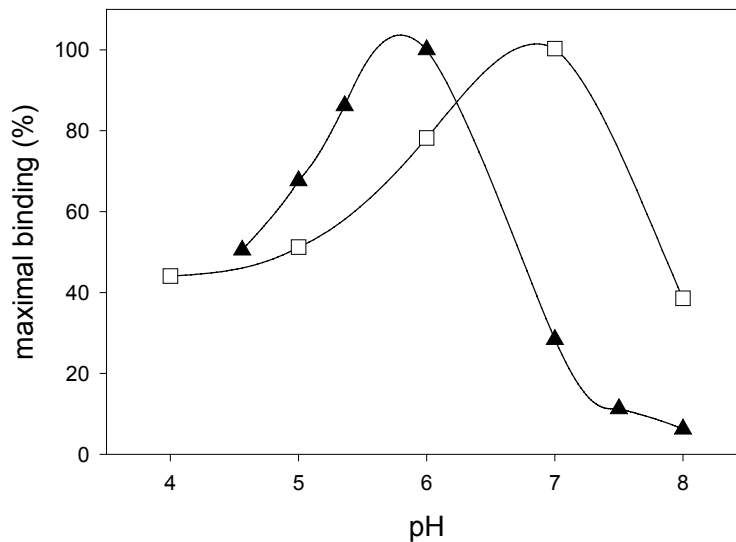
Biochemical purification of proteins is a common approach to identify the sequence of a protein. Comparison of the amino acid sequence with known protein sequences provides valuable information on the protein structure, that often allows conclusions on function, localization, origin, and other features. Additionally, pure protein fractions allow production of specific antibodies, that are a further important tool for characterization. It is especially worthwhile to purify proteins of *A. thaliana*, since in contrast to other plant model organisms, its genome has been fully sequenced. Protein purifications utilize biochemical characteristics of proteins that distinguish them from other proteins. Such differences can be for instance molecular weight, charge or hydrophobicity. A highly efficient purification method is affinity chromatography, where the protein of question either serves as a ligand or as a binding site. Here, we tested different methods for their efficiency in the purification of the flagellin binding site.

### I.3. Results

#### I.3.1.Characterization of the flagellin binding site

##### I.3.1.1. Flg22-binding depends on pH and salt concentration

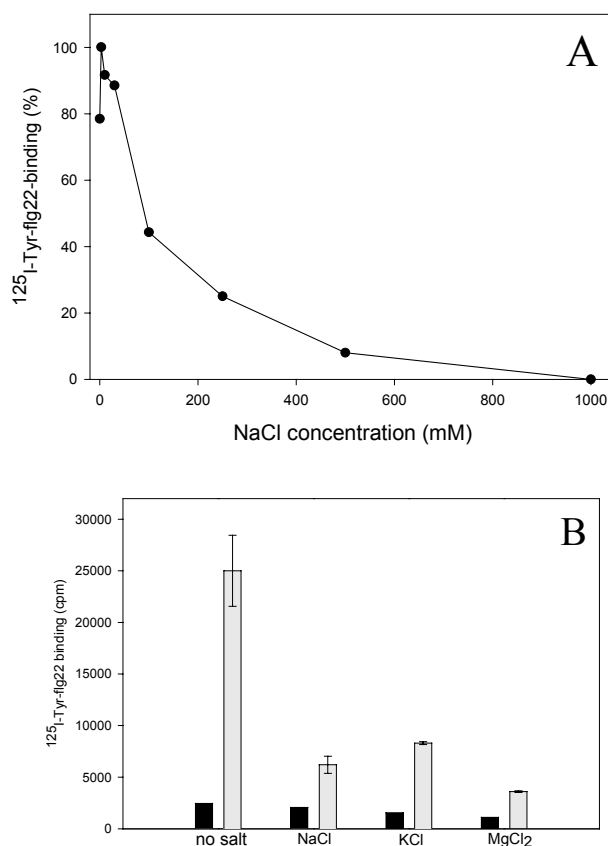
$^{125}\text{I}$ -Tyr-flg22, a radioactive derivative of flg22, has been used to establish a binding assay for flagellin in tomato suspension cell cultures (Meindl et al, 1998). We attempted to apply this assay to *A. thaliana*. Initially, we failed to detect specific binding sites in cells and membrane preparations. Variation of the experimental parameters showed that binding in *A. thaliana* had a pH optimum between pH 5 and 6, and buffering to pH > 7, as used for assays with tomato, reduced binding by >90% (Fig I/1). Similarly, binding decreased with increasing salt concentrations



**Fig. I/1: pH-dependence of  $^{125}\text{I}$ -Tyr-flg22 binding of *A. thaliana* and tomato.** Binding of 0.5 nM  $^{125}\text{I}$ -Tyr-flg22 to microsomal membranes containing 100  $\mu\text{g}$  protein was determined at various pH-values in MES and Tris buffers. The results are calculated as the average of two measurements and are presented as the percentages of maximal total binding, that was 30,000 cpm for tomato (white squares) and 14,000 cpm for *A. thaliana* (black triangles).

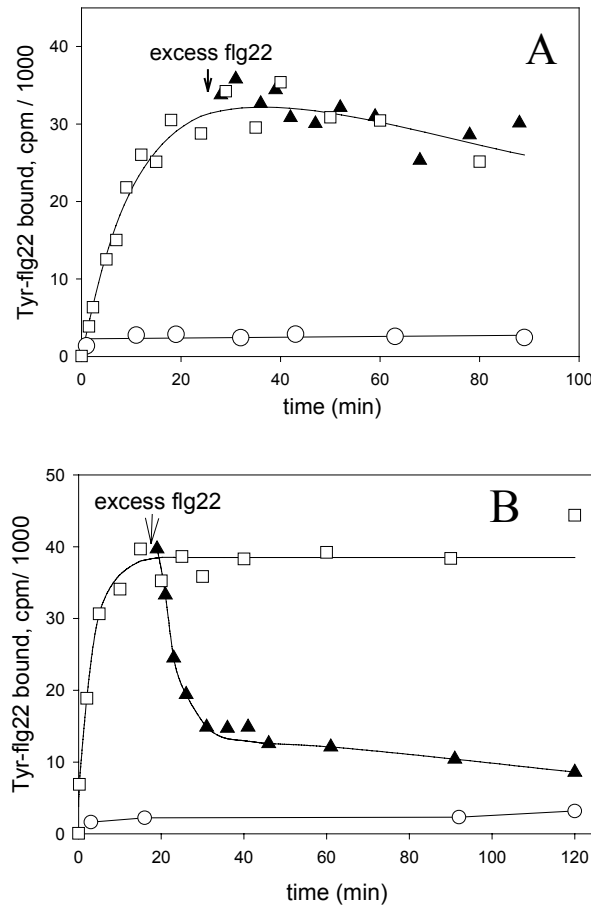
(Fig I/ 2a). This salt-sensitivity existed to all NaCl, KCl and  $\text{MgCl}_2$  salts (Fig I/ 2b). After replacing the high-salt buffer (0.5 M) against a low-salt buffer (10 mM), or

readjusting the pH from 8 to 6, binding was fully restored (data not shown). Therefore, ionic interactions between the binding site and flagellin seems to play a crucial role, but the interference of salts and high pH is reversible.



**Fig. I/2: Salt-dependence of flg22-binding.** A, Aliquots of *A. thaliana* microsomes were incubated with 0.5 nM  $^{125}\text{I}$ -Tyr-flg22 in buffers containing various concentrations of NaCl and used for binding studies. The plot shows specific binding. Maximum specific binding (16,000 cpm) was set as 100%. B, 100  $\mu\text{l}$  *A.thaliana* cell homogenates were supplied with 0.4 nM  $^{125}\text{I}$ -Tyr-flg22 alone (total binding, grey bares) or in combination of 10  $\mu\text{M}$  flg22 (nonspecific binding, black bars) in the absence or presence of 0.25 M of various salts. The bars represent one (nonspecific binding) or the average of two measurements (total binding).

Under appropriately modified and optimised assay conditions, buffering at pH 6.0 and lowering the concentration of NaCl to 10 mM, specific binding of  $^{125}\text{I}$ -Tyr-flg22 to *A. thaliana* cells and membrane preparations could readily be detected (Fig.I/ 3A and B).



**Fig. I/3: Time course and reversibility of binding of  $^{125}\text{I}$ -Tyr-flg22 to cells and microsomal membranes.** A, Binding kinetics of  $^{125}\text{I}$ -Tyr-flg22 to intact *A. thaliana* cells at 4 °C. Aliquots of the cell suspensions (60 mg fresh weight) were supplied with 0.4 nM  $^{125}\text{I}$ -Tyr-flg22 alone (open squares) or in combination with 10  $\mu\text{M}$  flg22 (added at  $t = 0$  min, open circles, or at  $t = 25$  min, closed triangles). B, Binding kinetics of  $^{125}\text{I}$ -Tyr-flg22 to microsomal membranes. Aliquots containing 100  $\mu\text{g}$  protein supplied with 0.4 nM  $^{125}\text{I}$ -Tyr-flg22 (open squares) alone or in combination with 10  $\mu\text{M}$  flg22 (added at  $t = 0$  min, open squares, added at  $t = 20$  min, closed triangles).

#### I.3.1.2. Time course and reversibility

Both, in intact (living) cells and in microsomal membrane preparations, maximal binding was reached within 20 min at 4°C (Fig. I/ 3). Label associated with cells and membranes remained stable for at least 90 minutes. Nonspecific binding, assayed in the presence of an excess of 10  $\mu\text{M}$  flg22, remained low throughout the experiment.

Binding to intact cells appeared nonreversible (Fig. I/ 3A) because the addition of excess flg22 in midcourse did not result in a decrease of label associated with the cells. This irreversibility of binding was found in repetitions with different batches of intact cells (n>4). Binding appeared to be irreversible also in cells pre-incubated in 10 mM NaN<sub>3</sub> or 10 mM NaF (data not shown), indicating that non-reversibility of binding was not due to internalisation or other processes dependent on energy or membrane flow.

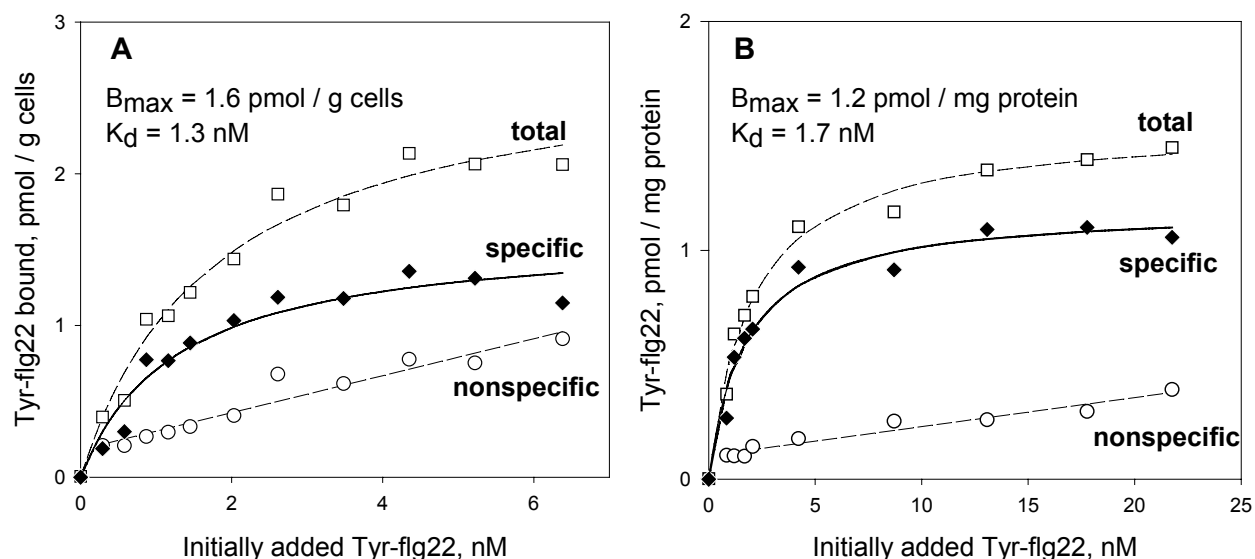
In contrast, binding of <sup>125</sup>I-Tyr-flg22 to microsomal membranes (Fig. I/ 3B) proved to be reversible to a large extent, and 60 % +/- 10 % of label was replaced after 20 min in six independent membrane preparations.

### *I.3.1.3. Flagellin binding site is saturable and shows high-affinity*

In order to test saturability, and to estimate the affinity of the binding site, we incubated increasing concentrations of <sup>125</sup>I-Tyr-flg22 with intact cells and microsomes (Fig. I/ 4A and B). Fitting the data of specific binding to rectangular hyperbola (solid lines in Fig. I/ 4A and B) resulted in an apparent K<sub>d</sub>\* of 1.3 nM for intact cells and 1.7 nM for microsomal membranes, respectively. In the experiments shown in Figure I/4 the number of binding sites (B<sub>max</sub>) corresponded to 1.6 pmol binding sites /g of cells (fresh weight) and 1.2 pmol / mg of microsomal protein. In three repetitions of the saturation experiments with microsomes and in two repetitions with intact cells K<sub>d</sub> values reproducibly ranged between 1 and 3 nM and the values for B<sub>max</sub> varied 2- to 3-fold in different batches of cells and membranes (data not shown). The *A. thaliana* cells used in these assays contain approximately 4 x 10<sup>4</sup> cells per mg fresh weight, thus there are 2-6 x 10<sup>4</sup> receptor sites per cell.

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\* The equilibrium dissociation constant (K<sub>d</sub>) represents the concentration of the ligand that half-maximally occupies the receptor at equilibrium. An increase in K<sub>d</sub> correlates with a decrease in affinity.



**Fig. I/4: Saturation of binding to intact cells and microsomal membranes.** Various concentrations of  $^{125}\text{I}$ -Tyr-flg22, diluted with nonlabeled Tyr-flg22 to a specific radioactivity of 710 Ci / mmol, were incubated with aliquots of intact cells (A) (9mg fresh weight) or microsomal membranes (B) (15  $\mu\text{g}$  protein) at 4°C for 25 min in the absence (total binding, open squares) or the presence (open circles) of 10  $\mu\text{M}$  flg22. To determine the specific binding (filled diamonds), nonspecific binding was subtracted from total binding.  $K_d$  and  $B_{\max}$  were determined by curve fitting to rectangular hyperbola ( $y = B_{\max} * x / (K_d + x)$ ), where  $y$ =bound ligand and  $x$ =free ligand.

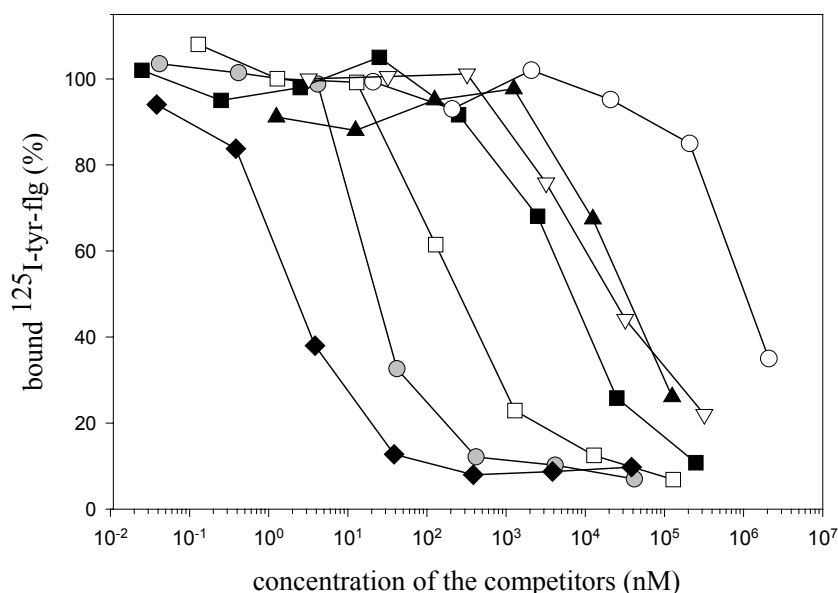
Apart from the apparent change in reversibility, the binding characteristics in cells, crude extracts, P1 and microsomal membrane fraction P2 were indistinguishable with respect to affinity, measured with saturation kinetics, and specificity, tested in competition assays with different flagellin-derived peptides (data not shown). This indicates that binding activity detected in these different fractions all represent the same binding site.

#### *I.3.1.4. The binding site is specific for biologically active flagellin-derived peptides*

The specificity of binding was tested in competitive binding assays with increasing concentrations of flagellin protein or various flagellin-derived peptides as competitors of  $^{125}\text{I}$ -Tyr-flg22. Examples for competition by different peptides (Tyr-



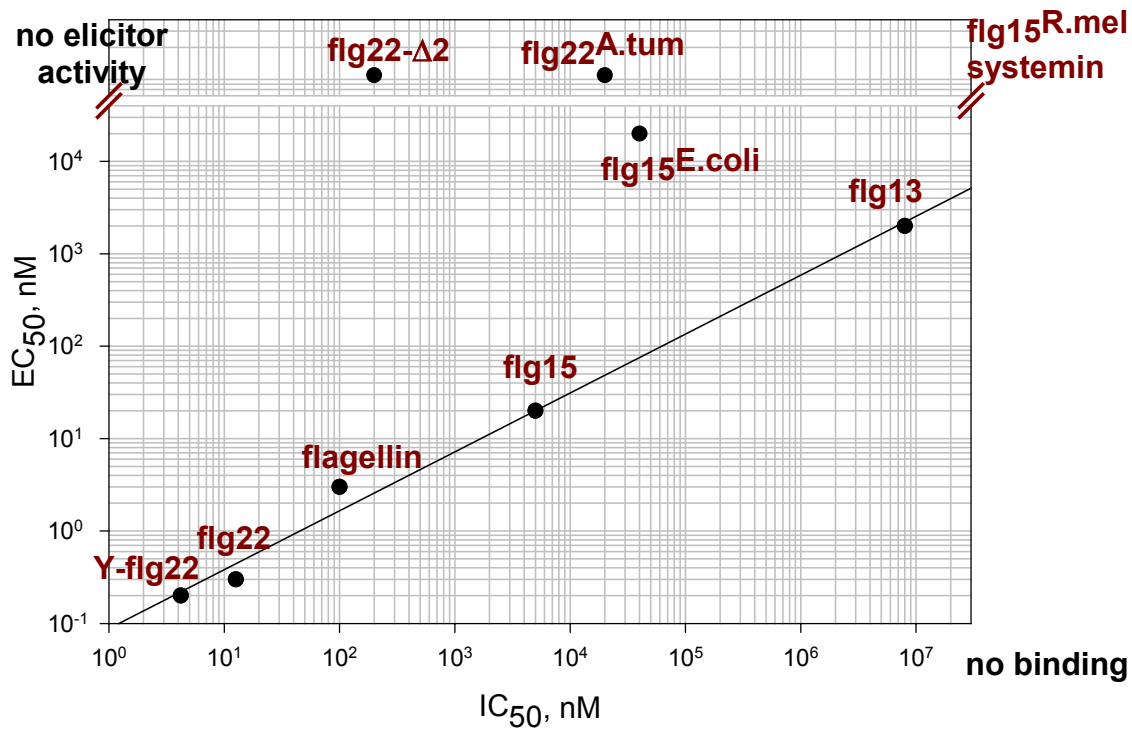
flg22, flg22, flg15, flg13, flg22-Δ2, flg15<sup>E.coli</sup> and flg22<sup>A.tum</sup>) in binding assays with microsomal preparations are shown in Figure I/5. In binding competition assays



Peptide	Sequence
◆ Y-flg22	YQRLSTGSRINSAKDDAAGLQIA
● flg22	QRLSTGSRINSAKDDAAGLQIA
■ flg15	RINSAKDDAAGLQIA
○ flg13	NSAKDDAAGLQIA
□ flg22-Δ2	QRLSTGSRINSAKDDAAGLQ
▲ flg15 <sup>E.coli</sup>	RINSAKDDAAGQAIA
▽ flg22 <sup>A.tum</sup>	ARVSSGLRVGDASDNAAYWSIA

**Fig. I/5: Competition of <sup>125</sup>I-Tyr-flg22- binding by flagellin and flagellin- derived peptides.** Binding assays with <sup>125</sup>I-Tyr-flg22 (0.6 nM) and various concentrations of Tyr-flg22, flg22, flg22-Δ2, flg15, flg15, flg13, flg15<sup>E.coli</sup>, and flg22<sup>A.tum</sup>. The results were obtained with different batches of microsomal membrane preparations using 60μg protein and are presented as the percentages of total binding. Total binding (100%) ranged between 14000 and 18000 cpm, and nonspecific binding (5-10%) between 1000 and 2000 cpm.

with intact cells, the peptides tested exhibited the same relative order with similar IC<sub>50</sub> values (the concentration required to inhibit binding of the radioligand by 50 %)(data not shown). In Figure I/6, the IC<sub>50</sub> values for flagellin protein and various



**Fig I/6: Correlation between biological activities and binding affinities for flagellin and flagellin-derived peptides.** Relative activities of flagellin and flagellin-derived peptides for induction of the alkalization response (EC<sub>50</sub> values) in *A. thaliana* cells are plotted against their activities (IC<sub>50</sub>) in binding competition assays with microsomal membranes

peptides, deduced from dose-competition curves such as the ones shown in [Figure I/5](#), were plotted against their respective activity for induction of a half maximal alkalization response (EC<sub>50</sub> values), determined by Jürg Felix. Most efficient competition and highest biological activity were observed for Tyr-flg22 and its iodinated form I-Tyr-flg22 (values for IC<sub>50</sub> of 4 nM and for EC<sub>50</sub> of 0.2 nM, respectively). Flg22 was ~3- to 5-fold less efficient in both assays, whereas intact flagellin protein was ~20-fold less active. Peptides shortened at the N-terminus, flg15 and flg13, showed decreasing binding affinity in parallel to dropping elicitor activity. Flg22-Δ2, acting as antagonist for biological activity, strongly competed for binding with an IC<sub>50</sub> only 10-fold lower than flg22. Weak competition of binding was observed for flg15-Δ2 when added in mM concentrations. Peptides corresponding to the homologues of flg15 from *A. tumefaciens* and *R. meliloti*

(flg15<sup>A.tum</sup> and Flg15<sup>R.mel</sup>) were previously reported to be inactive as inducers of alkalization (Felix et al 1999), and Flg15<sup>R.mel</sup> also did not show measurable activity in binding competition. In contrast, the homologue of flg22 from *A. tumefaciens*, flg22<sup>A.tum</sup> competed for binding with an IC<sub>50</sub> of 20  $\mu$ M but did not induce alkalization in concentrations up to 30  $\mu$ M. Structurally unrelated peptide, able to induce alkalization, such as the 18 amino acid systemin did not compete binding at any of the concentrations tested.

In summary, the binding site detected exhibited clear specificity for flagellin-derived peptides with biological activity as agonists or antagonists.

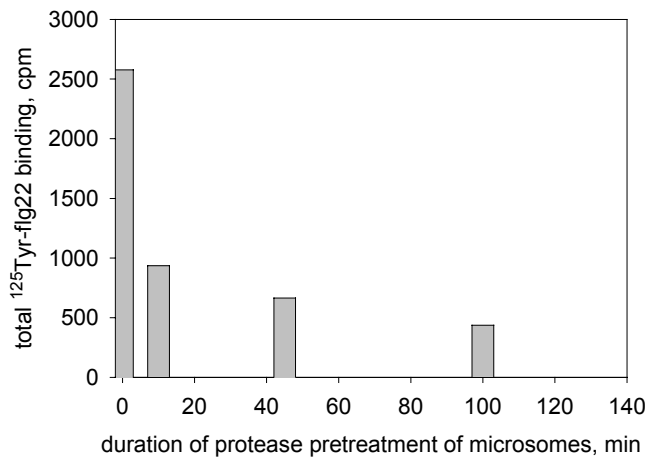
### *I.3.1.5. Localization of the binding site*

In order to localize the binding site within the plant cell, flg22-binding was determined in all centrifugal fractions. Specific binding was compared in crude homogenates, the first (10.000 x g) centrifugal pellet (P1), the second ultracentrifugal (100.000 x g) pellet (microsomal fraction) and the supernatant (soluble fraction) in three independent experiments. 70-95% of total binding activity observed in cell homogenates was associated with P1, 5-30% with microsomes and none with the soluble fraction. In terms of specific activity (binding per mg protein), the microsomal fraction showed a 3- to 5-fold higher binding activity than P1 (data not shown). We speculated that the binding site is a membrane protein, that is part of a bigger complex and only binding sites separated from this bigger complex reach the microsomal fraction. One possibility is that the binding site is attached to the cell wall. To test this hypothesis, cell wall was enriched by repeated filtration of the rapidly sedimenting pellet P<sub>0</sub>. The enriched cell wall showed a loss of binding activity compared to the original cell homogenate in two repetitions (data not shown). Another approach to demonstrate, that the binding site resides below the cell wall within the cell was degradation of the cell wall by fungal enzymes to create protoplasts. Interestingly, emerging protoplasts exhibited no significant binding in many repetitions, even when a mix of protease inhibitors was added. It is possible that proteolytic digestion occurs nevertheless and is the actual reason for the loss of binding activity in protoplasts. Separation of the plasma membrane from the cell

wall by plasmolysis with 1 M mannitol did not impair response to flg22 (data not shown), that refutes the idea, that the binding site is attached to the cell wall.

### *I.3.1.6. The binding site is sensitive to proteolytic digestion*

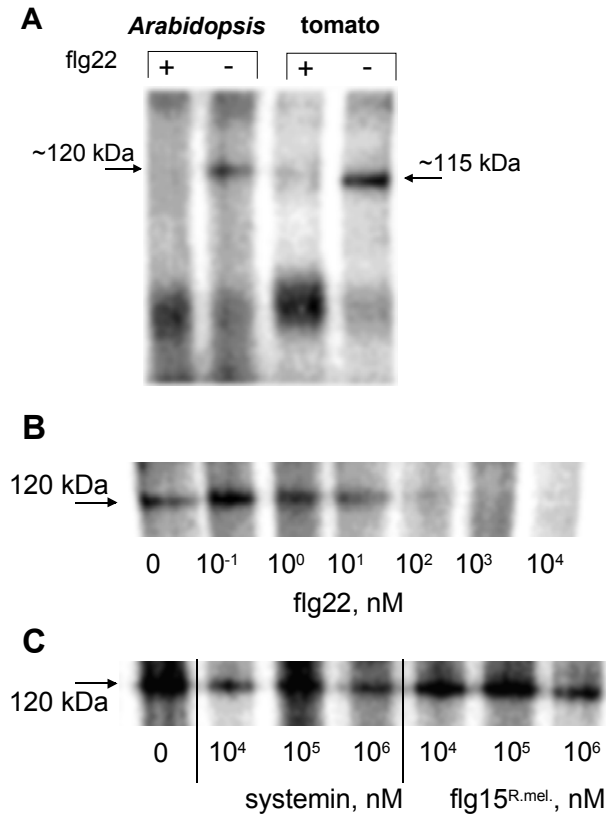
In order to ensure that the binding site is a protein, we tested if the binding site is sensitive to proteolytic digestion. For protecting  $^{125}\text{Tyr}$ -flg22-peptide from digestion, protease inhibitors were added prior to binding assays. To initiate proteolytic digestion, microsomes or P1 were supplemented with proteinase K. After 30 min incubation, the proteinase K inhibitors PMSF and Pefabloc were added to protect flg22 from degradation. Binding assays with proteinase K treated microsomes showed no significant binding in contrast to nontreated microsomes, indicating that the binding site was degraded upon protease treatment (data not shown), even though proteolytic degradation of flg22 peptide could not be fully inhibited, as shown by control experiments. Similar experiments were conducted with the protease trypsin. Trypsin digestion of microsomes and P1 was stopped after various incubation periods with a mixture of protease inhibitors (TLCK, EDTA, Pefabloc and leupeptin), and then the membranes or P1 were used for binding assays. We observed a significant reduction of binding activity in response to trypsin treatment, which was proportional to the duration of this treatment (Fig 1/7, and data not shown). This time dependent reduction of flg22 binding was attributed to digestion of the binding site and not of the flg22 peptide, since the peptides exposure to a potentially remaining proteolytic activity during the binding reaction was the same in each case (20 min). We conclude that the flagellin binding site is sensitive to protease digestion and therefore, it consists of a polypeptide.



•**Fig I/7: Flagellin binding site is sensitive to proteolytic digestion:** Microsomal fraction of *A. thaliana* cell extracts was incubated with trypsin for various periods before a mixture of protease inhibitors was added. In the case of  $t=0$ , protease and protease inhibitor were mixed before addition to the microsomes. <sup>125</sup>Tyr-flg22 was incubated with the pretreated microsomes for 20 min. Nonbound label was separated by filtration.

#### *I.3.1.7. Affinity crosslinking labels a polypeptide with an apparent molecular weight of ~120 kDa*

In previous work with microsomal membranes from tomato, covalent chemical crosslinking of <sup>125</sup>I-Tyr-flg22 to a polypeptide of ~115 kDa has been observed (Meindl et al, 2000). In microsomal membranes of *A. thaliana*, in comparison, crosslinking with the homo-bifunctional crosslinker dithiobis(succinimidyl-propionate) labeled a polypeptide migrating with an apparent molecular mass of ~120 kDa (Fig. I/8 A). Labeling of the 120 kDa band was specifically inhibited by unlabeled flg22 with a dose-dependence similar to the one observed for binding in competition assays (Fig. I/8 B). Competitive inhibition was specific for flg22 and labeling of the 120 kDa band was not inhibited by the addition of the inactive peptide flg15<sup>R.mel</sup> or the structurally unrelated peptide systemin at concentrations up to 10  $\mu$ M (Fig. I/8 C). Crosslinking was also performed with P1 in addition to the microsomes. There too, a band at around 120 kDa was observed, and labelling of this band was competed by flg22.



**Fig. I/8: Chemical crosslinking of <sup>125</sup>I-Tyr-flg22 to microsomal membranes:** A, Aliquots of *A. thaliana* and tomato microsomal membranes were incubated with 0.5 nM <sup>125</sup>I-Tyr-flg22 in absence or presence of 10  $\mu$ M unlabeled flg22. B, Aliquots of *A. thaliana* microsomal membranes were incubated with 0.5 nM <sup>125</sup>I-Tyr-flg22 with increasing concentrations of flg22 or (C) systemin and flg15<sup>R.mel.</sup>. In all three experiments, 5 mM Dithiobis(succinimidylpropionate) was added after 30 min incubation on ice and further incubated for another 30 min at room temperature. Aliquots were separated on 8% SDS-Gel.

The nonspecifically labeled band between 50 and 60 kDa was suspected to be BSA, which was used in 0.1 % concentrations in all peptide dilutions and was therefore the most abundant protein in the assay. In order to test this possibility, BSA in concentrations present in the radioactive assays, was crosslinked to flg22. Coomassie blue stained SDS-gel of this sample showed a diffuse band between 50 and 60 kDa that resembled the band observed in radioactive crosslinking experiments with microsomes and P1 (data not shown). Interestingly, the BSA-band became broader and more diffuse upon flg22-crosslinking, probably due to covalent attachment of various numbers of flg22-units.

In order to find out, if, due to disulfide bridges, reducing conditions change the migration of the specifically labelled band in SDS-PAGE, 5%  $\beta$ -mercaptoethanol (v/v) or 1 mM DTT was added to the crosslinking sample prior to loading on the SDS-gel. Since the crosslinker DSP (dithiobis(succinimidylpropionate)), which was used in previous experiments, is thio-cleavable by reducing reagents, different crosslinkers were used, such as EGS (Ethylene glycolbis(succinimidylsuccinate)) or DSS (Disuccinimidyl suberate), which were not sensitive to reduction. In these experiments, we observed the same specific band at 120 kDa as under nonreducing conditions with the crosslinker DSP (data not shown). We conclude that the labelled band representing the putative binding site does not contain disulfide bridges.

### **I.3.2. Partial Purification of the flagellin binding site**

#### *I.3.2.1. Solubilization of flagellin binding sites*

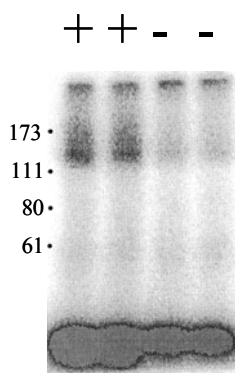
An essential requirement for protein purification is that the protein of question is in a soluble state. This is generally no problem for cytoplasmic proteins, but in this study, there was no binding activity in the soluble fraction (S2) of *A. thaliana* cell culture extracts, that corresponded to the second supernatant during differential centrifugation. Most of the activity (>95%) was found in P1, the first centrifugal pellet, and < 5% in the microsomal fraction (see I.3.1.5.). Treatment of P1 and microsomes with 0.5 M NaCl, a treatment known to bring peripheric membrane proteins in solution (Steck and Yu, 1973), did not solubilize the binding site (data not shown). Detergents provide means of disrupting the integrity of biological membranes, and thus allow the characterization of membrane proteins. Proteins may be solubilized in a native-like state with retention of functional properties. However, many detergents interfere with the biological function of proteins. The flagellin binding site of tomato was successfully solubilized with 1% octylglycoside (Meindl, 2000). Although 50 % of overall binding activity was lost, 50-80% of the remaining activity was found in the solubilized fraction (e.g. ultracentrifugal supernatant). Similarly, addition of 1% octylglycoside or Zwittergent reduced binding in *A. thaliana* cell suspensions by approximately 60 %. By contrast, addition of 1% Triton X-100 reduced binding under similar conditions by maximally 20 % (data not shown). Thus, we used Triton X-100 for solubilization, using microsomes or P1. Both, with octylglycoside or Triton X-100, only 1-5% of the binding activity was

found in the solubilized fraction, and the rest of the binding sites remained in the ultracentrifugal pellet. Up to date, no conditions could be identified that increased the efficacy of solubilization, thus we worked with this low efficacy.

### *1.3.2.2. Characterization of the solubilized binding site*

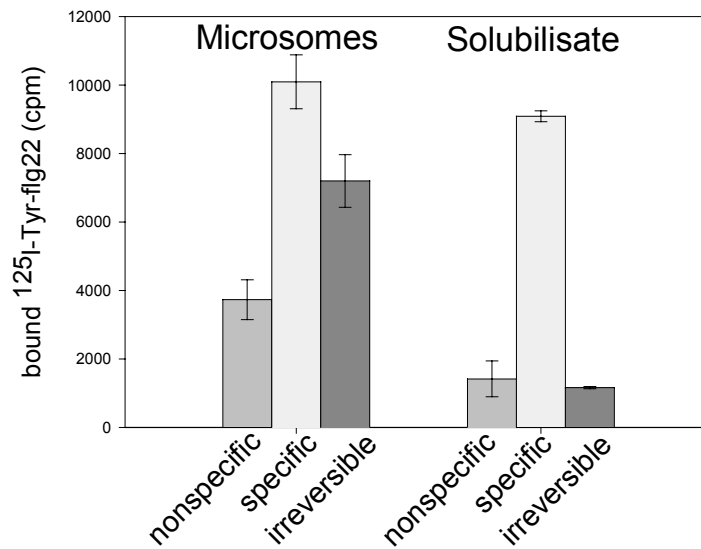
The fact that only a small fraction of the binding sites gets solubilized opens the possibility that this fraction represents only a subclass of the binding site. Competition binding assays with detergent solubilized flagellin binding sites revealed that the  $IC_{50}$  for flg22 (15 nM and 80 nM in two repetitions, data not shown) remained in the same order of magnitude as observed for whole cells and microsomal membranes (~20 nM, Fig I/5). To further investigate the characteristics of the solubilized binding site, we performed chemical crosslinking experiments according to those performed with microsomal membranes and P1 (Fig. I/8). However, no specific bands were labelled on radiograms of SDS-gels with 100  $\mu$ l of solubilized fraction crosslinked to  $^{125}$ I-Tyr-flg22 (data not shown), presumably due to the low concentration of binding sites in this fraction. Therefore, we attempted to concentrate the flagellin binding sites by precipitation with Con A-agarose beads and used these precipitated binding sites for crosslinking experiments. In order to separate the binding sites from the Con A beads for loading onto the SDS-gel, the sample was boiled in SDS-gel sample buffer and centrifuged. The supernatant containing the binding sites was loaded on an SDS-gel and subjected to radiography. However, the radiograms did not show any specific labeling and showed a strongly reduced radioactive background in addition. We speculated, that the Con A beads fully absorbed the radioactive flagellin peptides preventing their binding to the flagellin binding site. Thus, we first performed flg22-binding and crosslinking with a 100-fold volume of solubilized fraction (10 ml), maintaining the original concentration of all other components, and precipitated the binding site with Con A sepharose beads after the crosslinking procedure. On this gel a specific band around 120 kDa was detected (Fig. I/9), though, labelling was broad and blurred, possibly due to overloading or interference of Triton X-100 with SDS. This result supports the assumption that the same 120 kDa band which specifically crosslinks to flg22 in the microsomal fraction and P1, is present in the soluble fraction, and that it represents the flagellin binding site.





**Fig I/9: Chemical crosslinking of  $^{125}\text{I}$ -Tyr-flg22 to the solubilized fraction.** 0.5 nM  $^{125}\text{I}$ -Tyr-flg22 was added to 10 ml solubilized fraction containing ~1,000,000 cpm specific binding with (+) or without (-) competitor (10  $\mu\text{M}$  flg22) and incubated for 30 min on ice. After addition of 12 mg DSP to each sample, they were incubated for another 30 min at room temperature, and the crosslinking reaction was stopped by addition of 20 mM Tris/Hcl pH 7.5. The binding sites were collected by precipitation of Con A-sepharose beads and washed. Aliquots of SDS-boiled ConA precipitates were loaded on 7% SDS-gels.

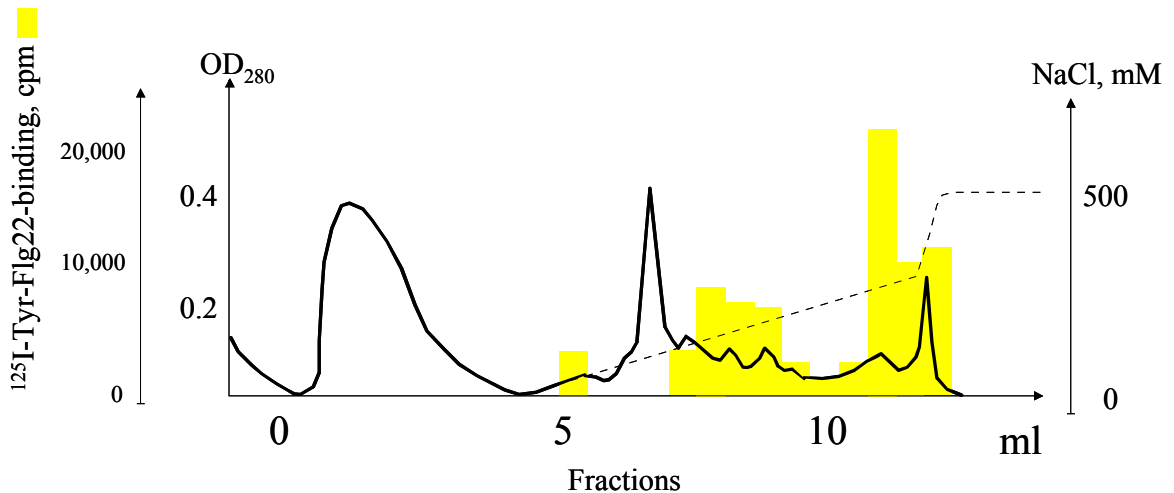
In contrast to intact cells that exhibited irreversibility and microsomal preparations that exhibited approx. 60 % reversibility (see I.3.1.2.), binding after solubilization was fully reversible in three independent repetitions (Fig. I/10). This fact is an optimal prerequisite for purification by flagellin ligand chromatography. As already stated (I.3.1.2.), reversibility increased gradually during cell fractionation, indicating, that disassembly of the receptor complex or loss of cofactors take place. Interestingly, Thomas Meindl reported full irreversibility for solubilized flagellin binding sites in tomato (Meindl, 2000). Some preliminary experiments with the flagellin binding sites of tomato on my own did not support this finding (data not shown). I found similar reversibility for tomato as for *A. thaliana* solubilized binding sites.



**Fig. I/10: Reversibility in the solubilized fraction compared to microsomes.** Aliquots of microsomes and solubilized fractions were incubated with 0.5 nM  $^{125}\text{I}$ -Tyr-flg22 for 40 min at 4°C in presence (middle grey bars: nonspecific binding) or absence (light grey bars: specific binding) of 10  $\mu\text{M}$  flg22, or 10  $\mu\text{M}$  flg22 was added after 20 min (dark grey bars: irreversible binding). Bars end error bars represent averages and standards deviations of three replicates.

#### *I.3.2.3. MonoQ anion exchange chromatography*

MonoQ anion exchange chromatography was tested for its suitability as a pre-purification step. Unfortunately, the flagellin binding site was eluted over a broad area (Fig I/11). Therefore, purification was not achieved with this method. Strikingly, the elution pattern in two major peaks was the same for the tomato binding site (Meindl, 2000), the second peak being slightly larger than the first one. This finding might reflect the presence of two distinct classes of the flagellin binding site.

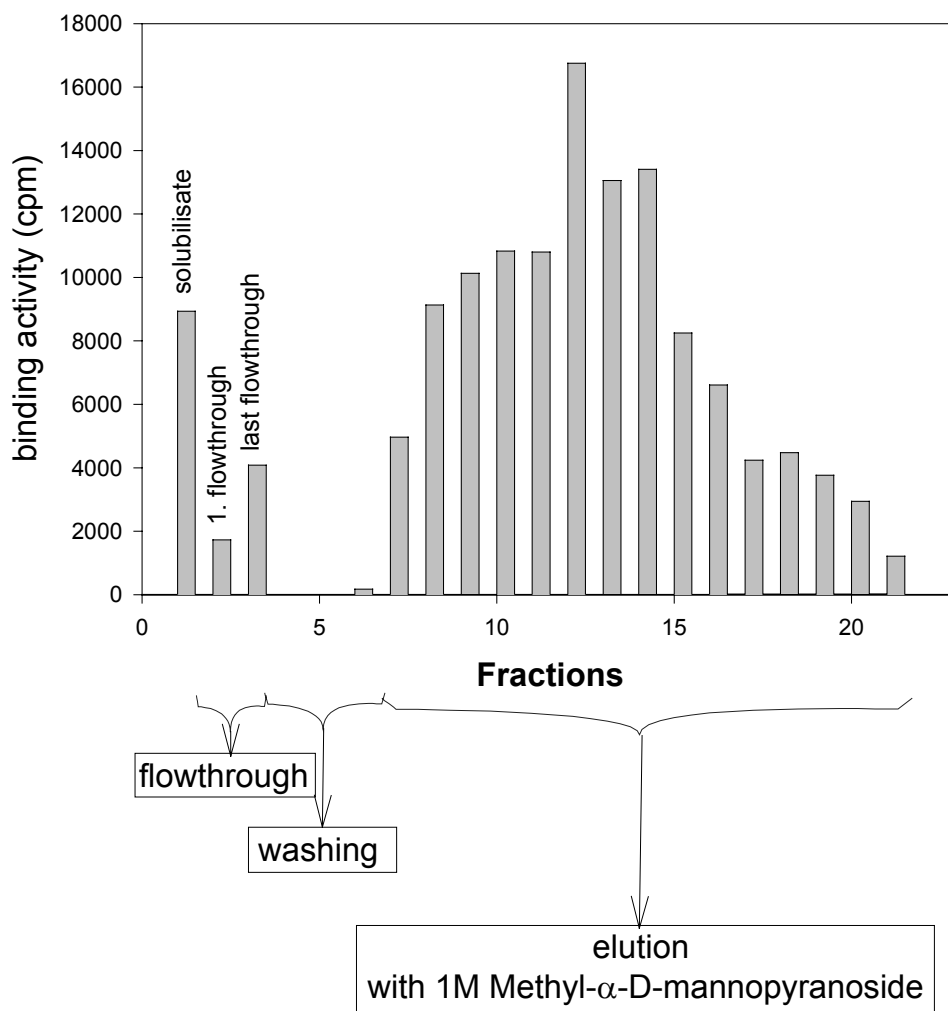


**Fig. I/11: MonoQ anion exchange chromatography of flagellin binding sites:** 6 ml Triton X-100 solubilized and filtered binding sites with 6000 cpm/ 100  $\mu$ l binding activity were loaded on a MonoQ column and eluted in 500  $\mu$ l fractions with a 0-500 mM NaCl gradient using the SMART system (Pharmacia Biotech Upsala Sweden; flow rate: 150  $\mu$ l/ min). 5  $\mu$ l of the fraction was diluted with 90  $\mu$ l 1% Triton X-100 in binding buffer and used to determine flagellin binding (integrated bar graphics).

#### *I.3.2.4. Con A affinity chromatography*

The lectin Con A binds glycoproteins with  $\alpha$ -D-mannosyl and  $\alpha$ -D-glucosyl residues at nonreducing ends or within the glycosyl-chain, in presence of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  (Kalb and Lustig, 1968; Gunther et al, 1973; Bittiges and Schnebli, 1976). Con A exhibits the highest affinity to methyl- $\alpha$ -D-mannopyranoside. In order to test if the flagellin binding site carries glycosyl residues that allow the utilization of ConA affinity chromatography, solubilized binding sites were applied on a Con A-sepharose-column. When the flow rate was small, the flow through of this column did not contain any detectable binding activity suggesting that the Con A column completely absorbed the binding sites. To test if this absorption was due to binding to functional Con A proteins or to some nonspecific binding to the sepharose column, we measured absorption of solubilized binding sites by sepharose control column without Con A, as well as Con A sepharose beads boiled in 1% SDS prior to use. In these two cases full binding activity was recovered in the flow through showing, that intact Con A is indeed required for retaining the binding site. Elution of binding activity from Con A-sepharose started with addition of 1 M methyl- $\alpha$ -D-

mannopyranoside (Fig. I/12). In total, however, only a small fraction of the originally loaded binding sites could be eluted from the column, and this eluted fraction contained less specific flagellin binding per volume than the originally loaded fraction. Binding assays with aliquots of the column revealed that the rest of



**Fig. I/12: Concanavalin A affinity chromatography of flagellin binding sites.**

13 ml solubilized fraction with 10,000 cpm /100  $\mu$ l was loaded on a 0.5 ml Con A-sepharose column at 4°C with a velocity of 200 $\mu$ l/min. The column was washed with 10 ml 1% Triton X-100 in binding buffer with the same velocity at 4°C. The binding site was eluted with 1 M Methyl- $\alpha$ -D-mannopyranoside in 1% Triton in binding buffer at room temperature with a velocity of 10  $\mu$ l/min.

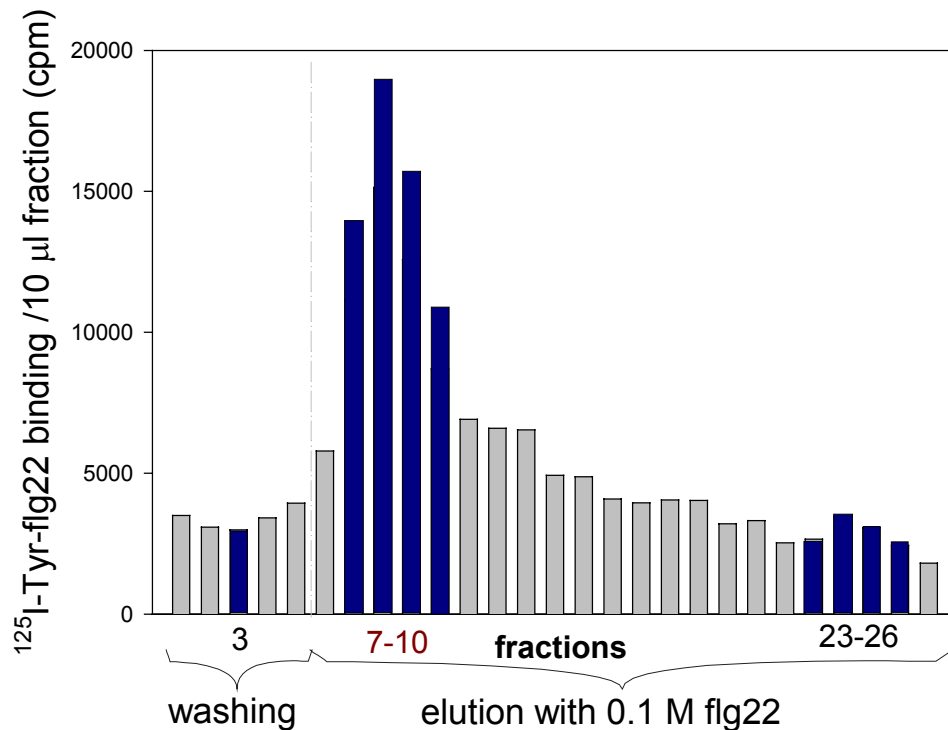
binding activity was still retained by the column. Addition of 1M NaCl, 4 M MgCl<sub>2</sub>, 1 M Ammoniac, 6 mM DTT, 20% ethylenglycol, as well as performing the elution at pH 4.5 or at temperatures of 50°C, did not improve the yield of elution. Complete

elution of Man<sub>9</sub>GlcNAc<sub>2</sub>Asn derivatives, that bind very tightly to Con A and are difficult to elute, could be achieved by allowing the column to stand in elution buffer containing 1 M methyl- $\alpha$ -D-mannopyranoside for 1 hour at room temperature (Deras et al, 1998). Analogous experimental conditions for the flagellin binding site improved elution 2-3 fold, but the majority of the binding sites (70% in this experiment) still remained on the column. Even under these improved conditions, Con A affinity chromatography did not result in a concentration of binding activity, but rather in a dilution. We conclude, that Con A binds the flagellin binding site with high affinity and poor reversibility, suggesting, that the binding site carries multiple glycosyl groups with  $\alpha$ -D-mannosyl and  $\alpha$ -D-glucosyl residues at nonreducing ends or within the glycosyl-chain. However, due to this fact, Con A affinity chromatography was not found to be useful for purification of the flagellin binding site. Interestingly, Thomas Meindl found that the tomato flagellin binding site was not retained on a Con A column and the total binding activity was found in the flow-through (Meindl, 2000). Repetitions of this experiment by myself did not confirm these results. Under experimental conditions used for *A. thaliana*, tomato binding sites were highly concentrated on the Con A column, similarly to the flagellin binding site of *A. thaliana* (data not shown).

#### *I.3.2.5. Flagellin affinity chromatography*

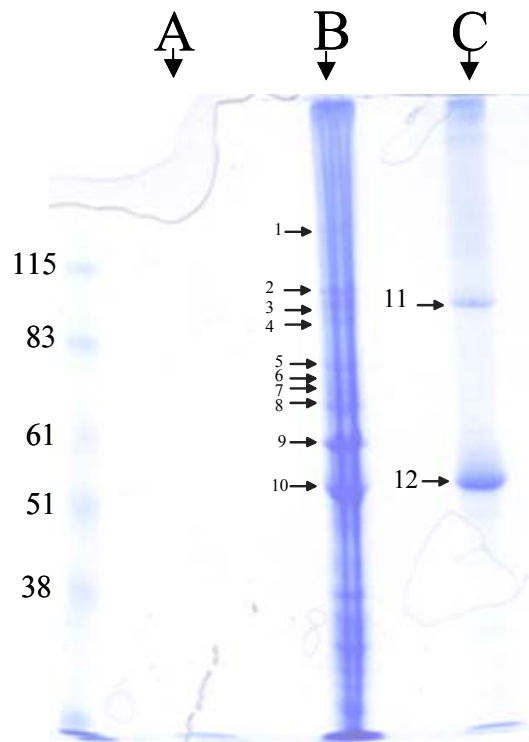
Ligand affinity chromatography is a highly selective method that allows effective purification of receptor binding sites. In order to prepare a ligand affinity column, we coupled flg22 peptide to Affi 15 derivatized agarose gel, by crosslinking primary amino groups of the peptide to N-hydroxy-succinimide esters of the column. However, Affi 15-flg22 columns did not retain any binding activity, possibly because arginine or lysine in the flg22 peptide was involved in the coupling to the column, or because the coupling prevented correct accessibility of the peptide for the flagellin binding site by steric hindrance. In order to prevent these difficulties, instead of flg22, the whole flagellin protein of *Pseudomonas syringae* pv. *tabaci* was purified and coupled to the Affi 15 gel support. This flagellin protein column absorbed most of the binding activity when solubilized fraction was loaded. Determination of the elution efficiency from ligand affinity columns is not simple, since elution requires breakage of specific binding, e.g. by an excess of ligand, high

pH or salt concentrations. We solved this problem by absorbing the binding sites to Con A agarose beads after elution and removing the elution reagents by repeated washing. Subsequently, binding activity could be determined. Elution of binding sites was found to be successful at pH 8, high salt concentrations (200-1000 mM NaCl), and 0.1 M flg22. Specific elution with flg22 peptide is an advantage over nonspecific elution with high pH or salt concentrations, since it represents a second purification step. The purification factor (binding activity / total protein) could not be determined, since flg22-peptide used for elution influences the determination of the protein content. Nevertheless, the concentration of the binding site could be determined: Namely, the original solubilized fraction, loaded on the affinity column, contained in one experiment (Fig. I/13) 10,6300 cpm/ 100  $\mu$ l specific binding. In the second elution fraction (Fig. I/13), we measured 130,000 cpm specific binding in



**Fig I/13: Flagellin affinity chromatography of the flagellin binding site.** Forty ml solubilized binding sites with 4,252,000 cpm total binding were loaded on a precolumn (0.4 ml ethanolamine-treated Affi 15 gel) and a main flagellin column (0.4 ml Affi-15 with 2.7 mg flagellin protein). The loading took 7 hours (Pump velocity: 0.25) at 4°C. 1,692,600 cpm were found in the flow through. The column was washed for 1 hour with 1% Triton X-100 in binding buffer (Pump Velocity: 1.0) at 4°C. Washing fraction volume: 0.5 ml. The binding sites were eluted with 4 ml of 0.1 M flg22 in 1% Triton X-100 in binding buffer within 1 hour 40 min at room temperature (Pump velocity: 0.1). Elution fraction volume: 167  $\mu$ l. The binding sites of 10  $\mu$ l of each fraction were precipitated by Con A-agarose beads and washed to remove flg22. The precipitated binding sites were used for binding activity determination. 653055 cpm were eluted in fraction 7-10. Dark labelled fractions were collected, methanol-chloroform precipitated and applied on an SDS-PAGE (see Fig. I/14).

100  $\mu$ l. Therefore, flagellin affinity chromatography achieved a maximum of 12-fold concentration. SDS-PAGE of methanol-chloroform precipitated eluates revealed (Fig. I/14), that a high number of contaminating proteins was co-purified, creating a strong background, though several distinct bands were visible. These bands were cut out and analyzed by MALDI-TOF mass spectrometry by Dr. Daniel Hess (Friedrich Miescher Institute). The band at 120 kDa (Fig. I/14, Nr. 1) was the main candidate for the flagellin binding site, since chemical crosslinking labeled a polypeptide at this molecular weight (see I.3.1.7.). Unfortunately, this band could not be identified. The identified proteins further down were as follows: heat-shock protein (Nr. 5), luminal binding protein (Nr. 6), HSP70 (Nr. 8), calnexin (Nr. 9), ATPase b-subunit (Nr. 10), and BSA (Nr. 10, 11 and 12). The carrier protein BSA

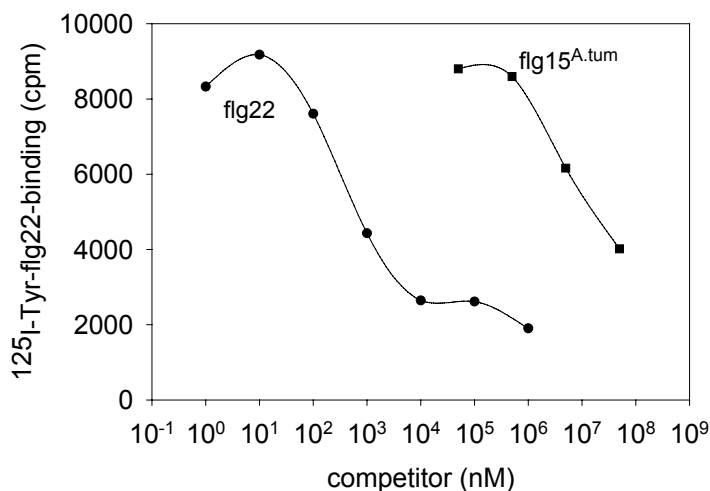


**Fig. I/14: SDS-gel of eluates of flagellin affinity chromatography.** Collected fractions from flagellin-affinity chromatography (previous figure) were applied on 8 % SDS-gel and stained with colloidal coomassie blue. A: wash step (fraction 3); B: elution with flg22 with high binding activity (collected fractions 7-10); C: late elution with flg22 with no binding activity (collected fractions 23-26). Selected bands were cut out and analyzed by MALDI-TOF MS by Dr. Daniel Hess: 1-4: not identified; 5: heat-shock protein; 6: luminal binding protein; 7: not identified; 8: HSP 70; 9: Calnexin (lectin chaperones in ER); 10: ATPase b-subunit / BSA; 11: BSA; 12: BSA. Bands in the lower Mw range were excluded from further investigation, since the binding site was expected in the high Mw range.

was present at two sizes: ~50 kDa and ~100 kDa. The 100 kDa band might represent a dimeric form of BSA. The presence of BSA was a surprise until we realized, that the flg22 peptide used for elution was dissolved in 0.01 % BSA and 0.01 M NaCl. The proteins co-purified by flagellin affinity chromatography might not be connected to flagellin perception, they rather are members of highly abundant, nonspecifically bound proteins. The presence of BSA might be an explanation for the fact that so many proteins were eluted.

In summary, flagellin affinity chromatography is a powerful method for partial purification of the flagellin binding site. However, due to the high background and low quantity of purified binding sites in these experiments, up to date this method has not led to the identification of the flagellin binding site.

To assess binding characteristics of partly purified binding sites, eluted with high salt concentrations, competition binding studies were performed with flg22 and flg15<sup>A.tum</sup> (Fig I/15). In this experiment IC<sub>50</sub> for flg22 was one order of magnitude higher than that found on intact cells and microsomal fractions. Nevertheless, the preference of these partly purified binding sites for flg22 over flg15<sup>A.tum</sup> was retained.



**Fig. I/15: Competition binding assays with affinity purified flagellin binding sites.** Triton X-100 solubilized flagellin binding sites were loaded on an Affi 15-flagellin protein column and eluted with 1 M NaCl pH 8.5. The eluted binding sites were precipitated by aliquots of Con A-sepharose beads and washed several times with 1% Triton X-100 in binding buffer to remove elution buffer and used for binding assays.



## **I.4. Discussion**

### **I.4.1. The high-affinity binding site in *A. thaliana* exhibits characteristics expected for a functional flagellin receptor**

The binding site for flagellin studied in this report shows characteristics expected for a receptor with respect to affinity (I.3.1.3.) and specificity for flagellin-derived ligands with activity as agonist or antagonist of elicitor responses (I.3.1.4.). For all flagellin-derived peptides tested, the apparent affinity for the binding site in competition assays correlated with their relative ability to induce or inhibit flagellin-dependent responses in *A. thaliana* cells (I.3.1.4.). Binding was saturable and exhibited high-affinity with an apparent  $K_d$  of  $\sim 1.3$  nM (I.3.1.3.). As previously observed in tomato (Meindl et al, 2000), occupancy of the binding site and the strength of the alkalinization response do not show a linear, one-to-one, relationship (I.3.1.4.). At least for the alkalinization response, used to quantify elicitor activity, a full response appears to be triggered when only a small percentage of binding sites are occupied. The presence of “spare receptors” is characteristic for many receptor mediated signaling processes in animals and has been observed before for perception of chitin fragments in tomato cells (Baureithel et al, 1994). The presence of a relatively high number of functional receptor sites (several ten thousands per cell; I.3.1.3.) might also explain the extreme sensitivity of flagellin perception on one hand and the high molar excess of antagonist over agonist required for complete inhibition of responses.

### **I.4.2. Localization of the binding site**

In contrast to viruses that can spread symplastically, bacteria invading plant tissues usually remain outside the cells. *Rhizobium* spec., that is taken up by the plant cell, represents an exception. Therefore, it is essential to perceive the presence of bacteria on the cell surface, outside the plasma membrane. On the other hand, the signal has to be conducted into the cytoplasm through the plasma membrane. Many elicitor binding sites have been localized to the cell surface or the plasma membrane of the cells (Nürnberg et al, 1994; Baureithel et al, 1994; Cheong et al, 1993; Kooman-Gersmann et al, 1996; Shibuya et al, 1996; Bourque et al, 1999). Exceptions to this

localization have been described for bacterial elicitors and avr-products known to be injected into the plant cytoplasm by export systems of bacterial pathogens (Alfano and Collmer, 1996; Van den Ackerveken and Bonas, 1997). For two of these elicitors, AvrPto (Tang et al, 1996) and syringolide (Ji et al, 1998), soluble, cytoplasmic proteins acting as receptors have been identified. Up to date, the only membrane associated receptor for which successful biochemical purification and cloning has been described is the binding site for the fungal glucan elicitor (Umemoto et al, 1997; Mithöfer et al, 2000). Interestingly, the functional form of the binding protein appeared associated with the plasma membrane but much of the protein, as demonstrated immunologically, appeared to reside in the soluble fraction in an apparently non-functional form (Mithöfer et al, 2000). We expected the binding site for flagellin to be exposed at the cell surface since neither intact flagellin nor peptides like flg22 have apparent characteristics of membrane permeable molecules. Consistent with an exposure of the binding sites to the cell surface, we observed the same number of sites in assays with intact cells and cell homogenates, prepared from these cells (I.3.1.5.). Additionally, solubilized binding sites strongly bound to the lectin Concanavalin A and could be partially eluted from it by  $\alpha$ -D-mannopyranoside (I.3.2.4.). This suggests that the binding site contains a glycoprotein which usually occur on the extracellular cell surface. Binding studies with supposedly membrane-associated binding sites usually neglect binding sites remaining in rapidly sedimenting cell debris (P1). In this work we found, that 70-95% of total binding sites remained associated with P1 in all cell homogenates tested (I.3.1.5.). Microscopical inspection indicated that this was not due to incomplete breaking of cells in the Parr bomb (Data not shown). Nevertheless, a 3 to 5-fold enrichment of binding activity per mg of protein was observed for the microsomal fraction compared to P1 (I.3.1.5.), which indicates membrane localization of the binding site. In the literature membrane proteins are divided into three classes: the integral membrane proteins, the proteins associated by means of glycosylphosphatidylinositol anchor (GPI), such as NDR1 of *Arabidopsis*, that is required for the activity of several CC/NBS/LRR resistance genes (Repetti P, Coppinger JP, Dahlbeck D, Staskawicz BJ, 2001 meeting report), or peripheric membrane proteins associated by noncovalent interactions with other membrane proteins. Many peripheric membrane proteins can be released by exposures to solutions of very high or low ionic strength or extreme pH. Treating the microsomes

with 0.5 M NaCl did not release the binding site into solution (I.3.2.1), excluding peripheral ionic association of the binding site with the membrane fraction. 1-5 % of the total binding activity could be solubilized by detergents such as Triton X-100 or octylglycoside, thus supporting membrane-association (transmembrane or lipid-anchored) of the binding site. 95-99 % of the binding sites remained unsolubilized but intact (I.3.2.1.).

A connection to the cell wall would be a plausible explanation for remaining of the binding site in P1 and the nonsolubilized pellet. There are about 20 wall-associated receptor kinases (WAKs), that have a wall-associated domain, likely to be involved in ligand recognition, and a cytoplasmic serine/threonine kinase domain. (Kohorn, 2001; He et al, 1999; Lally et al, 2001; Wagner and Kohorn, 2001; Park et al, 2001). The association with the wall is very strong, as release from the cell wall requires enzymatic digestion of the carbohydrate or boiling in detergent and reductant (He et al, 1996). Under conditions that collapse the turgor of a plant cell so as to separate the membrane from the wall (plasmolysis), the WAK wall association is so strong that they remain in the cell wall, presumably in lipid vesicles. In this work, partial purification of the cell wall of *A. thaliana* suspension cultured cells showed reduced binding activity compared to crude cell homogenates, and separating the plasma membrane from the cell wall by plasmolysis did not interfere with proper flg22 recognition, demonstrated by medium alkalization assays (I.3.1.5.). These data speak against a cell wall connection of the binding site. Therefore, the question of the exact flagellin binding site localization has not completely been answered yet.

### **I.4.3. Comparison of flagellin perception in tomato and *A. thaliana***

Perception of flagellin in tomato and in *A. thaliana* shows clear overall similarity but exhibits characteristic differences in detail. Perception in both species occurs with specificity for the same conserved domain of the flagellin molecule (Felix et al, 1999; Meindl, et al, 2000, I.3.1.4.). However, *A. thaliana* exhibited preference for peptides spanning a somewhat larger domain than tomato. For the N-terminal side this is exemplified by the larger differences in activities for flg22 and flg15 in *A. thaliana* compared to tomato. For the C-terminal side, this is most striking for

peptides that lack the two amino acid residues present at the C-terminal end (flg15- $\Delta$ 2 and flg22- $\Delta$ 2). Whereas these peptides retained nearly full elicitor activity in tomato they proved completely inactive as elicitors in *A. thaliana* (I.3.1.4.). In tomato, abrupt loss of elicitor activity occurred for peptides lacking four amino acid residues (flg15- $\Delta$ 4, Felix et al, 1999). Interestingly, in both species these C-terminally truncated peptides that lack agonist activity were found to act as competitive inhibitors or antagonists of flagellin responses, suggesting a common mechanism of signal perception. Thus, as proposed for tomato (Meindl et al, 2000), activation of flagellin receptor in *A. thaliana* appears to occur as a two step process according to the address-message concept with the N-terminal part required for binding (address) and the C-terminal part for activation (message). Interestingly, this address-message concept was also found for the systemin binding site of tomato (Meindl et al, 1998).

Cultured cells of tomato and *A. thaliana* showed similar number of binding sites (1 to 3 pmol per g fresh weight) and affinity for flg22 ( $K_d$  values or half saturation at 1 to 3 nM)(Meindl et al, 2000; I.3.1.3.). Affinity-crosslinking specifically labeled proteins with similar molecular masses of 115 kDa in tomato and 120 kDa in *A. thaliana* (Meindl et al, 2000; I.3.1.7.). Binding to intact cells of both species appeared non-reversible (Meindl et al, 2000; I.3.1.2.). In tomato, non-reversibility of interaction persisted in membrane preparations (Meindl et al, 2000), excluding internalization as explanation for this phenomenon. Rather, it has been hypothesized that the two-step mechanism discussed above involves binding as a first step (reversible) and a process of intra- or inter-molecular isomerization leading to “locking” of the ligand as a second step (Meindl et al, 2000). In *A. thaliana*, interaction of radioligand with binding sites in membrane preparations and the fraction P1 was partially (I.3.1.2.), and in solubilized membrane preparations even fully reversible (I.3.2.2.). With respect to the two step mechanism proposed for receptor activation and the multi-component character of the functional receptor discussed below, one can speculate that this change in reversibility of binding could be due to disassembly of the receptor complex during cell disruption. Indeed, in assays with cells killed by freezing and thawing or in cell homogenates prepared by disruption with a polytron blender, reversibility of binding varied between the one of intact cells and the one of microsomal membranes. Clearly, further experiments

aimed at the purification and identification of the elements involved in flagellin perception are required to clear this point.

### **I.4.4. Partial purification of the binding site**

Purification of the flagellin binding site was started inspite of very low efficacy of solubilization (I.3.2.1.). It is possible, that only a supgroup of the binding site becomes solubilized that differs in certain characteristics from the major fraction of the binding site. For example, the major fraction might covalently be attached to the cell wall or be part of a big membrane complex that is insoluble by detergents. Whatever reason caused insolubility of the binding site by detergents, it might be the same reason as the one that prevented the binding site to be associated with the microsomal fraction (I.3.1.5.). Since we did not find the experimental conditions that had improved solubilization, we had no other choice then to proceed with this low efficacy. Higher volumes of the solubilized fraction were expected to help in obtaining sufficient amount of binding site.

We tested several chromatography methods for their suitability for purification of the binding site. Fath (1995) purified the glycopeptide receptor by anion exchange chromatography with an purification factor of 67. However, for the *A. thaliana* flagellin binding site this method was found not useful for purification, since the binding site was eluted in two broad peaks (I.3.2.3.), as it was already observed for the tomato flagellin binding site (Meindl, 2000). This finding might have a biological relevance, since it might indicate, that there are two distinct classes of the binding site. For example, there might be a difference in the glycosylation.

Another purification method we tested was the Con A affinity chromatography, which had been successfully applied for several receptor purifications before. As a paradigm, the insuline-receptor was purified in two different laboratories using lectin-chromatography (Jacobs et al, 1977; Harrison und Itin, 1980). However, the flagellin binding site bound very strongly to the Con A column, and its elution was inefficient under all tested conditions (I.3.2.4.). This feature excluded Con A chromatography as a purification method for our purpose. Also this finding has a biological relevance: It confirms, that the flagellin binding site is a glycosylated protein. Furthermore, strong binding of the binding site to Con A-sepharose beads

could be exploited for concentration of the solubilized binding site (I.3.2.2.;II.3.3.6.). Additionally, Con A absorption of the binding site was used to separate it from the elution reagents used for ligand affinity chromatography (I.3.2.5.). That way, determination of the binding activity was possible.

The most promising purification method was the ligand affinity chromatography performed with flagellin protein. Mithöfer et al purified the  $\beta$ -glucan elicitor-binding protein in a one-step affinity chromatography about 9000 fold. Single step purification by affinity chromatography was also accomplished for the binding protein of the syringolide elicitor (Ji et al, 1998). Also the receptor for phytosulfokine was purified with ligand-based affinity chromatography (Matsubayashi et al, 2002). In this work, flagellin protein turned out to be an optimal ligand for affinity chromatography. Binding sites were fully absorbed by the column, when applied slowly. Efficient elution could be performed with high pH or high salt concentrations, that were found to disrupt binding previously (I.3.1.1.). Specific elution could be performed with high concentrations of flg22 peptide. However, this fraction still contained many contaminating proteins but relatively little binding site (I.3.2.5.). Thus, up to date this method did not lead to the identification of the flagellin binding site. Still, flagellin affinity chromatography is potentially a very powerful method for purification of the binding site, that could be improved to success in future. The purification factor could not be determined, since the binding site was eluted with high concentrations of a peptide, and the determination of the purification factor is based on the comparison of the protein content in the crude and the purified fractions.

Crosslinking with the affinity-purified binding sites would also have been interesting in order to see if the same specific band at ~120 kDa is labeled in this fraction as found for P1-microsomes and solubilized fractions. Unfortunately, this question could not be answered due to the following reason: In order to remove elution buffer, the affinity purified binding sites had to be collected and washed by precipitation of Con A beads. However, Con A beads, as described before, prevented successful crosslinking when added prior to binding (I.3.2.2.).

In conclusion, purification attempts of the flagellin binding site did not lead to homogenous fractions but resulted in an enrichment of the binding site and revealed interesting features.

## **I.4.5. Suggestions for future experiments**

### *I.4.5.1. Crosslinking*

Chemical crosslinking of  $^{125}\text{I}$ -Tyr-flg22 to microsomal membranes and P1 has specifically labeled a 120 kDa band in many independent experiments (I.3.1.7.). Interestingly, when these crosslinking experiments were repeated and further developed by Martin Regenass with intact cells, several specifically crosslinked bands were observed (Preliminary data, not shown). In *A. thaliana* there were about six bands specifically labeled via EGS, Sulfo-EGS, BS<sup>3</sup> and ANB-NOS with two major bands at  $\geq 160$  and 120 kDa and four smaller bands. It would be interesting to find out, which experimental condition was the reason for this different result. One intriguing possibility is that the additional bands occur only on intact cells but not in extracts and that they represent members of a bigger receptor complex that is disrupted by cell-extraction.

A general disadvantage of chemical crosslinking experiments is that the pH optimum of flg22-binding and the crosslinking reaction greatly differ. Under conditions adapted for optimal flg22 binding (pH 6), chemical crosslinking, that had a pH-optimum between 7 and 9, had low efficiency. We assessed that only several percentages or promilles of ligand-receptor pairs were crosslinked. In order to increase efficiency of the crosslinking reaction, photo-affinity labeling, as performed for the phytosulfokine receptor (Matsubayashi et al, 2002) or the systemin receptor (Scheer and Ryan, 2002) could be advantageous. In this case, the labeling would not depend on the availability of primary amino groups of the binding site.

### *I.4.5.2. Flagellin affinity chromatography*

Indirect methods in this work, such as characterization of an epitope-tagged FLS2 (II.3.2.), failed to unequivocally clarify if FLS2 was identical to the binding site. A very promising approach to identify the binding site remains its purification using

flagellin affinity chromatography (I.3.2.5.). Although high enrichment of the binding site has been achieved, this approach has been discontinued, since it was somewhat cumbersome and characterization of tagged FLS2, an obvious candidate for the flagellin binding site, seemed faster and more straightforward. Here I would like to specify the problems I encountered during my attempts to purify the binding site via affinity chromatography and their possible solutions.

As already described, flagellin affinity chromatography only worked when whole flagellin protein was crosslinked to the chromatography support gel (I.3.2.5.). Affinity columns with flagellin peptides failed to retain the binding site. Therefore, flagellin protein had to be purified first. However, purification of flagellin was very inefficient, due to poor separation of flagellin from the bacteria by either a blender or a vortex (data not shown). Repeated passing the bacterial suspension through a tight syringe was found to be very effective but also tedious with higher quantities (data not shown). Difficulties of flagellin purification were therefore a major limit for repetitions of the flagellin affinity chromatography. Another difficulty with flagellin affinity chromatography was the high background by nonspecific binding of unrelated proteins (I.3.2.5., [Fig. I/14](#)). In my experiments background was increased by using BSA (0.1 mg/ml final concentration; I.3.2.5.) in the elution solution that was routinely added to any peptide-solution in low concentrations to prevent adherence to the reaction vessel. That would, however, not have been necessary for flg22-concentrations used for elution (100  $\mu$ M). Instead I would suggest to dilute flg22 peptides for elution only in detergent and in binding buffer without BSA. I also would suggest a control I have forgotten: There were several proteins that were abundant in the eluate from flagellin column: it would be interesting to know if they were also abundant in solubilisate concentrated in the same way as the eluate.

Another matter for consideration is the way how to concentrate the eluted proteins for loading on the SDS-gel: Precipitation by Concanavalin A-sepharose beads is theoretically the best method, because it was shown to completely precipitate the binding site and it theoretically achieves a subsequent purification step. However, when Con A beads were boiled and applied on SDS-gel, very high protein background over the whole range of molecular weight was observed, possibly due to



impurities in the Con A preparation (data not shown). On the other hand, I found that elution of the binding site from Con A by other methods than boiling in 1 % SDS is very inefficient (I.3.2.4.). Therefore, instead of ConA-precipitation I used methanol-chloroform precipitation for concentrating proteins in the eluate (I.3.2.5.). In this case, however, there is no experimental evidence, that the binding site indeed was present in the precipitate and not discarded with the aqueous phase. Therefore it would be worthwhile to try several different ways to concentrate the binding site in the eluate (for instance ultrafiltration, as described in Matsubayashi et al, 2002).

Given a molecular weight of 100 kDa as an order of magnitude, there must be 1 pmol binding site loaded in a lane of an SDS-gel to give a band of 100 ng, that is the detection limit for visualization with colloidal coomassie blue staining. If 100.000 cpm represent 0.05 pmol  $^{125}\text{I}$ -Tyr-flg22, as it was indicated on the product specification sheet for  $^{125}\text{I}$ -Tyr-flg22 from Anawa (the company that radioactively labelled Tyr-flg22), then 2,000,000 cpm give 1 pmol. Therefore, at least 2,000,000 cpm specific binding must be loaded on an SDS-gel to provide a visible band. In order to collect sufficient purified binding sites, it will probably be necessary to run multiple flagellin chromatographies. According to my experiences, flagellin columns lose their capacity during the run, probably due to proteolytic digestion, so reloading after elution is hardly worthwhile. Freshly prepared flagellin should be therefore used. As a rule of thumb, 10 to 30 mg available purified flagellin with 1-3 mg flagellin used for one run would be a good start.

In spite of many challenges flagellin affinity purification might be the most effective method to identify the flagellin binding site and, on the top of that, it might identify some further proteins that are closely associated with it. Transgenic *FLS2:myc* cell cultures (II.3.2.) would be practical to work with, since in parallel the co-purification of our major candidate could be monitored.

### *I.4.5.3. Ion-exchange chromatography*

Ion-exchange chromatography exhibited two peaks of the flagellin binding site (I.3.2.3.). Very interestingly, Thomas Meindl found similar elution pattern with two peaks for the tomato binding site (Meindl, 2000). This suggests that the flagellin binding site occurs in two distinct classes. It would be very interesting to

consolidate the investigation of these two classes. It is possible, that they show different binding characteristics such as affinity or specificity, or different molecular weight in crosslinking experiments.

(D<sub>II</sub>) Chapter II: Genetic and biochemical approaches to demonstrate that FLS2 is the flagellin binding site

## II.1. Summary

FLS2 has been shown to be essential for flagellin sensitivity, and its protein structure exhibits the characteristics of a receptor (Gómez-Gómez and Boller, 2000). Therefore, it is the first candidate for the flagellin receptor or an element thereof. To determine the role of FLS2 for flagellin binding, flg22-binding was determined in several flg22-insensitive mutants and ecotypes of *A. thaliana*. One of these mutants, called *fls2-24*, showed a point mutation in the *FLS2* leucine-rich-repeat domain (Gómez-Gómez and Boller, 2000). *Fls2-17* showed a mutation in the *FLS2* kinase domain (Gómez-Gómez and Boller, 2000). The ecotype Ws-0 and two further mutants *fls1-2* and *fls1-19* were believed to be altered in *fls1*, a locus distinct from *fls2* (Gómez-Gómez et al, 1999; Gómez-Gómez and Boller, 2000), but later results (see next paragraph) revealed that these insensitive plants were actually altered in the FLS2 kinase domain as well. We found, that all identified flagellin insensitive mutants and ecotypes were strongly impaired in flg22-binding. Nine other flg22-sensitive ecotypes showed clear flg22-binding. The tight correlation between the presence of the binding site and elicitor response provides strong evidence that this binding site acts as the physiological receptor. Furthermore, we conclude, that FLS2 plays a pivotal role for flg22 binding, and that both the extracellular leucine-rich-repeat domain and the cytoplasmic kinase domain are required for it.

To further investigate the role of FLS2 for flagellin perception, a c-myc-tagged version was introduced in tomato and *A. thaliana*. *FLS2:myc* restored flagellin binding and responsiveness not only in *fls2-24*, but also in Ws-0 and *fls1-19*. Sequencing revealed that all presumed *fls1* mutants actually were mutated in *FLS2* (Silke Robatzek, unpublished results).

Immunoblots revealed an apparent molecular weight of FLS2:myc of approx. 175 kDa. Chemical deglycosylation reduced the apparent molecular weight of FLS2:myc to 135 kDa, suggesting that it is glycosylated. Differential centrifugation of homogenates obtained from *FLS2:myc*-transgenic suspension cells suggested, that FLS2:myc is membrane associated. The characteristics of FLS2:myc were compared to that of the flagellin binding site. Immunoprecipitated FLS2:myc showed no flagellin binding, and Con A-sepharose bead-precipitation of the binding site out of solubilized homogenates,

did not show co-precipitation of FLS2:myc. Enrichment of the binding site by flagellin affinity chromatography out of *FLS2:myc*-transformed tomato cells was not paralleled by the enrichment of FLS2:myc. However, none of these results disprove definitively that FLS2 is the flagellin binding site.

Specificity of *FLS2:myc* transgenic tomato cell cultures for flagellin-derived peptides carried characteristic traits of *A. thaliana* binding and elicitor-response. This finding suggests that FLS2 determines specificity of flagellin perception. Another interesting aspect of this finding is that FLS2 seems compatible with tomato signal transduction components.

Part of the results presented in this chapter have been published in Bauer et al 2001 and Gómez-Gómez et al, 2001. The majority of the results presented here are unpublished.

## II.2. Introduction

The *FLS2* gene, encoding a leucine-rich-repeat receptor like kinase, is the only gene identified to date that is indispensable for flagellin perception and signaling (Gómez-Gómez et al, 2000). Moreover, it carries all the characteristics expected for a receptor: the extracellular leucine-rich-repeat is a putative ligand-binding domain (Kobe and Deisenhofer, 1994) and the putative serine/threonine kinase domain in the cytoplasmic region (Gómez-Gómez et al, 2000) could be involved in signal transmission. The putative plasma membrane localization of FLS2 (Gómez-Gómez et al, 2000), deduced from the presence of a putative transmembrane domain and glycosylation sites on the LRR-domain, is ideal for detection of bacterial invaders in the intercellular space and transmission of the signal into the cell inside. Accordingly, LRR domains of resistance gene products are hypothesized to create fast adapting recognition surfaces in order to survey molecules for rapidly evolving pathogens (Jones and Jones, 1996). By analysis of *in vivo* and *in vitro*-generated recombinants between different flax L alleles, Ellis and colleagues confirmed experimentally that the LRRs constitute the principal determinant of specificity for Avr products (Ellis et al, 1999). Also, the fact that other RLKs, such as BRI1, CLV1 and the phytosulfokine receptor were demonstrated to act as receptors in hormone signaling by ligand binding supports by analogy a role of FLS2 in flagellin perception (Wang et al, 2001, Trotochaud et al, 2000, Matsubayashi et al, 2002). Therefore, FLS2 is an obvious candidate for the flagellin binding site. Still, direct evidence for the role of FLS2 in flagellin binding has to be demonstrated. Detailed characterization of the flagellin binding site (see I.3.1.) provided a basis for analogous studies with FLS2 in order to find out if these two molecules are identical.

In the first part of this Chapter we performed flg22-binding analysis of flg22-insensitive mutant plants in order to see if there is a correlation between impairment of binding and sensitivity. The other purpose of these experiments was to study if FLS2 mutations would disrupt flg22 binding.

In the second part of this chapter we investigated the role of FLS2 by introduction of an epitope tagged version in plants and cell cultures.

## II.3. Results

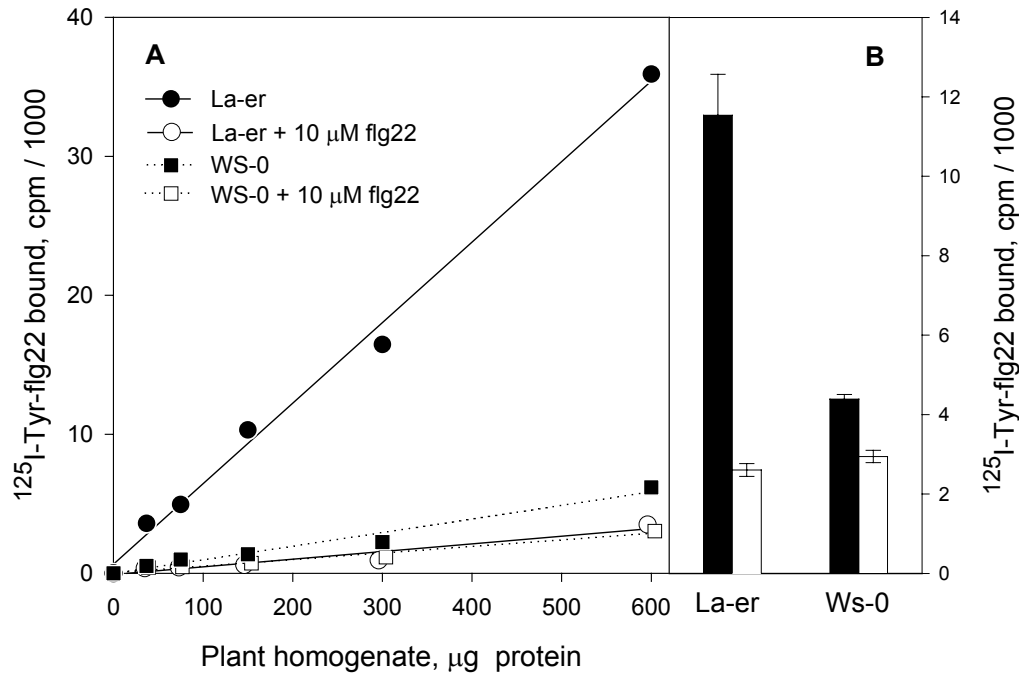
### II.3.1. Impairment in flagellin sensitivity and binding correlate in all *fls2* mutants

#### II.3.1.1. Correlation of binding and response in different ecotypes of *A. thaliana*

For studying flagellin binding sites in tissues of *A. thaliana* plants, we homogenized leaves in binding buffer, and assayed the crude homogenates for binding of <sup>125</sup>I-Tyr-flg22 as described for extracts of cell cultures. Specific binding, defined as the difference between total binding and nonspecific binding, was clearly detectable in homogenates of the flagellin sensitive ecotype *La-er*. Specific binding of radioligand increased linearly with the amount of homogenate applied and homogenates containing 100 to 200 µg protein, corresponding to ~10 mg plant tissue, were sufficient to detect significant binding (Fig. II/ 1A). Thus, the assay was sensitive enough to measure binding activity in homogenates of individual plants.

In contrast to the ecotype *La-er*, seedlings of the ecotype *Ws-0* exhibit no sensitivity to treatment with flagellin. This insensitivity was originally attributed to a single locus termed *FLS1* (Gómez-Gómez, 1999), but later turned out to be caused by a mutation in the *FLS2* kinase domain (see II.3.3.5.). When assayed for flagellin binding, homogenates of *Ws-0* showed greatly reduced specific binding compared to the flagellin sensitive ecotype *La-er* (Fig. II/ 1A). Although the difference between total- and nonspecific binding was close to the detection limit in *Ws-0* (Fig. II/ 1A), a very low specific binding activity was detectable in most repetitions with independent homogenates of *Ws-0* (n>6, data not shown). Mixtures of homogenates from *La-er* and *Ws-0* plants exhibited binding corresponding to the arithmetic of the mixtures, indicating that no soluble factors inhibit or enhance binding in the two homogenates (data not shown).

Reduced binding, as observed in homogenates of *Ws-0*, could indicate a reduced number of binding sites, or it could reflect a reduced affinity of these sites. A reduced affinity could lead to loss of bound radioligand during the washing step used to remove



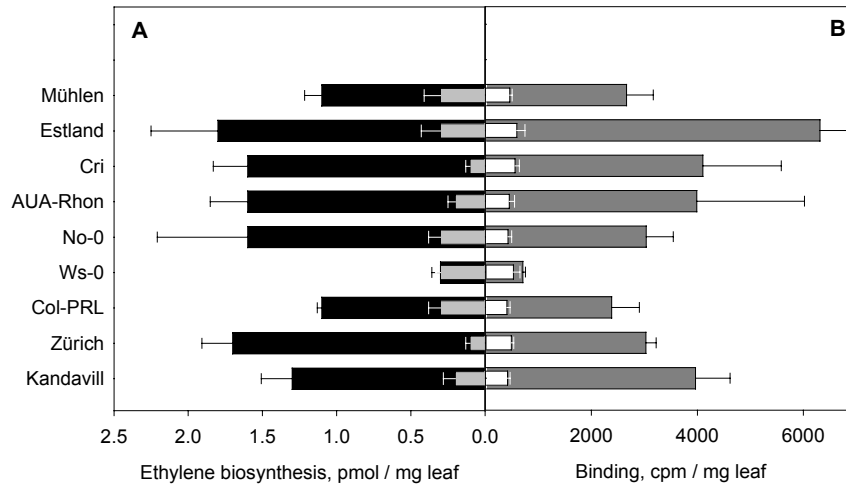
**Fig. II/1. Binding sites for  $^{125}\text{I}$ -Tyr-flg22 in the ecotypes La-er and Ws-0.** A, Different amounts of plant homogenates from soil grown plants of the ecotype La-er and Ws-0 were assayed for binding of  $^{125}\text{I}$ -Tyr-flg22 in the absence (closed symbols) or presence (open symbols) of 10  $\mu\text{M}$  unlabeled flg22. B, Equilibrium binding assays with plant homogenates of the ecotype La-er and Ws-0. Aliquots of homogenates containing 500  $\mu\text{g}$  protein were incubated in the absence (filled bars) or presence of 10  $\mu\text{M}$  unlabeled flg22 (open bars). Binding in the pellet P1 was determined after centrifugation and removal of supernatant. Bars and error bars represent average and standard deviation of experiments with 3 replicates.

unbound ligand in the binding assays. To test this possibility, we performed equilibrium binding assays with separation of bound and unbound ligand by centrifugation (Fig II/1B). Although background in assays with excess unlabeled flg22 was higher than in standard assays with washing on filters, it clearly demonstrated significant specific binding in La-er and a strongly reduced number of binding sites in ecotype Ws-0.

Several additional ecotypes of *A. thaliana* were assayed for the presence of flagellin binding sites and their response to treatment with flg22. In Figure II/2, binding activity in homogenates was compared to the flg22-dependent induction of ethylene biosynthesis in leaf tissues of these ecotypes. With the exception of Ws-0, all ecotypes



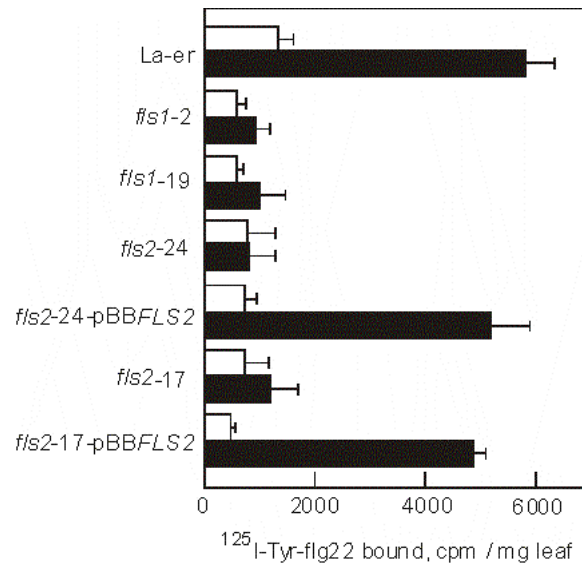
showed clear induction of ethylene biosynthesis and significant specific binding of  $^{125}\text{I}$ -Tyr-flg22.



**Fig. II/2. Correlation of binding activity and flagellin-dependent induction of ethylene biosynthesis in several ecotypes.** A, Ethylene production in leaf tissues of different ecotypes after incubation for 2 h in  $\text{H}_2\text{O}$  (controls, light bars) or  $\text{H}_2\text{O}$  supplemented with  $1\ \mu\text{M}$  flg22 (black bars). Bars and error bars represent mean and standard deviation of experiments with 3 replicates. B, Total binding (shaded bars) and nonspecific binding (open bars) of  $^{125}\text{I}$ -Tyr-flg22 were measured in homogenates of different ecotypes. Bars and error bars represent the mean and standard deviation of three homogenates obtained from three individual plants of every ecotype.

#### II.3.1.2. Flagellin binding sites in flagellin insensitive mutants

We assessed total binding versus non-specific binding in individual plants of wildtype and several mutant lines selected for flagellin insensitivity (Gómez-Gómez et al, 2000). The two mutants *fls2-24* and *fls2-17* carry two different point mutations in the *FLS2* gene (Gómez-Gómez et al, 2000). These mutants exhibited strongly reduced flagellin binding that was fully restored in plants complemented with the wt-*FLS2* gene (Fig. II/3). Two additional mutant lines, previously termed *fls1-2* and *fls1-19*, also exhibited point mutations in *FLS2* causing loss of a major part of the kinase domain at protein level (Silke Robatzek, unpublished results, details see II.3.3.5.). In the original approach, no complementation of these mutants by the wt-*FLS2* gene was found



**Fig. II/3.**  $^{125}\text{I}$ -Tyr-flg22 binding in homogenates of wild type and flagellin-insensitive mutants of the ecotype La-er. Total (black bars) and nonspecific binding (white bars) was measured in homogenates of 7 individual plants for wild type (La-er) and the mutant lines *fls1-2*, *fls1-19*, *fls2-24* and *fls2-17*. *fls2-24-pBBFLS2* and *fls2-17-pBBFLS2* refer to mutant plants complemented with wt-*FLS2* gene. Bars and error bars indicate mean and standard deviation.

(Gómez-Gómez and Boller, 2000; Lourdes Gómez-Gómez unpublished data). These mutants also exhibited strongly reduced total binding and little changes in non-specific binding, such that the specific binding was close to the detection limit (Fig. II/3). Mutant plants *fls2-24*, carrying a point mutation in one of the leucine rich repeats of *FLS2* (Gómez-Gómez and Boller, 2000), exhibited no detectable specific binding in all experiments. In contrast, as observed above for Ws-0, the mutants *fls1-2*, *fls1-19* and *fls2-17* (all affected in the kinase domain) appeared to have a little specific binding (Fig. II/3). From data of several independent repetitions ( $n > 3$ ) with different sets of plants, we estimated that these mutants contained significant but ~6.5 to 10-fold lower binding than La-er wild type plants.

### **II.3.2. Introduction of c-myc tagged *FLS2* into tomato and *A. thaliana* elucidates the role of FLS2 in flagellin perception**

#### *II.3.3.1. Engineering of FLS2:myc and transformation of plants and cell cultures*

Detection of FLS2 protein is crucial for answering the question if FLS2 is identical to the flagellin binding site. Unfortunately, antibodies raised against FLS2, produced in *E.coli*, and peptide antibodies raised against characteristic sequences in both, in the LRR and kinase domain, failed to recognize FLS2 specifically in immunoblots, possibly due to the high glycosylation of FLS2 as deduced from the amino acid sequence (Gómez-Gómez and Boller, 2000) and the existence of numerous highly homologous proteins (Shiu and Bleecker 2001). Therefore, we engineered an epitope-tagged form of FLS2 that could be detected by antibodies against the tag. We chose the 10 amino acid long sequence EQKLISEEDL of the human protooncogene c-myc, since it was a commonly used tag in biological research and commercial antibodies were readily available. Furthermore, less interference of myc-tag with the biological function of proteins was reported in the literature than for certain other tags, such as the His-tag. The triple version of this tag (GEQKLISEEDLN GEQKLISEEDLN GEQKLISEEDLN) was supposed to improve sensitivity for the detection of the chimeric protein with anti-myc antibodies (Piedras et al, 2000). The triple myc tag was fused to the C-terminal end of *FLS2* next to the stop-codon. The correctness of the fragment was verified by sequencing. Fig II/4. shows the nucleic acid sequence and Fig II/5 the amino acid sequence of FLS2:myc. DNA encoding *FLS2:myc* was inserted into the pCAMBIA 2300 P&T Vector between the 35S promoter and terminator (Fig II/6).

Cell cultures of *A. thaliana* and tomato and rice were transformed with 35S:*FLS2:myc* pCAMBIA 2300 by particle bombardment, and different ecotypes and mutants of *A. thaliana* plants were transformed via *Agrobacterium tumefaciens*. We obtained approximately 100 kanamycin-resistant *A. thaliana*, and four kanamycin resistant tomato cell culture calli. Out of them, we selected two *A. thaliana* (At 6 and At 8), and one tomato (Tom2) cell lines that exhibited FLS2:myc expression in immunoblots (see II.3.3.2.). The resistant calli of *A. thaliana* and tomato were converted to cell suspension cultures.

## Chapter II: Results

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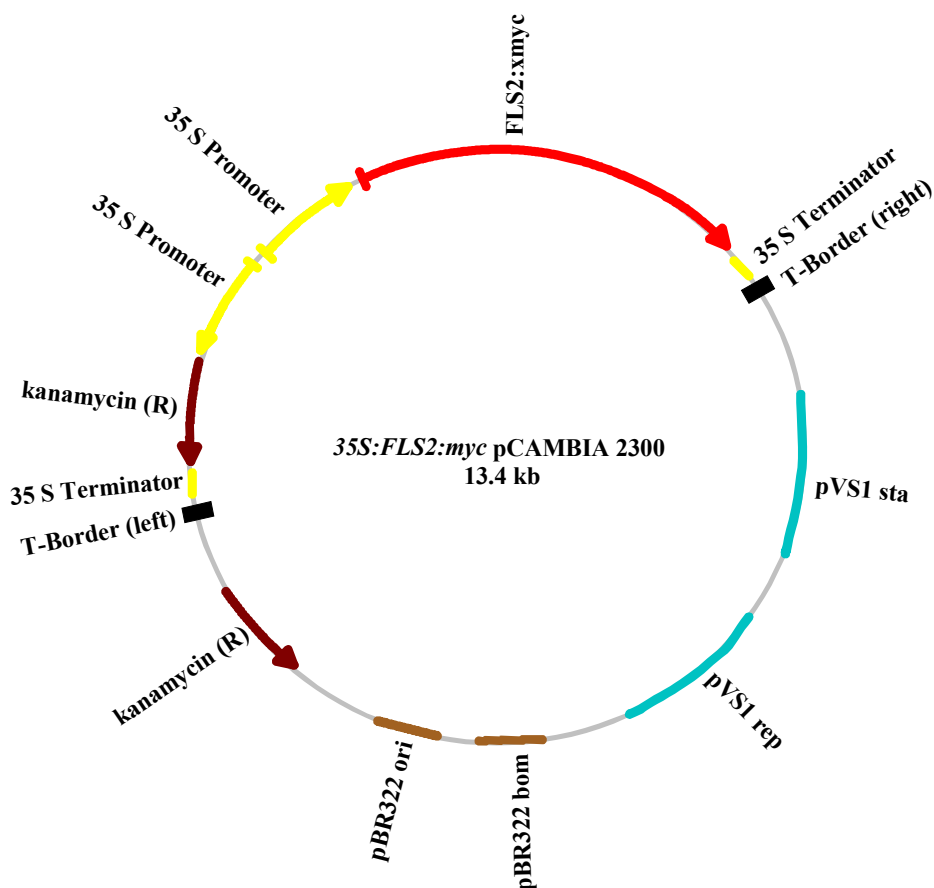
1 ATGAAGTTAC TCTCAAAGAC CTTTTTGATA TTAAGTCTCA CCTTCTTCTT
51 CTTTGGCATT GCACTAGCGA AACAGAGCTT TGAACACAGAG ATCGAAGCTT
101 TGAATCCTT CAAGAATGGT ATTTCCAACG ACCCTTTAGG AGTATTATCA
151 GATTGGACCA TCATCGGTTT GTTACGACAC TGTAATTGGA CCGGAATCAC
201 CTGCGATAGT ACCGGACATG TAGTCTCGGT TTCCTTGCTG GAGAAGCAAC
251 TTGAAGGTGT TCTGTCTCCA GCCATAGCGA ATCTCACCTA TCTCCAGGTT
301 CTTGATCTCA CTTCAAATAG TTTTACCGGC AAAATACCGG CTGAAATAGG
351 AAAGTTAACC GAGCTTAACC AGCTTATTCT GTACCTAAAC TATTTCTCTG
401 GTTCGATTCC TTCTGGAATC TGGGAGCTTA AGAATATTTT CTATCTTGAT
451 CTTAGAAATA ATTTGTTGTC CGGTGATGTT CCTGAGGAAA TCTGCAAAAC
501 CAGTTCTTTG GTATTGATTG GGTTTGATTA CAACAACCTA ACCGGGAAAA
551 TACCAGAATG CTTAGGAGAT TTGGTTCATC TCCAAATGTT TGTAGCAGCT
601 GGTAACCATT TAACCTGGTC GATTCCGGTA TCAATGGGTA CTCTGGCTAA
651 TTTAACCGAT TTAGACCTGA GTGGTAACCA GTTAACCGGA AAAATACCGA
701 GAGATTTTGG AAATCTCTTG AACTTACAGT CTCTCGTTTT AACTGAAAAA
751 TTGTTGGAAG GAGATATACC AGCTGAGATC GGAAACTGCT CGAGCTTTGGT
801 CCAACTTGAG CTTTACGATA ACCAGTTAAC CGGGAATAA CCAGCTGAAT
851 TAGGGAATTT GGTTCAGCTG CAAGCACTCC GGATATACAA GAACAAACTT
901 ACTTCTTCAA TTCCATCTTC ATTGTTCCGG TTAAGTCTAGT TAACCCATTT
951 GGGGTTATCA GAAAACCATT TGGTTGGACC GATATCAGAA GAAATCGGTT
1001 TTCTTGAGTC ACTTGAAGTC CTCACACTTC ATTCCAACAA CTTACAGGTA
1051 GAGTTTCCAC AGTCCATCAC AAAGTTGAGG AACTTGACAG TCCTAACGGT
1101 GGGGTTCAAT AATATTTCCG GTGAGCTCCC GCGGATCTA GGGCTTCTTA
1151 CAAACCTTCG GAACCTTTCA GCGCACGACA ATCTTCTTAC CGGACCAATA
1201 CCTTCCAGCA TAAGTAACTG CACCGGTCTT AAAGTCTCTG ACCTGTCTCA
1251 CAACCAATG ACTGGCGAGA TCCCGCGGGG TTTGGAAGG ATGAATCTTA
1301 CGTTCATTTT TATTGGGAGG AATCATTTCA CCGGTGAAAT TCCAGATGAT
1351 ATCTTCAACT GTTCAAACCTT GGAAGTCTT AGTGTGGCAG ATAACAACTT
1401 AACAGGAAGT CTCAAGCCAT TAATTGGGAA GCTTCAAAAA CTCAGGATTT
1451 TGCAAGTTTC ATATAACTCT CTCACTGGAC CGATTCTCTG AGAAATCGGG
1501 AATCTGAAAG ATTTGAATAT CTTGTACCTT CACTCTAATG GTTTCACAGG
1551 GAGAATCCCG AGAGAGATGT CGAATCTCAC TCTCTCTCAG GGGCTAAGGA
1601 TGTATTCAAA TGATCTTGAA GGTCCAATTC CTGAAGAAAT GTTTGATATG
1651 AAGCTACTCT CAGTTCTTGA TCTTTCCAAC AACAAATCTT CAGGTCAAAT
1701 TCCTGCCTTG TTCTCCAAGC TTGAATCGCT TACCTACTTG AGTCTTCAAG
1751 GAAACAAATT CAACGGGTCT ATCCCTGCAA GCCTTAAGTC GCTTTCGCTT
1801 CTCAACACAT TCGATATCTC CGACAATCTT CTCACTGGAA CCATCCCTGG
1851 AGAGCTGTGA GCTTCTTTGA AAAACATGCA GCTTTACCTC AACTTCTCAA
1901 ACAACTTGTT GACTGGAACC ATCCCAAAGG AGCTTGGAAA GCTTGAATG
1951 GTTCAAGAAA TCGACCTTTC AAACAATCTC TTTTCTGGGT CTATTCCAAG
2001 ATCTTTACAG GCCTGCAAAA ATGTGTTTCA ACTGGATTTT TCGCAGAAAC
2051 ATCTCTCGGG TCATATACCA GATGAAGTCT TCCAAGGCAT GGATATGATC
2101 ATAAGCTTGA ACCTTTCAAG GAACAGTTTC TCTGGAGAAA TCCCTCAGAG
2151 CTTGCGGAAC ATGACGCATT TGGTCTCCTT GGATCTCTCT AGTAACAATC
2201 TCACTGGTGA AATTCCAGAG AGTCTCGCCA ATCTTTCGAC TCTGAACAT
2251 CTCAAAC TAGTCTTCAA CCTCAAAGGC CATGTTCTCTG AATCCGGGGT
2301 GTTCAAAAAC ATCAACGCCT CTGATCTAAT GGGAAACACA GATCTCTGTG
2351 GTAGCAAGAA GCCTCTCAAG CCATGTACGA TCAAGCAGAA GTCCAGCCAC
2401 TTCTCGAAGA GAACAGAGT CATCTGATG ATTCTTGAT TCTGAGGAA
2451 TCTTCTTCTT GTCCTGCTTC TTGTTCTGAT TCTAACCTGT TGCAAGAAAA
2501 AAGAAAAAAA GATTGAAAAAT TCATCAGAGT CCTCATTACC GGATTTGGAT
2551 TCAGCTCTGA AACTGAAGAG ATTTGAACCA AAAGAGTTGG AGCAAGCAAC
2601 AGATTCAATC AACAGTGCCA ACATCATTGG CTCAAGCAGC TTAAGCAGAG
2651 TGTACAAAGG TCAGCTAGAA GATGGGACAG TGATTGCAGT AAAAGTATTG
2701 AATCTAAAGG AATTCTCTGC AGAATCAGAC AAGTGGTTCT ACACAGAAGC
2751 TAAAAACATTG AGCCAACTAA AACATCGAAA CCTGGTGAAG ATCTTAGGGT
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2851 AATGGAAGT TGGAGGACAC CATTACGGC TCTGCAGCAC CGATTGGGTC
2901 GCTTTTAGAA AAAATCGATC TTTGTGTTCA TATCGCAAGC GGAATCGATT
2951 ATCTTCATTC TGGATATGGT TTTCCCATCG TTCATTGTGA TCTGAAGCCA
3001 GCTAATATAC TCCTTGACAG TGACCGCGTT GCTCACGTAA GCGATTTTGG
3051 AACTGCTCGG ATTCTAGGTT TCCGCGAAGA TGGAAGCACC ACAGCTTCAA
3101 CATCAGCCTT CGAGGGTACA ATTGGATACT TAGCTCCAGG TAAATTACTC
3151 ACAACACTTA ATTTTCAAAA CCTTAATTAT GCCATCGACT TACTTGAAAT
3201 ACACTCTTAT TTTTCATGAA CAGAGTTTGC TTATATGAGG AAAGTGACAA
3251 CAAAAGCCGA TGTATTGAGC TTCGGGATCA TAATGATGGA GCTGATGACG
3301 AAACAGAGAC CAAGTTCTGT GAATGATGAA GATTACAAAG ACATGACTTT
3351 GCGCCAATTG GTGGAGAAAT CGATTGGAAG TGGAAGAAAA GGGATGGTTA
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3551 TTAGAGGCAA AGCGAATTCA TTTGAGAGAG ATCGTAACGA GGATCGAGAG
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3651 ACAAAGGCTA ATCTCCGAGG AAGACTTGAA CGGTGAACAA AAATTAATCT
3701 CAGAAGAAGA CTTGAACGGA TAG

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**Fig II/4: Nucleic acid sequence of *FLS2:myc*.** The intron is highlighted in green, the 3xmyc-tag in red. The correctness of the sequence was confirmed by sequencing.

1	MKLLSKTFLI	LTLTFFFFGI	ALAKQSFEPE	IEALKSFKNG	ISNDPLGVLS
51	DWTIIGSLRH	CNWTGITCDS	TGHVVSVSLL	EKQLEGVLSP	AIANLTYLQV
101	LDLTSNSFTG	KIPAEIGKLT	ELNQLILYLN	YFSGSIPSGI	WELKNIFYLD
151	LRNNLLSGDV	PEEICKTSSL	VLIGFDYNNL	TGKIPECLGD	LVHLQMFVAA
201	GNHLTGSIPV	SIGTLANLTD	LDLSGNQLTG	KIPRDFGNLL	NLQSLVLTEN
251	LLEGDIPAEI	GNCSSLVQLE	LYDNQLTGKI	PAELGNLVQL	QALRIYKNKL
301	TSSIPSSLFR	LTQLTHLGLS	ENHLVGPISE	EIGFLESLEV	LTLHSNNFTG
351	EFQSQITNLR	NLTVLTVGFN	NISGELPADL	GLLTNLRNLS	AHDNLLTGPI
401	PSSISNCTGL	KLLDLSHNQM	TGEIPRGFGR	MNLTFISIGR	NHFTGEIPDD
451	IFNCSNLETL	SVADNNLTGT	LKPLIGKLQK	LRILQVSYNS	LTGPPIPREIG
501	NLKDLNILYL	HSNGFTGRIP	REMSNLTLLQ	GLRMYSDNLE	GPIPEEMFDM
551	KLLSVLDLSN	NKFSGQIPAL	FSKLESLEYL	SLQGNKFNGS	IPASLKSLSL
601	LNTFDISDNL	LTGTIPGELL	ASLKNMQLYL	NFSNNLLTGT	IPKELGKLEM
651	VQEIDLSNNL	FSGSIPRSLQ	ACKNVFTLDF	SQNNLSGHIP	DEVFQGMDMI
701	ISLNLSRNSF	SGEIPQSFGN	MTHLVSLDLS	SNNLTGEIPE	SLANLSTLKH
751	LKLASNNLKG	HVPESGVFKN	INASDLMGNT	DLCGSKKPLK	PCTIKQKSSH
801	FSKRTRVILI	ILGSAAALLL	VLLLVLILTC	CKKKEKKIEN	SSESLPDL
851	SALKLKRFEF	KELEQATDSF	NSANIIGSSS	LSTVYKGQLE	DGTVIAVKVL
901	NLKEFSAESD	KWFYTEAKTL	SQLKHRNLVK	ILGFAWESGK	TKALVLPFME
951	NGNLEDTIHG	SAAPIGSLLE	KIDLCVHIAS	GIDYLSHGYG	FPIVHCDLKP
1001	ANILLSDRV	AHVSDFGTAR	ILGFREDGST	TASTSAFEGT	IGYLAPEFAY
1051	MRKVTTKADV	FSFGIIMMEL	MTKQRPTSLN	DEDSQDMTLR	QLVEKSIENG
1101	RKGMVRVLDL	ELGDSIVSLK	QEEAIEDFLK	LCLFCTSSRP	EDRPDMNEIL
1151	THLMKLRGKA	NSFREDRNE	REVHMGEQKL	ISEEDLNGEQ	KLISEEDLNG
1201	EQKLISEEDL	NG*			

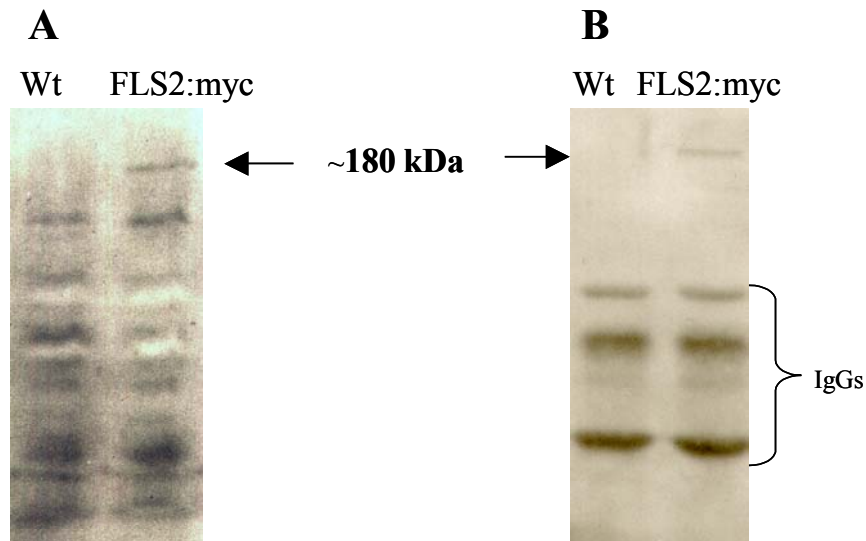
**Fig II/5: Amino acid sequence of FLS2:myc.** The LRR domain is highlighted in yellow, the kinase domain is highlighted in green and the triple myc-tag highlighted in red.



**Fig. II/6: Vector used for transformation of *A. thaliana* plants, and *A. Thaliana* and tomato cell cultures with 35S:FLS2:myc.**

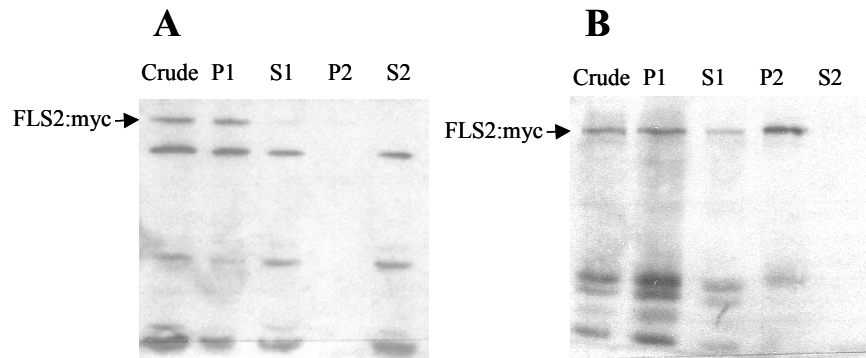
*II.3.3.2. Immunoblots of FLS2:myc transformed cell cultures reveal a band of an apparent molecular weight of ~175 kDa.*

Crude homogenates of a mixture of 20 FLS2:myc transformed kanamycin resistant *A. thaliana* and tomato cell cultures and control lines of both species were subjected to immunoblot with anti-myc antibodies. A cross-reacting band was detected in transformed plants that was not observed in the non-transformed control homogenate (Fig. II/ 7A). Comparison with pre-stained molecular weight markers showed that this specific band represented a 180 kDa protein. This result was valid for both species, *A. thaliana* and tomato, and was confirmed by both, 9E10 monoclonal and polyclonal anti-myc antibodies. Immunoprecipitation by these antibodies showed a single specific band at the same molecular mass (Fig II/ 7B). A more precise determination of the molecular weight with an unstained marker showed a Mw of ~175 kDa (data not shown). These results show, besides the molecular weight of FLS2:myc, that engineering and transformation of FLS2:myc was successful.



**Fig II/7: Western blots of transgenic FLS2: myc cell cultures reveal a specific cross-reacting band around 180 kDa.** Immunoblot of crude extracts (A) and immunoprecipitation (B) of the solubilized fraction of wildtype, and a mixture of 20 *FLS2:myc* transformed *A. thaliana* cell suspension cultures with 9E10 monoclonal anti-myc antibodies. Here, prestained molecular weight markers were used to estimate the molecular weight. More accurate, non-prestained markers showed slightly different Mw (see text). Low Mw bands in the immunoprecipitate represent the IgGs used for immunoprecipitation

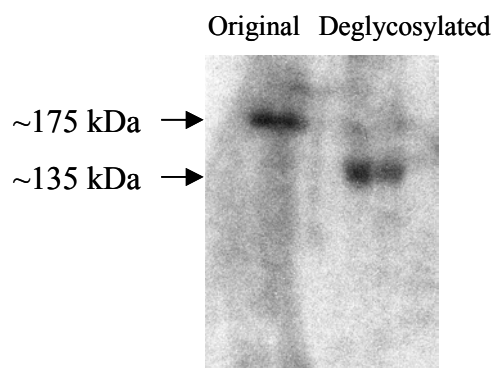
low-speed (10,000 g, 20 min) centrifugal pellet (P1) and only a small fraction reached the microsomal fraction (P2: 100,000 g, 45 min), but none was detectable in the soluble fraction (S2) (Fig II/ 8A). We observed a similar distribution of the flagellin binding site in *A. thaliana* (see I.3.1.5.). Further anti-myc-crossreacting bands, that also occurred in control cell lines (data not shown), were present in the soluble fraction but not in the microsomal reaction (P2). Parallel experiments with an FLS2:myc transgenic tomato line, termed Tom2 showed a different fractionation behavior (Fig. II/ 8B). Here, FLS2:myc only partially remained in P1, but the majority of it co-fractionated with the microsomal fraction (P2) but was not detectable in the soluble fraction. Thomas Meindl reported similar fractionation behavior of the flagellin binding site in tomato (Meindl et al, 2000) as we observed for FLS2:myc in Tom2. Additional anti-myc-crossreacting bands in Tom2 in the low-molecular weight range were not present in control cell lines (data not shown). These bands probably represent C-terminal degradation products of FLS2:myc. Taken together, our data suggest that FLS2:myc is a membrane-associated protein. It is still unknown, why the majority of the flagellin binding site and FLS2:myc remain in P1 in *A. thaliana* (for discussion see I.4.2.). Co-fractionation of FLS2:myc and the flagellin binding site suggests that they are co-localized.



**Fig II/8: Fractionation of FLS2:myc by differential centrifugation.** Crude extracts of *A. thaliana* (A) and tomato (B) *FLS2:myc* transgenic cell cultures were fractionated by a first low-speed (10,000 g, 20 min) and a second high-speed (100,000 g, 45 min) centrifugation. The pellets P1 and P2 were diluted in the original volume of the cell extracts. Aliquots of 20 µl were loaded on SDS-gel, western blot was performed with polyclonal anti-myc-tag antibodies.

## II.3.3.4. FLS2:myc is glycosylated

The FLS2-predicted protein has a molecular mass of 129 kDa (Gómez-Gómez, 2000), smaller than the observed mass of 175 kDa, even considering the 5 kDa added by the inserted triple myc. The difference between the observed and estimated molecular masses might be due to glycosylation, since FLS2 contains 23 putative N-glycosylation sites (N-X-S/T) (Gómez-Gómez, 2000). To test this possibility, enzymatic and chemical deglycosylation experiments were performed with crude extracts of FLS2:myc transgenic cell cultures. Treatment with Peptide-N-Glycosidase-F (PNGase F) following heat denaturation with SDS and  $\beta$ -mercaptoethanol did not result in a size shift on SDS-PAGE. However, enzymatic deglycosylation of proteins was often found to be little effective due to steric hindrance of the enzymes when approaching the glycan group. Therefore, we tested trifluoromethanesulfonic acid (TFMS), a chemical treatment reported to be much more effective (Fryksdale et al, 2002). This method resulted in a reduction of the apparent molecular weight by approximately 40 kDa (Fig. II/9). The molecular weight of the deglycosylated protein of approximately 135 kDa matches well with the predicted molecular weight of FLS2:myc.



**Fig II/9: Chemical deglycosylation of FLS2:myc in TOM2 crude extract by TFMS.** The Mw was determined by non-prestained Mw-marker.

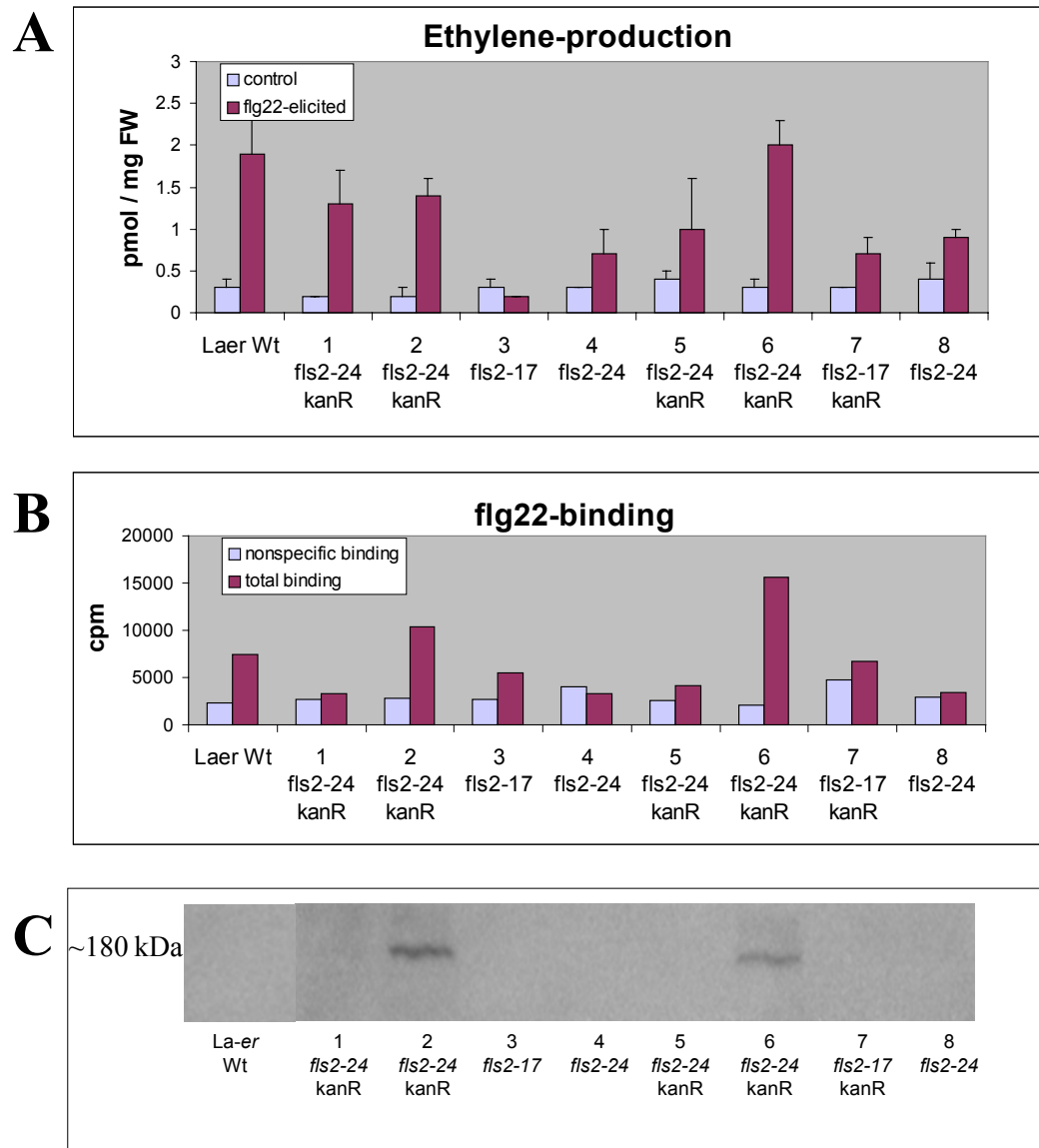


II.3.3.5. *FLS2:myc* is functional and complements both, *fls2* and *fls1*

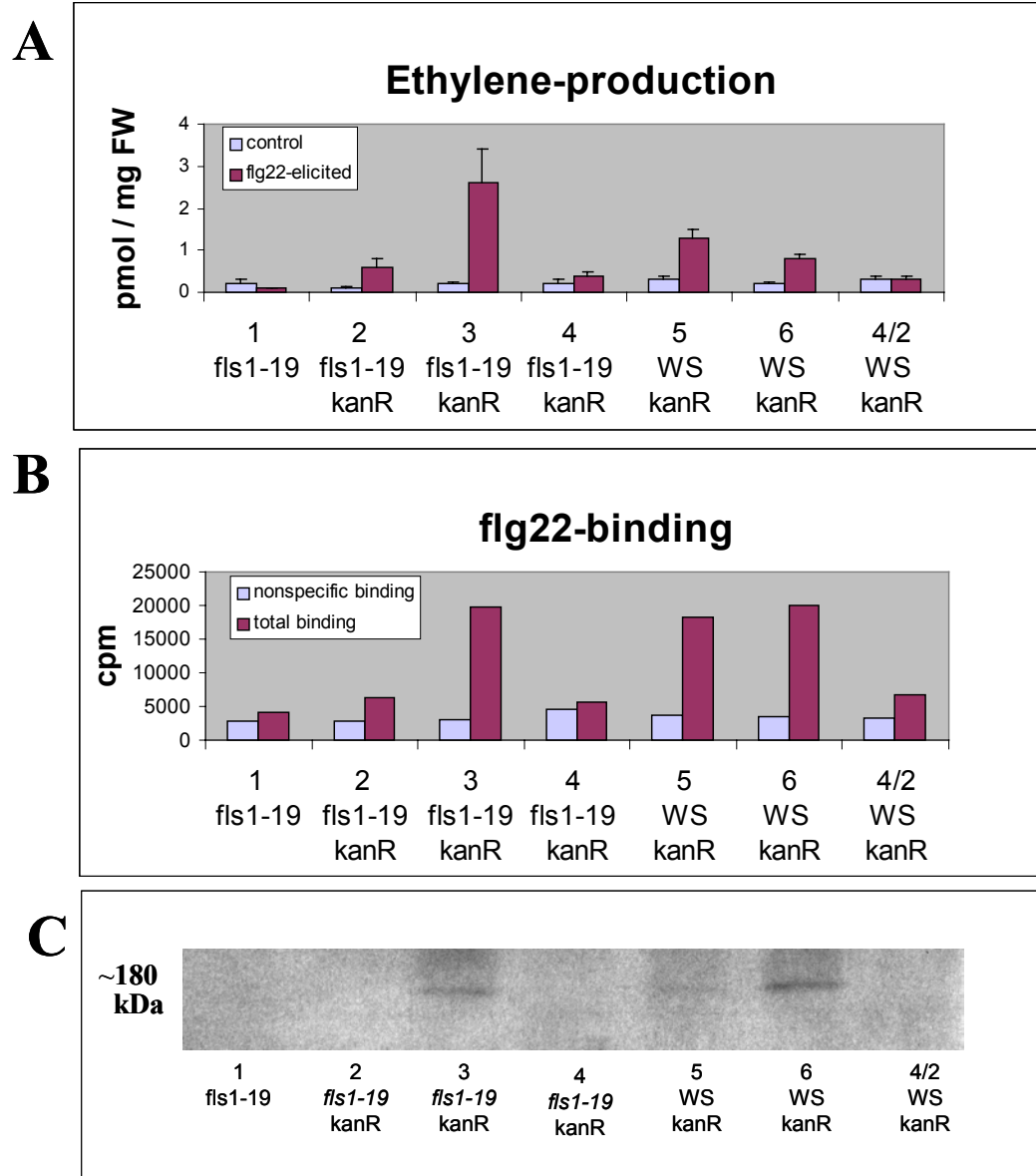
In order to see if *FLS2:myc* is functional, we tested if it can complement *fls2* mutants. We transformed *fls2-24* and *fls2-17* plants, and also *Ws-0* and *I-19* that were known to be altered in a non-allelic locus, termed *fls1* (Gómez-Gómez et al, 1999; Gómez-Gómez and Boller, 2000), with *35S:FLS2:myc* pCambia2300 containing the gene for kanamycin-resistance. We obtained four kanamycin resistant *fls2-24* plants, one resistant *fls2-17* plant, five resistant *Ws-0* plants and four resistant *I-19* plants. Selected kanamycin-resistant plants were tested for ethylene production, oxidative burst, flg22-binding and the presence for *FLS2:myc* by immunoblotting. Two of the four kanamycin-resistant *fls2-24* plants (Nr. 2 and Nr. 6) showed a restored reaction to flg22 in ethylene production (Fig. II/ 10A) and oxidative burst (data not shown), a restored flg22-binding (Fig II/ 10B) and, *FLS2:myc*-expression was readily detectable by immunoblots (Fig. II/ 10C). One kanamycin-resistant *fls2-24* plant (Nr. 5) and the only kanamycin-resistant *fls2-17* plants did not show any detectable bands by immunoblots, nor any response or binding. *Fls2-24* Nr. 1 showed a hardly detectable expression of *FLS2:myc*, that seemed to be sufficient for a restored ethylene-production but not for detectable flagellin binding (Fig II/ 10ABC). These results show, that *FLS2:myc* is functional and successfully complements *fls2-24*.

Surprisingly, *FLS2:myc* transgenic *fls1*-plants showed restored flagellin-response and binding, as well. Two out of three tested kanamycin-resistant *Ws-0* plants (Nr. 5 and Nr. 6) and one out of three tested kanamycin-resistant *I-19* plant (Nr. 3) showed a complementation in all tested reactions, such as ethylene production (Fig. II/ 11A), oxidative burst (data not shown) and flg22-binding (Fig. II/ 11B). Complementation strongly correlated with the expression of *FLS2:myc*, detected on immunoblots (Fig. II/ 11C).

These results show that, in contrast to former findings (Gómez-Gómez, 2000), *FLS2* complements not only *fls2*, but also *fls1*. To ensure that our results were not distorted by a contamination of non-responsive *fls1* seeds by responsive wildtype *La-er* or *Col-0* seeds, the ecotype of complemented *Ws-0* plants was determined via CAPS-markers. Restriction digestion of a gDNA-PCR-product with selected primers of the CAPS-marker RPS4-NT has shown *Ws-0*-specific cutting in the complemented lines



**Fig. II/10: Complementation of *fls2* mutants by *FLS2:myc*.** Ethylene production by leave peaces (A), flg22 binding (B) and immunoblot (C) of crude homogenates of wildtype and *FLS2:myc* transformed *A. thaliana* Laer *fls2* plants.



**Fig. II/11: Complementation of *fls1* mutants by *FLS2:myc*.** Ethylene production by leave peaces (A), flg22 binding (B) and immunoblot (C) of crude homogenates of wildtype and *FLS2:myc* transformed *A. thaliana* Laer *fls1* plants. The wildtype control is shown in the previous figure.

confirming that their ecotype was indeed Ws-0 (data not shown). Semiquantitative RT-PCR with *FLS2*-primers excluded that insensitivity in Ws-0 and *fls1-19* was due to decreased levels of *FLS2*-mRNA (data not shown). Re-sequencing of *fls2* in Ws-0, *fls1-19* and *fls1-2* by Silke Robatzek finally solved the mystery, why *FLS2:myc* complemented *fls1*. As shown in Fig II/12, the genomic sequence of *fls2* was found to be truncated in Ws-0, *fls1-19* and *fls1-2*, in the kinase domain. In Ws-0, a deletion at the codon 1006 caused a frame shift, which created a stop codon seven amino acids later, deleting half of the kinase domain. In *fls1-19* there is a G936A transition, creating a stop codon. Thereby, only one quarter of the kinase domain remains. Though, we did not obtain a kanamycin-resistant *fls1-2* plant, its *FLS2* kinase domain was sequenced as well, showing a C926T transition resulting in a stop-codon.

Wt	901	NLKEFSAESD	KWFYTEAKTL	<sup>CGA</sup> SQLKHRNLVK	<sup>TGG</sup> ILGFAWESGK	TKALVLPFME
Ws-0	901	NLKEFSAESD	KWFYTEAKTL	SQLKHRNLVK	ILGFAWESGK	TKALVLPFME
1-19	901	NLKEFSAESD	KWFYTEAKTL	SQLKHRNLVK	ILGFA <sup>★</sup>	<sup>TGA</sup>
1-2	901	NLKEFSAESD	KWFYTEAKTL	SQLKH <sup>★</sup>	<sup>TGA</sup>	
Wt	951	NGNLEDTIHG	SAAPIGSLLE	RIDLCVHIAS	GIDZLHSGZG	FPIVHCDLKP
Ws-0	951	NGNLEDTIHG	SAAPIGSLLE	RIDLCVHIAS	GIDZLHSGZG	FPIVHCDLKP
Wt	1001	<sup>CTTGAC</sup> ANILLDSDRV	AHVSDFGTAR	ILGFREDGST	TASTSAFEGT	IGYLAPEFAY
Ws-01001	1001	ANILL <sup>TVTALLT</sup> <sup>★</sup>				
		<sup>CT.GAC</sup>				

**Fig II/12: Partial amino acid sequences of the *fls2*-kinase domain in *La-er* Wt and *flg22*-insensitive lines.** The sequencing and the figure were done by Silke Robatzek. Red star prerepresents a stop codon.

Segregation analysis with the offspring of *FLS2:myc* transgenic plants was performed to determine, how the transgene was integrated into the genome of the individual plants. Oxidative burst in T2 plants, offspring of complemented T1 plants, was measured by Jürg Felix. 63 % of 11 seedlings of one *fls1-19* complemented plant were *flg22* responsive, which corresponds to a 3:1 segregation (Table II/1), meaning that *FLS2:myc* was integrated at a single locus. Seedlings of another *fls1-19* complemented plant showed 97 % responsiveness out of 30 seedlings, indicating a multiple integration of the

vector. Seedlings of one Ws-0 complemented plant showed 78 % resistance out of 31 seedlings, which corresponds to a 3:1 ratio.

Seedlings of two complemented *fls2* plants showed a responsiveness of 5 % of 21 plants and 19 % of 16 plants, respectively, suggesting that silencing might have occurred. However, these couple of responsive plants produced very few seeds. Interestingly, we observed a correlation between strong expression of *FLS2:myc* and lack of seeds also in Ws-0 background, although the plants looked otherwise healthy. This might be a coincidence, but we can not rule out that 35S:FLS2:myc or kanamycin resistance interferes with seed development. We often observed green/yellow patched leaves of complemented plant offspring on kanamycin plates that also indicates that silencing of the kanamycin resistance gene might occur.

**Table II/1: Segregation analysis** of flg22-responsiveness via oxidative burst measurements (in T2) or kanamycin resistance (in T3) in the offspring of *FLS2:myc*-complemented *fls1* and *fls2* plants. Unfortunately, we did not obtain sufficient number of T2 seeds of complemented *fls2* plants to investigate segregation in the T3 generation. N.d.= not determined

*fls1*

# KanR plant	<i>fls1-19</i>	(#2) <i>fls1-19</i>	(#3) <i>fls1-19</i>	(#4) <i>fls1-19</i>	(#5) Ws-0	(#6) Ws-0	(#7) Ws-0
Complementation in T1	n.d.	no	yes	no	yes	yes	no
Segregation in T2 resp./ all (oxidative burst)	7/11 63%		29/30 97%		no seeds	29/37 78%	
Segregation (KanR) in T3 of selected T2 plants (responsive: + /non-responsive: -)	#4 (-): 0% #5 (+): 100% #6 (-): 0% #7 (+): 80%		#2 (-): 0% #7 (+): 100% #9 (+): 73 % #13 (+):100% #14 (+):100% #18 (+): 77% #19 (+):100%			#1 (-): 0% #6 (+): 85% #8 (-): 0% #11 (+): 85% #12 (+): 78% #13 (+):100% #14 (+): 68%	

*fls2*

# KanR plant	(#1) <i>fls2-24</i>	(#2) <i>fls2-24</i>	(#5) <i>fls2-24</i>	(#6) <i>fls2-24</i>	(#7) <i>fls2-17</i>	(#8) <i>fls2-17</i>
Complementation in T1	No	yes	no	yes	no	no
Segregation in T2 resp./ all (oxidative burst)		1/21 5%		3/16 19%		

Seeds of T2 plants were grown on kanamycin plates to identify homozygote plants for further breeding. Resistant and nonresistant seedlings were counted. Seeds of T2 plants whose seedlings were 100 % resistant were preserved for subsequent experiments.

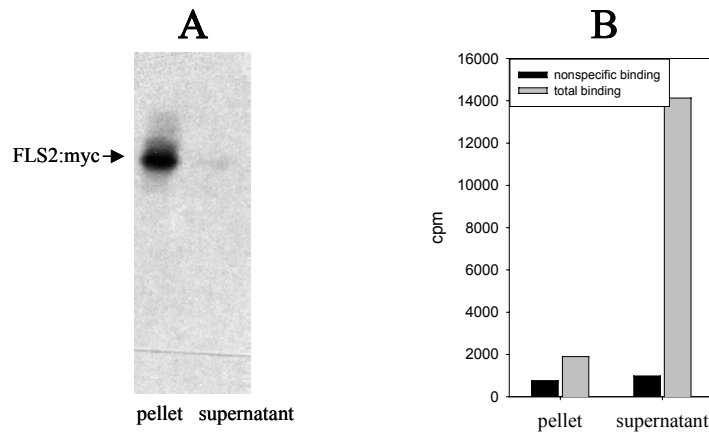
### *II.3.3.6. Solubilized FLS2:myc behaved differently than the flagellin binding site*

- Solubilization and immunoprecipitation of FLS2:myc from transgenic cell cultures and plants

For a number of applications, such as immunoprecipitation and affinity chromatography, it is essential to have FLS2:myc in a soluble form. To achieve solubilization, we treated the P1 fraction (the low-speed centrifugal pellet) of crude extracts with 1 % Triton X-100, since this method proved best for the flagellin binding site (see I.3.2.1.). However, no anti-myc-cross-reacting bands were visible in western blots of solubilized fractions, even after concentration of proteins by acetone or methanol-chloroform precipitations by up to 30-fold. Presumably, the amount of FLS2:myc still was under the detection limit of western blots. Immunoprecipitation with monoclonal and polyclonal anti-myc antibodies was performed, to concentrate FLS2:myc out of 500  $\mu$ l to 30 ml solubilized fraction. However, only four out of sixteen immunoprecipitation attempts (one with 500  $\mu$ l!) showed detectable FLS2:myc bands in immunoblots. One possible explanation for the failure of the immunoprecipitations is that FLS2:myc was not present in the solubilized fraction. Therefore, we tested several different detergents for solubilization, such as Triton X-114, octylglycoside, CHAPS and NP40 (1%). Alternatively, P1 or microsomal preparations were used as a substrate. Furthermore, we performed solubilization at 4°C and 37°C, or added 100 mM cyclodextran, that was reported to disrupt lipid rafts in mammalian cells (Boesze-Battaglia et al, 2002; Brown et al, 2000), 10 min prior to solubilization. Unfortunately, none of these conditions improved solubilization of FLS2:myc (data not shown). In parallel, solubilization of flagellin binding was tested. Also for the binding site, none of the described methods improved its solubilization significantly. Therefore, we had to draw our conclusions from the few immunoprecipitation experiments that worked.

- FLS2:myc did not co-precipitate with the flagellin binding site

When FLS2 represents the flagellin binding site, precipitation of the binding site should co-precipitate FLS2:myc in transgenic lines, and vice versa. The same results are expected, when the flagellin binding site and FLS2:myc are not identical but closely associated in a receptor complex. However, immunoprecipitation of FLS2:myc from solubilized extracts of transgenic tomato (Tom2) and *A. thaliana* cell cultures did not co-precipitate flagellin binding in three independent experiments (Fig II/13). In two of

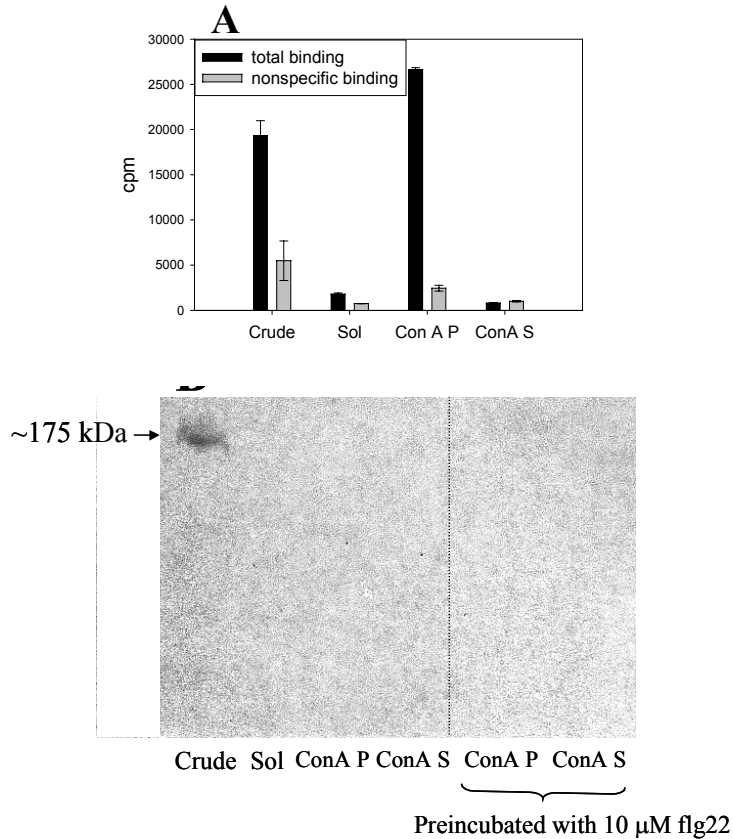


**Fig. II/13: Distribution of FLS2:myc and the flagellin binding site after anti-myc immunoprecipitation of the solubilized extract of *FLS2:myc* transgenic tomato cell culture (Tom2).** 500  $\mu$ l Tom2 solubilisate was immunoprecipitated with 26  $\mu$ g monoclonal  $\alpha$ -myc antibody. Aliquots of pellet and supernatant were subjected to western blot with anti-myc antibodies (A) and to flg22 binding assays (B). This result represents one of three successful immunoprecipitation experiments where flg22 binding in the supernatant and the pellet has been determined.

these experiments, immunoprecipitations were performed with control lines in parallel that did not express FLS2:myc. Specific binding was found the same in immunoprecipitates of transgenic and control lines (Data not shown). On the other hand, ConA-sepharose beads completely precipitated the flagellin binding site present in solubilized extracts of FLS2:myc transgenic cell cultures, as shown by binding assays (Fig. II/ 14A), but, in contrast to crude extracts, FLS2:myc was not detectable in the Con A precipitate by western blots, in three independent experiments, even when *fls2-19* background was used that showed a strong reduction of flg22-binding (Fig. II/ 14B). These data demonstrate, that FLS2:myc and the binding site do not co-precipitate. To test the possibility that flagellin might crosslink the binding site with FLS2, crude

extracts were incubated with 10  $\mu$ M flg22 for 20 min prior to solubilization. However, FLS2:myc could not be detected in the Con A-sepharose pellet and supernatant of the flg22-preincubated solubilisate, either (Fig. II/ 14B).

We were surprised that Con A did not bind FLS2:myc, though its high glycosylation was previously shown (Fig II/9). However, Con A does not bind all glycoproteins but only those with  $\alpha$ -mannosylresidues at nonreducing ends and within the glycosyl-chain.

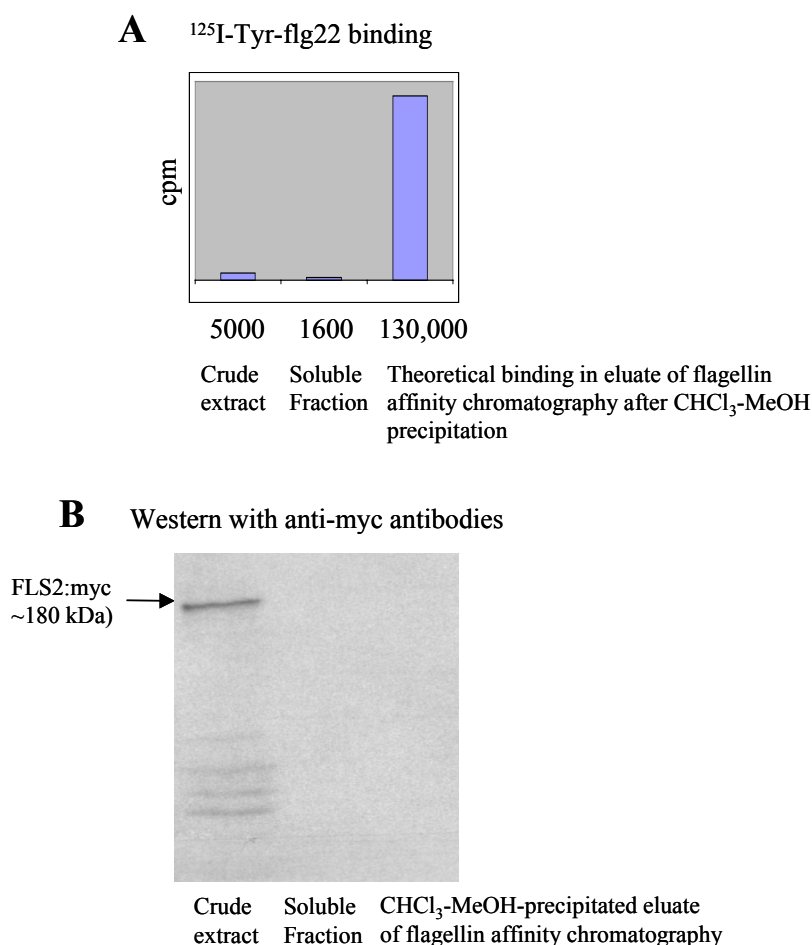


**Fig. II/14: Distribution of the flagellin binding site and FLS2:myc after ConA-sepharose precipitation of the solubilized extract of *fls2-19* FLS2:myc plants.** Two ml aliquots of solubilized extracts were precipitated with Con A sepharose beads. The crude extract (Crude), solubilized extract (Sol), Con A pellet (ConA P) and Con A supernatant (ConA S) were subjected to flagellin binding assay (A) and anti-myc western-blot (B). This experiment represents two further ones conducted with transgenic *FLS2:myc* tomato cell cultures (Tom2).



### FLS2:myc does not co-purify with the flagellin binding site

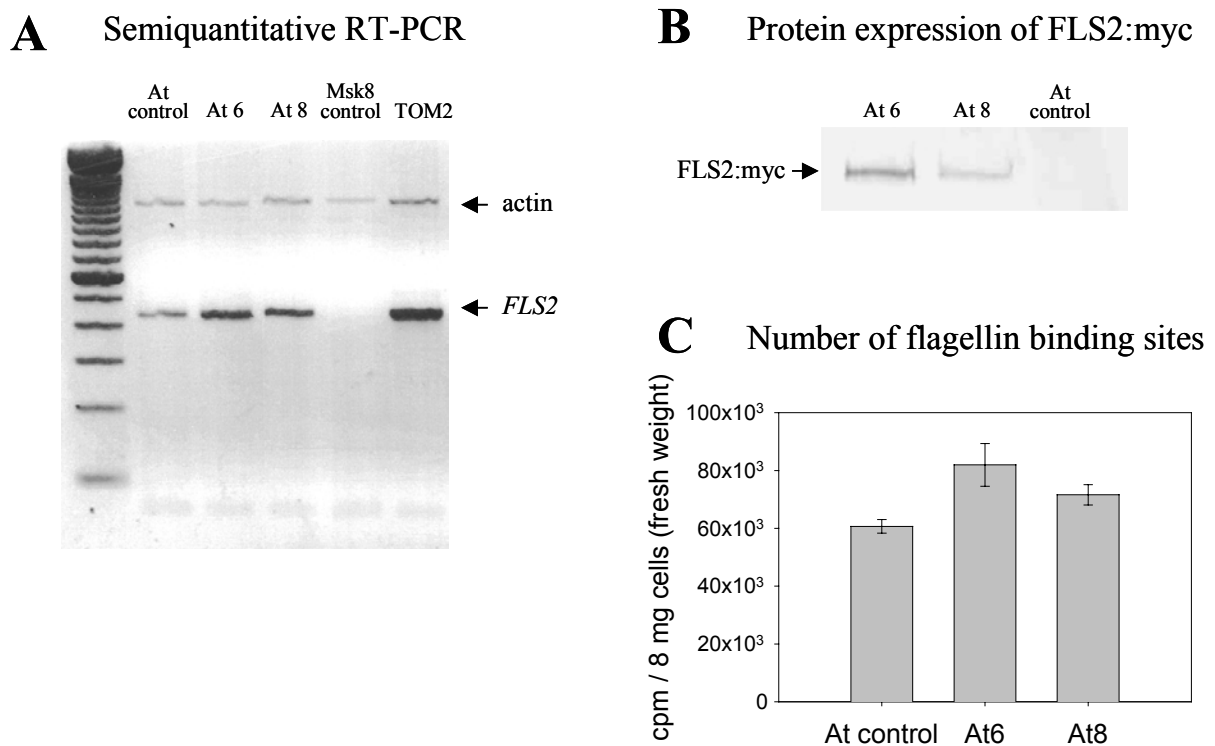
Purification of the binding site out of transgenic tomato cell culture Tom2 reveals, if the enrichment of FLS2:myc parallels the enrichment of the binding site or not. Thus, the binding site was partially purified according to the proceedings described before (I.3.2.5.), using flagellin affinity chromatography with solubilized P1 of Tom2. Successful enrichment of the binding site in the eluate was confirmed by determination of specific flg22-binding in the crude extract, in the solubilized fraction and in the affinity chromatography eluate (Fig II/ 15A). However, detection of FLS2:myc by immunoblots with anti-myc antibodies revealed no parallel enrichment of FLS2:myc (Fig II/ 15B).



**Fig II/15: Presence of FLS2:myc in crude extract and fractions of flagellin affinity chromatography used to enrich the flagellin binding site.** (A) Specific flg22-binding and (B) immunoblot with anti-myc antibodies in Tom2 crude extract, solubilized fraction and the eluate of flagellin affinity chromatography. To determine binding in the eluted fractions, small aliquots were Con A bead-precipitated and washed with binding buffer prior to binding assays. For western, all eluted fractions containing binding sites were pooled and chloroform-methanol-precipitated and theoretically concentrated thereby 50-fold.

### II.3.3.7. Overexpression of *FLS2:myc* was not paralleled by an increase of number of flagellin binding sites

Assuming that *FLS2* is the flagellin binding site, overexpression of *FLS2:myc* by the CMV 35S promoter should produce considerably more binding sites compared to the wildtype background. To test the level of *FLS2*-overexpression, semiquantitative RT-PCR with RNA extracted from *A. thaliana* control and 35S:*FLS2:myc* cell lines, At 6 and At 8 was performed, using primers that give rise to a 420 bp amplicon from the C-terminus of *FLS2*. The PCR-reaction was stopped after 28 cycles, prior to the saturation of the reaction. Figure II/ 16A shows that *FLS2* is overexpressed in transgenic lines,



**Fig. II/16: Comparison of *FLS2*-expression-level and number of binding sites in wt and transgenic *FLS2:myc* cell lines.** Quantification of *FLS2*-expression occurred via semiquantitative RT-PCR (A) and anti-myc western (B), and number of binding sites was determined by saturation binding studies (C) These radioactive binding studies were performed by Martin Regenass. Details see text.

where At6 shows a stronger overexpression than At8. Loading of equal amounts of cDNA was ensured using actin amplification. Comparison of the bands of PCR-products with several dilutions of the cDNAs (data not shown) suggests that *FLS2* is approximately 3-fold overexpressed in At 6 and about twice in At 8 (data not shown). This level of overexpression is much lower than generally reported for CMV 35S promoter-driven genes, but still can serve for comparison between overexpression and number of flagellin binding sites. Western with equal amounts of At6 and At8 crude extracts (10 mg fresh weight each) shows, similarly to the RT-PCR, that FLS2:myc is slightly stronger expressed in At6 than in At8 (Fig II/ 16B). This indicates that the *Fls2*-mRNA level reflects the FLS2-protein level.

Saturation binding assays were used to assess the number of binding sites in At6, At8 and control *A. thaliana* cell cultures using 12 different concentrations of radioactive  $^{125}\text{I}$ -Tyr-flg22 from 0.25 nM to 12 nM, in absence and presence of 10  $\mu\text{M}$  competitor Tyr-flg22, as described before (I.3.1.3., Fig. I/4).  $K_d$  and  $B_{\max}$  were determined by curve fitting to rectangular hyperbola ( $y=B_{\max} \cdot x/(K_d+x)$ ), where  $y$ =bound ligand and  $x$ =free ligand. Both  $K_d$  and  $B_{\max}$  did not differ substantially in the three cell lines:  $K_d$  was found to be 1.3 nM in the control cell line and 2.3 nM in At6 and 2.7 nM in At8, respectively. These results are equivalent with previous results (I.3.1.3.) where we stated, that the  $K_d$  ranged between 1 and 3 nM.  $B_{\max}$  was increased by 35 % in At 6 and by 18 % in At 8 (Fig. II/ 16C) compared to the control line. We conclude, that the level of FLS2 expression was not paralleled one-by-one by the number of flagellin binding sites. Although, *FLS2*-mRNA-expression is at least 8-fold higher in Tom2 than in *A. thaliana* control (Fig. II/ 16A and data not shown), the number of binding sites was not determined in Tom2 for several reasons: Firstly, optimal conditions for binding, such as salt concentration and pH, differ in Msk8 and *A. thaliana*. Secondly, intrinsic expression of the tomato FLS2-ortholog is not known.

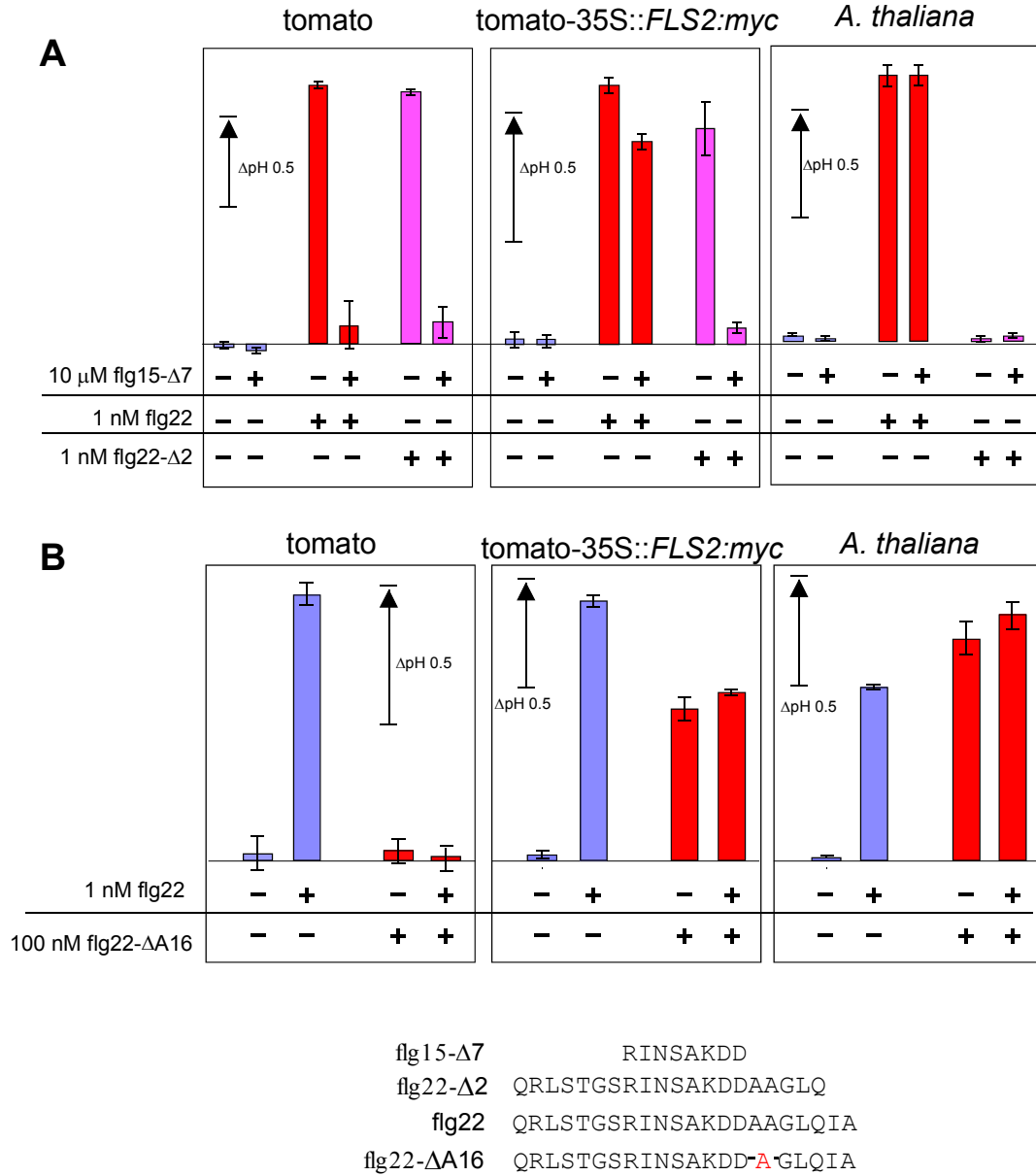
#### *II.3.3.8. FLS2:myc-transgenic tomato cell cultures show characteristics of A. thaliana in flg22 response and -binding*

In order to see if the presence of FLS2:myc changes specificity of flagellin-response in tomato, medium alkalization assays with various flg-peptides in FLS2:myc transgenic

Tom2 and tomato and *A. thaliana* control cell suspension cultures were performed by Jürg Felix.

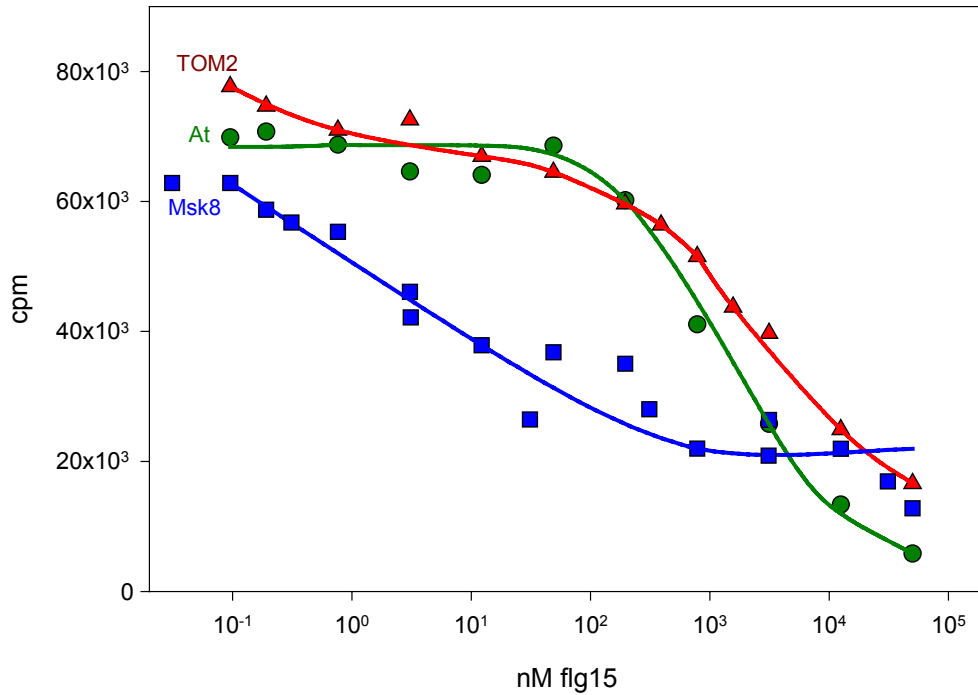
Basically, tomato shows a 10-fold lower  $EC_{50}$ -value for the most peptides, and shorter peptide fragments are in contrast to *A. thaliana* still sufficient for response (Jürg Felix, unpublished results). Thus, these differences are not useful for detection the functioning of FLS2:myc in tomato, since the tomato response "covers" the *A. thaliana* response. Luckily, there are certain peptides that act as antagonists in tomato but as agonists in *A. thaliana*, and vice versa, and peptides that are antagonists in tomato and are inactive in *A. thaliana*. These peptides can be used to determine the origin of flagellin response in the *FLS2:myc* transgenic tomato line Tom2. As shown in [Figure II/ 17A](#), flg15- $\Delta 7$ , that consists of only eight amino acids ([Fig II/ 17C](#)), is inactive in *A. thaliana* ([Fig II/ 17A](#), right panel) but acts as antagonist in tomato ([Fig II/ 17A](#), left panel), e.g. 10  $\mu$ M flg15- $\Delta 7$  completely inhibits response to 1 nM flg22. Whereas flg22- $\Delta 2$ , that lacks the last two C-terminal amino acids ([Fig II/ 17C](#)), acts as agonist in tomato ([Fig II/ 17A](#), left panel) but as antagonist in *A. thaliana* ([Fig. II/ 17A](#), right panel). In the *FLS2:myc* transgenic line Tom2 ([Fig II/ 17A](#), middle panel) we can see both: responses typical for tomato and for *A. thaliana*. Namely, flg15- $\Delta 7$  only partly inhibits flg22-response ([Fig II/ 17A](#), middle panel, red bars). This shows the presence of *A. thaliana*-specific response. The response to flg22- $\Delta 2$  shows clearly the tomato-specific response. This is confirmed by full inhibition of the flg22- $\Delta 2$ -response by flg15- $\Delta 7$  ([Fig II/ 17A](#), middle panel, purple bars). [Figure II/ 17B](#) shows responses to another set of flg-peptides: flg22- $\Delta A16$  lacks one alanine at the position 16 ([Fig. II/ 17C](#)). This peptide acts as antagonist in tomato but as agonist in *A. thaliana* ([Fig II/ 17B](#), left and right panels). Tom2 showed a clear reaction to flg22- $\Delta A16$  that refers to *A. thaliana*-specific response ([Fig II/ 17B](#), middle panel, red bars). However, this reaction exhibits a somewhat lower amplitude than the response to flg22. This finding can be explained by the inhibition of the tomato-specific response, since the response can be regarded as the sum of tomato and *A. thaliana*-specific responses.

These results demonstrate, that the *FLS2:myc* transgenic tomato line Tom2 exhibits combined recognition specificity of tomato and *A. thaliana*.



**Fig. II/17: Medium alkalization in Msk8, Tom2 and *A. thaliana* suspension cell cultures triggered by various flg-peptides.** The bars represent  $\Delta$ pH measured 20 min after addition of the peptides (concentrations are indicated in the figure). Bars and error bars represent average and standard deviation of  $n=4$  replicates. The experiment and the figure were made by Jürg Felix.

We wondered, if specificity of flg22-binding was altered in Tom2, additionally to specificity of response. To test this possibility, we chose competition binding assays of  $^{125}\text{I}$ -Tyr-flg22 binding by flg15, since its  $\text{IC}_{50}$  was clearly different in the two organisms. Flg15 competed  $^{125}\text{I}$ -Tyr-flg22-binding with an  $\text{IC}_{50}$  of about 7 nM in tomato (Meindl et al, 2000) and about 6  $\mu\text{M}$  in *A. thaliana* cell cultures (Fig I/5), e.g. there is a clear difference of  $\text{IC}_{50}$  of three order of magnitudes. Corresponding competition experiments using Tom2 cells showed an  $\text{IC}_{50}$  close to that of *A. thaliana* (Fig II/18). This finding suggests that FLS2 plays a critical role not only for the specificity of flagellin response but also for the specificity of flagellin binding.



**Fig. II/18: Binding competition assay to intact cells of *A. thaliana*, Msk8 and TOM2 cell cultures.** 100  $\mu\text{l}$  suspension cultured cells with  $\sim 17$  mg fresh weight were incubated with 1.5 nM  $^{125}\text{I}$ -Tyr-flg22 and increasing concentration of the competitor flg15. The experiment was performed by Martin Regenss.

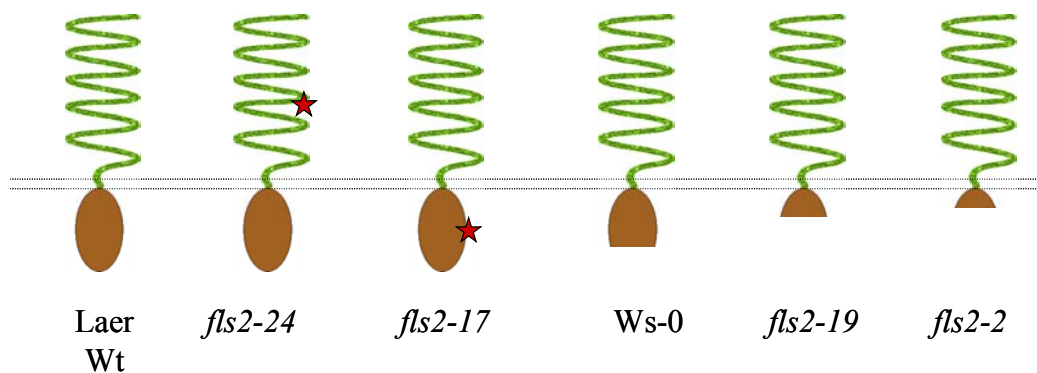
## II.4. Discussion

### II.4.1. The presence of binding site correlates with sensitivity to flagellin in *A. thaliana* plants

Binding assays established for cells and cell extracts from tissue culture (I.3.1) could be applied to study presence of flagellin binding sites in homogenates obtained from single *A. thaliana* plants (II.3.1.). All ecotypes and plants that exhibited physiological responses to treatment with flg22 also showed clear and significant binding activity. In contrast, both, the flagellin insensitive ecotype Ws-0 and all *fls*-mutants tested were impaired in binding of flagellin (II.3.1.). This correlation provides strong evidence for a functional role of this binding site as the physiological receptor for flagellin.

### II.4.2. FLS2 is essential for flagellin binding

Additionally, our data demonstrate, that FLS2 is essential for flagellin binding, since all of the flagellin-insensitive lines that showed reduction in flagellin binding in parallel, carried mutations in the *FLS2* sequence (II.3.1.). Currently, there are five different known sites within the *FLS2* locus, that were found to be essential for flagellin binding and sensitivity: one in the LRR, and four in the kinase domain (Gómez-Gómez and Boller, 2000; Lourdes Gómez-Gómez, Zsuzsa Bauer, Silke Robatzek, unpublished results (II.3.3.5. [Fig II/19](#))).



**Fig. II/19: Overview of currently known *FLS2* mutations causing flg22-insensitivity and binding reduction.** These mutations either result in amino acid substitutions (represented by red stars) or premature termination of the translation causing shortened C-terminus

#### *II.4.2.1. The LRR domain of FLS2 might be the ligand binding surface*

The extracellular domains of many receptors are members of the LRR protein superfamily that are expected to be responsible for protein-protein interactions (Kobe and Deisenhofer, 1994). The predicted site for this interaction is the solvent-exposed parallel  $\beta$ -sheet. The LxxLxLxx domain (x is any amino acid) within the LRR lies on the solvent face of the protein with the Leu facing away from the solvent face (Jones and Jones, 1996). The role of variable amino acids (x) is to confer specificity of protein binding to the LRR (Braun et al, 1991; Parniske et al, 1997). The *fls2-24* allele has a missense mutation in the 10<sup>th</sup> LRR domain in a variable position (Gómez-Gómez and Boller, 2000) indicating, that this residue is directly involved in this interaction.

#### *II.4.2.2. FLS2 kinase domain is essential for flagellin binding*

The kinase domain of *FLS2* is most closely related to the predicted receptor like kinases in higher plants such as CLV1 and SRK (Gómez-Gómez and Boller, 2000), which have the consensus amino acids characteristic for serine/threonine substrate specificity (Hanks and Quinn, 1992). Our data in this work demonstrated that flg22 binding not only requires an intact LRR domain but also an intact kinase domain (II.3.1. and II.3.3.5.). Four of the five known flg22 insensitive mutants, showing reduction of flg22 binding, are affected in the kinase domain: *Ws-0*, *fls1-2* (now *fls2-2*) and *fls1-19* (now *fls2-19*) lack major parts of the kinase domain (Silke Robatzek, unpublished results; II.3.3.5.) and *fls2-17* carries a point mutation in one of the 15 invariant residues conserved among all known members of the receptor kinase family in plants (Gómez-Gómez and Boller, 2000). In this case, the conserved glycine in the IX domain is changed to arginine (Gómez-Gómez and Boller, 2000). Mutations in this position also have been found in CLV1 alleles (Clark et al, 1998), in the BRI1 (Li and Chory, 1997) and in other serine/threonine protein kinases (Liu et al, 2000). Substitutions of this amino acid result in loss of kinase activity (Hanks and Quinn, 1991) and abolish autophosphorylation activity (Liu et al, 2000; Sessa et al, 2000). Indeed, Gómez-Gómez demonstrated that the *FLS2* kinase domain fused to glutathione S-transferase expressed in *Escherichia coli* can be autophosphorylated (Gómez-Gómez et al, 2001). In the same construct, the *fls2-17* mutation abolished autophosphorylation (Gómez-Gómez et al, 2001). We conclude, that autophosphorylation of *FLS2* itself or phosphorylation of



other components of the receptor complex by FLS2 are essential for flagellin binding activity. The mechanisms by which kinase activity is required for flagellin binding, are still unknown. It is possible, that kinase activity is necessary for receptor complex formation. The involvement of receptor phosphorylation in the formation of active receptor complexes has been studied extensively in the epidermal growth factor receptor (Lin et al, 1986) and transforming growth factor receptors (Luo and Lodish, 1997), in which activation requires the phosphorylation of a regulatory segment known as the GS region, which is located upstream of the serine/threonine kinase domain in the cytoplasmic portion of the receptor (Piek et al, 1999). Additionally, it has been shown that an active kinase is necessary for the assembly of the CLAVATA complex and for CLV3 binding (Trotochaud et al, 2000). Alternatively, it is conceivable that the kinase activity is needed for proper targeting or glycosylation of FLS2.

#### **II.4.3. *FLS1* is proved an experimental artifact**

Surprisingly, expression of *35S:FLS2:myc* restored flagellin responsiveness and binding in Ws-0 and the La-*er* mutant *fls1-19* (II.3.3.5.). Previously, flagellin insensitivity in Ws-0 was attributed to *fls1* (Gómez-Gómez et al, 1999), a locus distinct from *fls2*, since no mutations were found in the sequence encoding FLS2, responsiveness was not restored by ectopic expression of *FLS2*, and crossing between Ws-0 and La-*er fls2-24* revealed a co-dominance effect in that a part of the progeny had a full resistant phenotype and another part partial resistant phenotype in the growth inhibition bioassay (Gómez-Gómez et al., 2000). Similarly, *fls1-19* along with *fls1-2* showed no mutation in the *FLS2* sequence either and thus they were assumed to be allelic to *fls1* (Lourdes Gómez-Gómez, personal communication). Complementation of Ws-0 and *fls1-19* by *35S:FLS2:myc* (II.3.3.5., [Fig II/11](#)) put these previous results into question. Contamination of Ws-0 seeds by La-*er* or Col-0 seeds were excluded via CAPS markers. A clear correlation between the presence of a ~180 kDa crossreacting band detected by anti-myc antibodies and restoration of flagellin responsiveness and binding along with 1:3 segregation of T2 progeny provided compelling evidence that binding and flagellin-sensitivity in Ws-0, *fls1-2* and *fls1-19* was attributable to *FLS2:myc*-expression (II.3.3.5.). Re-run sequencing of *FLS2* open reading frame in Ws-0, *fls1-19* and *fls1-2* by Silke Robatzek revealed that indeed all three carried mutations in *FLS2*, that resulted in premature termination of the kinase domain ([Fig.II/12](#)). We conclude,

therefore, that *FLS1* was an experimental artifact, and insensitivity to flagellin was caused in all cases by changes in the sequence encoding *FLS2*. Namely, *fls2-24* carries a mutation in the *FLS2* leucine-rich-repeat domain, and *fls2-17*, Ws-0, and previously termed *fls1-2* (now *fls2-2*) and *fls1-19* (now *fls2-19*) carry mutations in the *FLS2* kinase domain (Fig. II/19).

Accordingly, our previous conclusion that several components are involved in flagellin binding and sensitivity (Bauer et al, 2001), are not supported by our recent results. Thus, we have to re-consider the interpretation of our results demonstrating significant reduction of flagellin binding in Ws-0, *fls1-2* and *fls1-19* additionally to *fls2-24* and *fls2-17*. Now, these findings supply further evidence that an intact *FLS2* kinase domain is indispensable for flagellin binding.

#### **II.4.4. Direct evidence that FLS2 is the flagellin binding site is lacking**

##### *II.4.4.1. RLKs might need accessory binding components for signal perception*

This present state of understanding, indicating that *FLS2* represents a major nonredundant component of flagellin perception and binding, does not exclude the involvement of accessory components. The lack of other components could be attributable to lethality, redundancy, or simply bad luck (i.e., not enough mutants). In the literature we find assumptions that LRR-receptor like kinases might require additional components for binding of the originally proposed ligand. Namely, it has been speculated, that the brassinolide receptor *BRI1* requires an accessory factor that directly binds brassinolides (Li et al, 2001), since leucine-rich-repeats, that compose the extracellular domain of *BRI1*, are believed to participate in protein-protein-interactions rather than in protein-steroid interactions. Additionally, sequencing of the Arabidopsis genome revealed a number of putative steroid binding proteins that are possible candidates (Arabidopsis Genome Initiative, 2000). Most recent work indicating that *tBRI1* (the tomato homolog of *BRI1*) perceives both brassinolides and systemin in tomato (Montoya et al, 2002; Scheer et al, 2003) implies, that additional components might be involved in direct hormone binding. Intriguingly, the putative *Avr9* receptor

Cf-9 does not directly interact with Avr9 (Luderer et al, 2001). Hence, the "Guard hypothesis", that is a newly emerging theory explaining the role of resistance genes (van der Biezen and Jones, 1998; Dangl and Jones, 2001) has been proposed for the Cf9 protein. As a guard, Cf9 would detect changes in the presence or in the phosphorylation status of the plant virulence target of Avr9 (probably the high-affinity binding site), as it was described for the resistance genes RPM1 and RPS2 in *Arabidopsis* (Mackey et al, 2003; Axtell and Staskawicz 2003). A further example for the requirement of several components for binding and signaling might be the  $\beta$ -glucan-perception. The structure of  $\beta$ -glucan elicitor-binding protein exhibited none of the expected characteristics for a receptor (Umemoto et al, 1997) and showed no clear membrane-spanning domains, although it was localized at the plasma membrane (Umemoto et al, 1997). These observation led to the suggestion that the  $\beta$ -glucan elicitor-binding protein might require other component(s) for localization in the membrane as well as for correct transmission of the elicitor response signal. Similarly, the syringolide-binding-protein P34 had no homology to receptor proteins but rather to thiol proteases (Ji et al, 1998). It has been speculated that P34 has multiple functions such as involvement in senescence (Kalinski et al, 1992) as well as in signal perception and its putative thiol protease activity might be important for syringolide binding and/or signaling (Ji et al, 1998).

In summary, at present we can not exclude that besides FLS2, accessory factors are required for flagellin binding. It is conceivable, that even though FLS2 is essential for flagellin binding, it is not the direct flagellin binding site itself.

#### *II.4.4.2. Characteristics of FLS2:myc and the flagellin binding site were found to differ in several aspects*

In order to demonstrate that FLS2 is the flagellin binding site, we compared biochemical characteristics of solubilized FLS2:myc, expressed in *A.thaliana* or tomato cell cultures or plants, with the characteristics of solubilized flagellin binding site. Additionally, we compared the number of binding sites with the level of FLS2 overexpression. We found, that (• *Result 1*) immunoprecipitated FLS2:myc did not bind flg22 (II.3.3.6.;Fig. II/13), and (• *Result 2*) FLS2:myc was not co-enriched with the flagellin binding site via Con A-sepharose beads, even when flg22 was added prior to solubilization (Fig II/14), or via flagellin affinity chromatography with solubilized cell

extracts of Tom2 (Fig II/15). Furthermore, (• *Result 3*) 2-3-fold overexpression of *FLS2* was not paralleled by a 2-3-fold increase of the number of binding sites (II.3.3.7; Fig II/16). Also, (• *Result 4*), the molecular weight found for FLS2:myc in western blots (~175 kDa, II.3.3.2; Fig II/8) did not match to the molecular weight of the binding site (~120 kDa, I.3.1.7; Fig I/8), as assessed via chemical crosslinking.

Taken together, these data refute the assumption that the myc-tagged FLS2 is the flagellin binding site. According to our data, separation of the myc-tagged FLS2 and the binding site is not prevented by previous flg22-binding. However, our data do not definitely exclude the identity of FLS2 with the flagellin binding site. For the FLS2:myc-flg22-interaction following experimental handicaps were possible:

• *Result 1*: Immunoprecipitated FLS2:myc did not bind flg22:

It is possible that the amount of FLS2:myc at the cell surface, that might constitute active binding sites, is negligible compared to the amount of endogenous binding sites of transgenic cell cultures. Even though FLS2 was found to be overexpressed in the transgenic lines (II.3.3.7.; Fig II/16), only a fraction of FLS2:myc might be active. This situation could explain, why no co-immunoprecipitation of FLS2:myc and the flagellin binding site was found.

It is also possible that only those FLS2:myc molecules can be precipitated by anti-myc-antibodies that occur in “wrong-side-out” micelles, exposing the C-terminus with the myc-tag on the micelle surface. These FLS2:myc molecules might be unable to bind added flg22, because their putative binding domain - the LRR domain - is inaccessible within the micelle. In the literature, immunoprecipitations - using antibodies raised against C-terminal epitopes - successfully showed interaction of plant RLKs and their cognate ligands. For instance, immunoprecipitated BRI1-GST using anti-GST antibodies showed, in contrast to wildtype control, clear brassinolide binding activity with the same  $K_d$  that was observed for BL binding sites in the wildtype (Wang et al, 2001). Since brassinosteroids are lipophilic compounds they might cross detergent micelles in contrast to flagellin. Also, interaction of CLV3 with CLV1 could be proven by immunoprecipitation: CLV1 was immunoprecipitated with antibodies raised against the C-terminus of the RLK. CLV3, the putative peptide ligand of CLV1, was found to co-immunoprecipitate in the 450 kDa complex but not in the 180 kDa complex

(Trotochaud, 2000). The advantage of this experiment was, that the binding took place prior to solubilization, so the CLV3 peptide had not had to cross detergent micelle barriers. Analogous experiments could have been performed with FLS2:myc (i.e. flg22-binding prior to solubilization and immunoprecipitation), but unfortunately, immunoprecipitations could not be successfully repeated. Also, the interaction of SCR (peptide ligand) and SRK (an RLK) of the self-incompatibility system of Brassicaceae was shown by co-immunoprecipitation (Kachroo et al, 2001). In this case, only the ectodomain of SRK was expressed in *E.coli*, thus problems with detergents were avoided a priori. Thus, none of the described successful co-immunoprecipitation experiments in the literature were performed under analogous conditions as our attempts.

- **Result 2:** Lack of co-purification of FLS2:myc and the flagellin binding site by Con A- and flagellin-affinity chromatography

Lack of co-purification of FLS2:myc with the flagellin binding site can be deceiving. For example, it is possible that the majority of FLS2:myc, visible on western blots of crude extracts (Fig II/14+ Fig II/15), represents mainly inactive, nonbinding forms, residing for instance in the ER (as mentioned above). Possible active forms, at the cell surface for instance, that are purified by Con A (Fig II/14) or flagellin affinity chromatography (Fig II/15), might be below the detection limit of western blots. Western blots and radioactive binding assays are highly different in their sensitivity. Also, it is possible, that the 50-fold concentration of the flagellin binding site/FLS2:myc was not achieved by chlorophorm-methanol precipitation, as assumed (Fig II/ 15A). Another possibility that explains why FLS2:myc did not co-purify with the flagellin binding site in this experiment (Fig II/15) is, that the tomato binding site was solubilized but not FLS2:myc, and therefore only the tomato binding site was enriched by affinity chromatography.

- **Result 3:** Overexpression of FLS2:myc was not paralleled by an increase in the number of flagellin binding sites

In the literature, there are examples, where overexpression of RLK receptor-candidates significantly increased the number of binding sites. CMV 35S promoter-driven overexpression of the phytosulfokine receptor candidate in carrot cells increased more

then 12-fold the number of phytosulfokine binding sites, deduced from saturation binding assays (Matsubayashi et al, 2002), while the  $K_d$  was not effected. Similarly, overexpression of BRI1 in *A. thaliana* correlated with a dramatic (~10-fold) increase in BL binding activity with similar  $K_d$  (Wang et al, 2001). However, high overexpression of *FLS2*, that was achieved in these two other examples of LRK receptors, could not be found for *FLS2:myc* (II.3.3.7.). It is difficult to definitely exclude a correlation between *FLS2*-expression level and number of binding sites with only 2-3-fold overexpression. But still then, lack of correlation might be explained by the requirement of a limiting additional component or limited traffic to the cell surface. As discussed in the previous paragraphs, it is possible, that only a fraction of transgenic *FLS2:myc* is active. When ~10 % of the overexpressed *FLS2:myc* would constitute active binding sites, clear correlation of the number of binding sites ( $B_{max}$ ), determined by saturation binding assays (Fig II/16), could be found. Namely, At6 was found to be ~3-fold overexpressed and the  $B_{max}$  was increased by 35 %, and At8 was ~2-fold overexpressed, and the  $B_{max}$  was increased by 18 % (II.3.3.7.).

- *Result 4:* Molecular weight of the flagellin binding site, based on chemical crosslinking, was different then the molecular weight of *FLS2:myc*

Chemical crosslinking might label other molecules then the actual binding site, that are in close vicinity to the ligand and provide good reaction partners. The 120 kDa band found in crosslinking experiments using P1, microsomes or solubilized microsomes (I.3.1.7.) might be an additional component in the receptor complex. For instance, it can represent the second partner in the receptor heterodimer. Such heterodimers, consisting of a bigger RLK and a smaller second protein was reported for the *CLAVATA*, the self-incompatibility and the brassinosteroid receptor complexes. *CLV2* was found to lack a kinase domain, and *SLG* was a soluble extracellular protein (Clarke 2001; Takayama et al, 2001). *BAK1*, the second component of the brassinosteroid receptor was a LRR-RLK, but significantly smaller, then *BRI1*, the first component.

In summary, our data do not support the hypothesis, that *FLS2:myc* represents the flagellin binding site. Nevertheless, they are not sufficient to definitely refute this hypothesis. Namely, it can be argued that the right experimental conditions have not

been met. However, it is striking that four independent approaches show different characteristics of FLS2:myc and the binding site.

#### II.4.5. FLS2 confers specificity of flagellin binding and perception

We investigated recognition and binding specificity in *FLS2:myc* transgenic tomato cell culture (Tom2)(II.4.5.). Our results demonstrated, that the FLS2:myc transgenic tomato line exhibits combined recognition specificity of tomato and *A. thaliana* (Fig II/17). This means, that FLS2:myc is functional in tomato and that it is able to take over the role of the tomato flagellin receptor and communicate with tomato downstream signaling elements leading to ion-fluxes across the plasma membrane. Furthermore, these data indicate that FLS2 confers specificity of flagellin-response.

Competition binding experiments using Tom2 cells showed a shift of  $IC_{50}(\text{flg15})$  from the  $IC_{50}$  characteristic for tomato to the  $IC_{50}$  characteristic for *A. thaliana* (Fig II/18). This finding suggests that FLS2 also plays a critical role for the specificity of flagellin binding.

#### II.4.6. Considering all data: Is FLS2 the flagellin binding site?

Models of other plant receptor systems give limited support for constituting a model for flagellin perception. Perception of general elicitors (PAMP's) is little elucidated at present, but it might differ from the perception of hormones and effectors whose perception is better understood. The data presented in this work have therefore priority for model building. Currently, there is no single model that can explain all emerged data. I propose three alternative models that attempt to integrate most of the current results (see Fig II/20):

- Model A: FLS2 is identical to the binding site

As already stated, FLS2 is an “ideal” candidate for the flagellin binding site, regarding its putative localization and its receptor-like structure, containing an extracellular LRR domain (a putative binding domain) and a cytoplasmic kinase domain, as found for other receptors for hormone signaling. Additionally, all to date identified flagellin-insensitive mutants and ecotypes affected in *FLS2* (*fls2-24*, *fls2-17*; *fls2-2* (former *l-2*),

*fls2-19* (former *l-19*) and *Ws-0*) showed strongly reduced binding and, vice versa, at present no additional loci to FLS2 found responsible for flagellin binding have been identified. Complementation of these mutants by Wt *FLS2* restored response and binding. However, the most important experimental evidence for FLS2 being the flagellin binding site are the findings with the Tom2 cells. In these tomato cells, the transgenically introduced FLS2:myc was found to determine the specificity of flagellin response and binding. This finding can only be explained when FLS2 comprises the binding site. Since the LRR domain is regarded as the direct binding surface, the indispensable role of the FLS2 kinase domain for flagellin binding is not unambiguously explained by this model. As an explanation, (auto)phosphorylation might influence the conformation, intracellular localization or complex formation of FLS2. In this model, change of reversibility during fractionation could be due to disruption of FLS2-homodimers.

As discussed above, there are also several data that do not support that FLS2 is the binding site. Two further models attempt to integrate these data:

- **Model B: FLS2 needs an accessory binding component**

According to this hypothesis, the 170 kDa FLS2 alone would not be able to bind flagellin, as suggested by our results showing different characteristics for the flagellin binding site and FLS2:myc. A 120 kDa accessory molecule, that was labelled by crosslinking, would be necessary for binding. Therefore, overexpression of FLS2:myc alone was not found to be sufficient to increase the number of binding sites. The 120 kDa accessory component would be separated from FLS2 after solubilization, since FLS2:myc did not coprecipitate and copurify with the binding site. However, in intact cells FLS2 would interact with the 120 kDa molecule in a complex and would be crucial for determining specificity. Change of reversibility during fractionation could be due to disruption of heterodimers, consisting of FLS2 and a 120 kDa accessory component. The FLS2 kinase domain could be important to direct the 120 kDa binding site to the cell surface and to retain its proper conformation, but after this step, FLS2 would not be necessary for flagellin binding any more. This model could fit well to the address-message hypothesis that claims that the N-terminus of flg22 is necessary for binding (for instance, by the 120 kDa binding site) and the C-terminus for activation of response (for instance, by FLS2). The weak point of this theory is that FLS2:myc also seems to



change binding specificity additionally to response specificity and seems to recognize the whole length of flg22 and not only the C-terminus (Jürg Felix, personal communication). It would be interesting to know, if solubilized binding sites in Tom2 show binding specificity of *A. thaliana* or of tomato. This could further clarify if FLS2:myc represents the binding site (this would be the case when solubilized Tom2 extract showed *A. thaliana*-specific binding), since in the solubilized form FLS2 and the binding site were found to be separated.

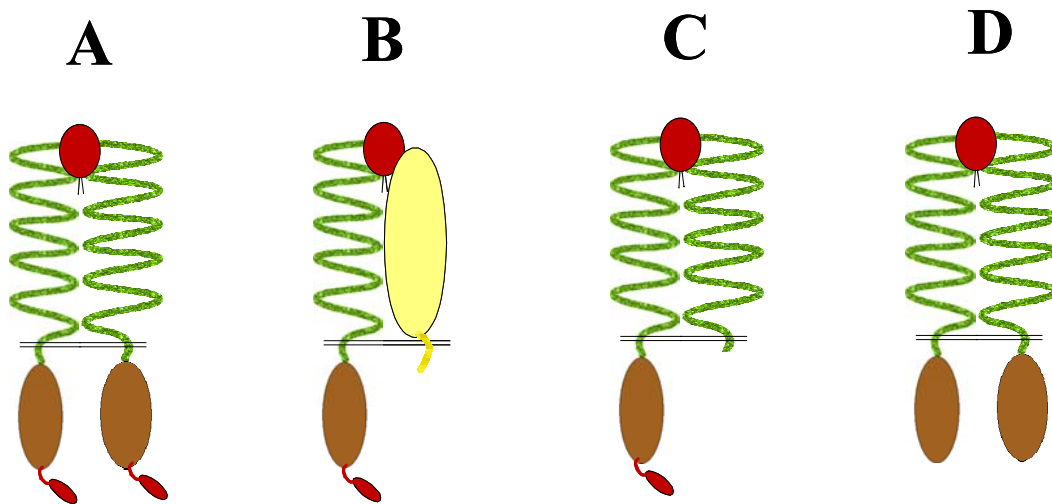
- **Model C: The C-terminus of FLS2:myc containing the kinase domain and the myc-tag is removed to produce an active binding form**

According to this theory, the 170 kDa FLS2 would be processed to produce a 120 kDa flagellin binding site by cutting off the C-terminus at protein or mRNA level containing the triple myc-tag. This would explain why crosslinking studies labelled a 120 kDa protein instead of a 170 kDa protein, why co-precipitation and co-purification of FLS2:myc and flagellin binding site did not occur and why FLS2:myc still conferred specificity of flagellin response and binding. Inexplicable posttranslational modifications that changed the migration in SDS-gels, were found for the phytosulfokine receptor (Matsubayashi et al, 2002) and overexpressed BRI1 (Wang et al, 2001). On SDS-gels, the phytosulfokine receptor (both, endogenic or transgenic) migrated in two distinct (120 and 150 kDa) bands. Deglycosylation shifted both bands by 10 kDa and the calculated molecular weight of the receptor was ~110 kDa. These data fit well to the 120 kDa form. The occurrence of the 150 kDa form could not yet been explained (Matsubayashi et al, 2002). Furthermore, antibodies raised against the N-terminus of BRI1 crossreacted in western blots of overexpressed BRI1-GFP extracts with an N-terminal cleavage product, that was 70-80 kDa smaller then the additional intact BRI1-GFP (Wang et al, 2001). Similarly, in western blots of Tom2 extracts, we observed C-terminal cleavage products of FLS2:myc. Certainly, the cleavage products might not have biological relevance.

- **Model D: C-terminal trimming of FLS2 leaves the kinase domain intact but removes the triple myc-tag.** My results show that an intact kinase domain at the C-terminus of FLS2 is essential for flagellin binding. However, it is possible that the C-terminus of the protein must be proteolytically trimmed in order to activate this

kinase domain. Thus, model D suggests that the primary translation product, FLS2:myc, is inactive. Only removing of the C-terminal end containing the myc-tag would allow activation of the kinase domain and thus restore flagellin binding. Therefore, FLS2:myc, detectable by western blots, would represent an inactive form of FLS2. Similarly to model C, this model explains why co-precipitation and co-purification of FLS2:myc and the flagellin binding site did not occur and why FLS2:myc still conferred specificity of flagellin response and binding. If this model is correct, it remains to be clarified why covalent crosslinking labelled a band of only 120 kDa. Assuming that this band represents the active, trimmed form of FLS2, we would have expected a molecular weight of about 160 kDa, taking glycosylation into consideration.

There are new experiments needed for deciding which of the current models is next to the reality. However, in the current state of understanding I consider it very likely, that FLS2 comprises the flagellin binding site, with or without C-terminal processing.



**Fig. II/20: Alternative models of the flagellin receptor.** A, FLS2 alone comprises the receptor. B, a 120 kDa flagellin binding site is part of the receptor complex. C, an N-terminal cleavage product of FLS2 is necessary for flagellin binding, lacking the major part of the kinase domain. D, C-terminal trimming of FLS2 leaves the kinase domain intact but removes the triple myc-tag.

## II.4.7. Suggestions for future experiments

### II.4.7.1. Antibodies and immunoprecipitation

Antibodies are a key tool to investigate characteristics of known proteins. For this purpose, FLS2 was expressed in *E.coli* by Lourdes Gómez-Gómez and injected into rabbits to produce anti-FLS2 antibodies. However, sera of immunized rabbits were unable to detect any proteins in crude extracts of *A. thaliana*. In microsomes and Concanavalin A-purified fractions we observed crossreacting bands smaller than 60 kDa in western blots, probably unrelated to FLS2. These anti-FLS2 antibodies were not able to detect FLS2:myc, that showed a strong crossreacting band with anti-myc antibodies in the same blot (data not shown). Lourdes then sent the FLS2-construct to a company to produce antibodies, but also the company's attempt failed. As an alternative, anti-peptide antibodies were raised against two unique peptides occurring in the N- and C-terminal region of FLS2. Also, these antibodies were not able to detect FLS2 or FLS2:myc. On western blots a broad smear was visible in the upper molecular weight range of Tom2 crude extracts (data not shown). To allow specific detection of FLS2, an epitope tag (c-myc) was therefore engineered to the C-terminus of *FLS2* next to the stop codon, and the construct, driven by the CaMV 35 S promoter, was transformed into cell cultures and plants (II.3.2.). Myc-tagging of FLS2 indeed turned out to be very useful for several applications. For example it facilitated the correlation between expression of FLS2:myc in flagellin-insensitive mutants and restoration of flagellin response and binding (II.3.3.5.), and it also enabled us to show a correlation between the alteration of flagellin response and binding in tomato suspension culture towards *A. thaliana*-specific response and binding - and the expression of FLS2:myc (II.3.3.8.). The FLS2:myc construct also made possible to assess the molecular weight of FLS2 and its glycosylation status (II.3.3.2 and II.3.3.4.). In several other aspects, however, the 35S:FLS2:myc construct did not fulfill our expectations. We were very surprised to see for example, that the 35S promoter did not cause a strong overexpression as in many reported cases (II.3.3.7.). That made difficult to draw a valid conclusions from a comparison of the number of binding sites in 35S-FLS2:myc and control lines.

For future experiments, a native promoter would be more preferable compared to the 35 S promoter for engineering transgenic FLS2-tag-plants or cell cultures, for example for

investigating upregulation of *FLS2*-expression after elicitation of flagellin or some other elicitors or hormones, as it was postulated and shown by Cyril Zipfel on the mRNA level (Cyril Zipfel, personal communication). Also, a native promoter would be advantageous for the determination of FLS2-localization within the cell. To show, that FLS2 is a plasma-membrane protein with the LRR-region on the cell surface, an N-terminal myc-tag would be more suitable than a C-terminal tag to allow accessibility of the anti-myc antibodies to the myc-tag without disrupting the cell. Obviously, this N-terminal tag would have to be inserted after the signal peptide. Alternatively, such an evidence could also be possible with the current *FLS2:myc* construct: For this purpose, cell-surface proteins could be biotinylated and tested if FLS2:myc is biotinylated or not (for example by solubilization of cell extracts in 1 % SDS, dilution to 0.1 % SDS and precipitation of biotinylated proteins with Avidin-agarose beads and detection of myc-tagged FLS2 in the precipitate and supernatant by western). Furthermore, it would be very interesting to find out, if *fls2*-mutants truncated in the kinase domain possibly fail to reach the cell surface or loose glycosylation, which could explain why an intact kinase domain is essential for flagellin binding. An N-terminal myc tag also might have been advantageous for flagellin binding measurements in immunoprecipitated FLS2:myc via the anti-myc antibody. We assume that immunoprecipitated FLS2:myc failed to bind flagellin, because its putative binding domain - the LRR-domain- is inaccessible within the micelle, when the C-terminal kinase domain with the myc tag is exposed to the micelle surface, accessible for immunoprecipitation. An N-terminal epitope tag could avoid this problem.

Immunoprecipitation of FLS2:myc seemed a particularly useful tool for several applications. Enrichment of FLS2:myc by immunoprecipitation would make up the first step to determine flagellin binding, to prove phosphorylation of the kinase domain upon elicitation, and to determine the phosphorylated residues. Also, we hoped to identify interacting partners that are closely associated with solubilized FLS2. Accordingly, a lot of effort has been invested into immunoprecipitation of FLS2:myc by anti-myc antibodies. Interestingly, the initial experiments were more successful than the later following experiments. Westerns with immunoprecipitations by 9E10 monoclonal anti-myc antibodies, kindly provided by Lothar Lindemann, FMI, using several ml of solubilized extracts, have shown clear crossreacting bands in the initial experiments, though the immunoprecipitated bands were fainter than the analogous bands in the

crude extract suggesting, that immunoprecipitation did not contribute to enrichment of FLS2:myc (data not shown). Commercial (Sigma) 9E10 antibodies were also successful inasmuch some very faint crossreacting bands were visible. Silver- and colloidal coomassie blue stained gels of these immunoprecipitated bands revealed, that FLS2 was not visibly stained, but there was a very high background of stained nonspecific proteins (data not shown), suggesting, that much optimization is needed to allow identification of interacting proteins. Later on, many immunoprecipitation attempts have been unsuccessful with a new batch of 9E10 antibodies that were produced in bulk volumes by Susanne Schenk and Michel Siegmann (FMI, monoclonal antibodies) and purified by myself on protein A columns. These antibodies were successful in western blots. In the face of unsuccessful immunoprecipitation attempts I also tried to perform immunoaffinity chromatography with several mg of these antibodies, with no more success, either. It is not clear if these antibodies were less suitable for immunoprecipitation, or if the problem was connected to solubilization difficulties. In fact, binding experiments revealed that recent solubilizations were much less effective than previous solubilizations. Only about one tenth of the binding sites got solubilized relative to previous experiments. In the recent batches specific binding was at the detection limit. Still, when I re-used an old solubilized extract stored at -80°C for several month with high concentration of binding sites, with which I had carried out successful immunoprecipitation ([Fig II/13](#)), a repeated immunoprecipitation was unsuccessful. Other labs working with monoclonal and polyclonal anti-myc antibodies often complained that the quality of the antibodies greatly changed from one batch to the other and that they often were completely useless (lab George Thomas (FMI) and Tina Romeis (Colonne; MPI), personal communication).

Polyclonal anti-myc antibodies (Upstate) were very useful for western blots. Whereas 9E10 could be diluted only 1000 fold to allow detection of FLS2:myc, polyclonal antibodies (Upstate) had an optimal signal/background ratio when they were diluted 1:10,000. However, two attempts to immunoprecipitate FLS2:myc with these polyclonal antibodies failed as well.

In summary, immunoprecipitation of FLS2:myc with anti-myc antibodies was a very frustrating experience, that did not come up to our expectations. I would not recommend carrying on with these experiments. We do not know if difficulties were due to

problems with detergents or with the used batch of antibodies. Since immunoprecipitation did not contribute to significant enrichment of FLS2:myc in any - even successful- experiments, it might not be reasonable to try to optimize immunoprecipitation with anti-myc antibodies. When somebody chooses to carry out similar experiments, it would be advisable to engineer a new transgenic protein with a tag that is reported to be useful for purifications (for instance, the tandem affinity purification (TAP) tag, consisting of a protein A tag, a cleavage site for the TEV protease, and the FLAG epitope), to add the tag to the N-terminus and to drive the construct by the native FLS2 promoter. Co-transfection of such a construct with the old FLS2:myc-construct could elucidate, if FLS constitutes homodimers, constitutively or upon elicitation, when co-precipitation of differentially tagged FLS2 molecules can be proven.

I think, it is still worthwhile to try another time to raise antibodies against FLS2 itself, since this was possible for other RLKs. For example, there is a functional anti-BRI1-antibody raised against the first 106 amino acids excluding the signal peptide, that was expressed in *Escherichia coli* and purified as a maltose binding protein (MBP) fusion. This MBP-BRI1N fusion protein was used as an antigen for generating the anti-BRI1N antibodies in rabbits, and for affinity purification of the antibodies (Wang et al, 2001). Silke Robatzek identified a null allele of FLS2 containing a t-DNA insertion in the FLS2-promotor region (Silke Robatzek, personal communication). This null-allele could serve as a negative control to confirm that the anti-FLS2-antibody crossreacts with FLS2.

### *II.4.7.2. Heterologous expression of FLS2:myc*

Heterologous expression of FLS2 would be, additionally to immunoprecipitation of tagged FLS2, a great tool to show specific flagellin binding on cells, that formerly did not bind flagellin. Such attempts with FLS2 (without tag) have been carried out with yeast, *E. coli* and rice cells by Lourdes Gómez-Gómez and Thomas Meindl (personal communication), but flagellin binding was not detectable. However, since no antibodies for detection were available, it was not clear, if expression of FLS2 was successful. Therefore, it would be worthwhile to repeat and optimize these experiments with tagged FLS2. Indeed, two attempts have been carried out, to express FLS2:myc in rice cells.

## Chapter II: Discussion

Unfortunately - in contrast to *A. thaliana* and Msk8 cells-, kanamycin did not have a selective effect on these cells. Meanwhile we identified another rice cell culture line that is more sensitive to kanamycin and will provide the prerequisite to retry this approach.

## (E) Final discussion

At the time when this work was started, a highly sensitive perception system for bacterial flagellin in plant cells had been described (Felix et al, 1999), and *FLS1* and *FLS2*, had been identified in *A. thaliana* as essential loci for flagellin perception (Gómez-Gómez et al, 1999; Gómez-Gómez and Boller, 2000). In addition, radioactive binding studies had been established for tomato to characterize the flagellin binding site (Meindl et al, 2000). It became necessary to establish binding studies for *A. thaliana* as well, in order to assess if mutations in *FLS2*, causing insensitivity to flagellin, correlated with impairment in binding. Beyond the biochemical characterization of the flagellin binding site in *A. thaliana*, the goal of this work was to find out if the putative flagellin receptor *FLS2* is the flagellin binding site.

The *A. thaliana* flagellin binding site was found to exhibit the biochemical characteristics of a bona fide flagellin receptor: Binding was saturable and exhibited high affinity. It exhibited specificity for flagellin-derived peptides with biological activity as agonists or antagonists of the elicitor responses. Activation of flagellin receptor in *A. thaliana* appeared to occur as a two step process according to the address-message concept with the N-terminal part required for binding (address) and the C-terminal part for activation (message), as proposed for tomato before (Meindl et al, 2000). Additionally, sensitivity to salt and pH were determined, and reversibility was found to increase during cell fractionation, indicating that disassembly of a receptor complex or loss of cofactors take place. Furthermore, it was concluded that the flagellin binding site is localized at the plasma membrane. Comparison between the characteristics of flagellin binding in *A. thaliana* and tomato revealed that they show clear overall similarity but exhibit characteristic differences in detail, for instance in specificity, reversibility and sensitivity to pH and salts.

The elution pattern from the ion exchange column indicated that two subclasses of the binding site occur. Concanavalin A chromatography showed, that the binding site is glycosylated, and optimization of ligand affinity chromatography paved the way for the identification of the binding site in future.



Binding assays in extracts of different ecotypes and *La-er FLS2* mutants showed a tight correlation between the presence of the binding site and sensitivity to flagellin, providing further evidence that the characterized binding site acts as the physiological receptor. Moreover, these results showed the pivotal role of FLS2 for flagellin binding. One mutation causing impairment of flg22 binding was localized in the LRR domain, indicating that this site might be involved in direct flagellin binding. Surprisingly, four mutations that disrupted binding, affected the cytoplasmic kinase domain. The significance of this finding is not understood yet. We speculated that the kinase activity might be important for proper targeting of FLS2 or for formation of a functional receptor complex.

In order to prove that FLS2 is the flagellin binding site, epitope-tagged FLS2 was introduced into *A. thaliana* and tomato cell cultures and plants. When introduced into *fls2* mutants, this construct complemented the mutation. However, properties of FLS2:myc protein, detected by immuno blot techniques, clearly differed from the properties of the flagellin binding site. Nevertheless, specificity of *FLS2:myc* transgenic tomato cell cultures for flagellin-derived peptides carried characteristic traits of *A. thaliana* binding and elicitor-response. This finding suggests that FLS2 determines specificity of flagellin perception. Another interesting aspect of this finding is that FLS2 seems compatible with tomato signal transduction components. It will be interesting to find out which part of FLS2 is responsible for the differences of flagellin perception, and which part is conserved in the two species.

Although the results presented in this work clearly demonstrate an essential role of FLS2 for flagellin binding, direct evidence that FLS2 is the flagellin binding site is still lacking. Binding studies with heterologously expressed FLS2 could contribute to clearing this point. Also, optimization of the experimental conditions in order to prove flagellin binding by solubilized FLS2 could be reasonable. Alternatively, purification of the flagellin binding site would represent an independent approach. This method, like partial purification of FLS2, could provide further information's about additional components of the receptor complex. It is likely that several components are needed for flagellin perception, as found for the CLAVATA and the self-incompatibility perception systems (for review see introduction).

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## (G) Appendix

### Abbreviations

Avr	avirulence
bp	base pair
BSA	bovine serum albumine
cDNA	complementary DNA
CMV	cauliflower mosaic virus
Col-0	ecotype Columbia
Con A	Concanavalin A
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1]decan}-4-yl)phenyl phosphate
Da	Dalton
ddH <sub>2</sub> O	bidistilled H <sub>2</sub> O
DEAE	diethylaminoethyl
DMSO	dimethylsulfoxide
DSP	dithiobis(succinimidylpropionate)
DSS	Disuccinimidyl suberate
DTT	DL-dithiothreitol
ECM	extracellular matrix
EDTA	ethylenedinitro-N,N,N',N'-tetraacetic acid
EGS	ethylene glycolbis(succinimidylsuccinate)
EGTA	ethyleneglycerol-bis-(β-aminoethyl)-N,N,N',N'-tetraacetic acid
EMS	ethyl methan sulfonate
flg	flagellin peptide
FLS	flagellin sensing
FW	fresh weight
Laer	ecotype Landsberg
LRR	leucine rich repeat
MES	2-Morpholinoethanesulfonic acid Monohydrate
MSMO	Murashige and Skoog Minimal Organics
P	pellet
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEI	polyethyleneimine
PMSF	phenylmethylsulfonyl fluoride
R	resistance
RLK	receptor-like kinase
rpm	rotation per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
T-DNA	transferred DNA
TFMS	trifluoromethanesulfonic acid
Tris	tris[hydroxymethyl]aminomethan
S	supernatant
TLCK	N-alpha-tosyl-L-lysyl-chloromethyl ketone

v/v	volume per volume
Ws-0	ecotype Wassilevskija
Wt	wildtype
w/v	weight per volume

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