Identification of receptor complex components and receptor activation mechanisms in plant innate immunity

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Prof. Dr. Thomas Boller, Prof. Dr. Georg Felix

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Prof. Dr. Eberhard Parlow, Dekan

Table of contents

List of abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ACS	ACC-synthase
ARF	ATP-ribosylation factor
BAK1	BRI1 associated kinase 1
BRI1	Brassinosteroid intensive 1
BIK1	Botrytis-induced kinase
BIR1	BAK1 interacting receptor-like kinase
CDPK	Calcium-dependent protein kinase
CC	Coiled-coil
CERK1	Chitin elicitor receptor kinase 1
CEBiP	Chitin elicitor binding protein
CLV1	CLAVATA 1
CTR1	Constitutive triple response 1
DAMP	Damage-associated molecular pattern
dpi	Days post infiltration
EFR	EF-Tu receptor
EF-Tu	Elongation factor Tu
EIX	Ethylene-inducing xylanase
ET	Ethylene
ETI	Effector-triggered immunity
ETR1	Ethylene receptor 1
FLS2	Flagellin-sensing 2
FIL	FLS2 interacting LRR-RLK
FRK1	Flg22 induced receptor like kinase 1
GBP	Glucan-binding protein
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HA	Hemagglutinin
HR	Hypersensitive Response
IRAK	Interleukin-1 receptor-associated kinase
ISR	Induced systemic resistance
IP	Immunoprecipitation
JA	Jasmonic acid
KAPP	Kinase associated protein phosphatase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MALDI	Matrix-assisted laser desorption/ionization
MAMP	Microbe-associated molecular pattern
MAP-kinase	Mitogen-activated protein kinase

MEKK	Mitogen-activated protein kinase kinase
MBB	Myeline basic protein
MPK	Mitogen-activated protein kinase
MS	Murashige and Skoog
MyD88	Myeloid differentiation primary-response gene 88
NBS	Nucleotide-binding site
NOD	Nucleotide binding and oligomerization domain
PAMP	Pathogen-associated molecular pattern
PEPR	Pep-receptor
PG	Peptidoglycan
PR	Pathogenesis-related
PRR	Pattern-recognition receptor
PTI	Pattern-triggered immunity
RAB	Ras-related in brain
RALF	Rapid alkalinization inducing factor
RbohD	Respiratory burst oxidase homologues D
RIP	Receptor-interacting protein
RK	Receptor kinase
RLK	Receptor-like kinase
SA	Salicylic acid
SAR	Systemic acquired resistance
SERK	Somatic embryogenesis receptor-like kinase
SNARE	Soluble NSF attachment protein receptors
Syp	Synataxin of plants
TIR	Toll/Interleukin receptor
TLR	Toll-like receptor
VAMP	Vesicle associated membrane protein
YFP	Yellow fluorescent protein

1 Summary

Plants rely on an innate immune system which successfully recognizes and restricts pathogenic microbes. The key for this defense is the detection of pathogen derived non-self signatures and endogenous elicitors released during a microbial attack.

Here we report the identification of PEPR2, a new receptor for endogenous elicitors in Arabidopsis (chapter 1). Together with its homologue PEPR1 it functions redundantly in the recognition of *At*Pep1, a plant derived peptide released during wounding and pathogen defense. Our analysis showed that the defense signaling triggered upon *At*Pep1 stimulation exhibits strong similarity to the response to microbe derived elicitors.

For detection of pathogen derived elicitors the flagellin perception through the receptor FLS2 evolved as model system in plants. FLS2 is known to function together with an associated receptor-like kinase referred to as SERK3/BAK1. In an *in vitro* analysis of the FLS2-kinase and the BAK1-kinase we were able to show, that FLS2 is a substrate for the BAK1-kinase. This indicates that BAK1 acts as upstream kinase, which phosphorylates and activates the receptor upon dimerization (chapter 2). Using a mass spectrometric analysis on immunopurified FLS2 protein we identified one elicitor independent and one elicitor dependent putative phosphorylation site. The position of both sites suggests a role for phosphorylation in the regulation of ubiquitination and endocytosis.

We further analyzed the impact of receptor kinase activity by a characterization of a kinase inactive version of the EF-Tu receptor EFR (chapter 3). This analysis verified that also EFR functions through BAK1 and demonstrated that kinase activity of the receptor is not required for formation of the EFR/BAK1 complex. Strikingly, kinase inactive EFR was able to initiate an elicitor dependent ethylene accumulation and conferred partial resistance to *Agrobacterium tumefaciens*, while other signaling events were absent. This finding revealed a diverging signaling network in which not all pathways require receptor kinase activity to get activated.

By immunopurification and subsequent mass spectrometric analysis of FLS2 protein we further explored this signaling system and its components. Importantly we found not only BAK1, but also its paralogues SERK1, SERK2, SERK4 and SERK5 to co-purify with the flagellin receptor, which indicates a redundant function of these proteins (chapter 4). We also identified several isoforms of the family of 14-3-3 general regulating factors. This is in line with an *in silico* analysis of the FLS2 sequence, which predicted the putative phosphorylation site S-1078 to operate as 14-3-3 protein binding site. Another group of proteins which co-purified with FLS2 in an elicitor dependent manner comprises RAB-GTPases and SNARE proteins. These protein factors are known to control vesicle fusion events. Since bacterial infections trigger focal secretion, we speculate that the elicitor activated FLS2 complex might lead secretory vesicles directly to the site of infection.

Taken together this works provides new insight into different levels of plant immunity. This includes not only the identification of a new receptor and receptor associated proteins, but also adds new aspects to our understanding of receptor activation and downstream signaling. Therefore these results provide a basis to further investigate plant innate immunity on the whole.

2 General introduction

As green plants are the prime source of carbon and energy, plant health is of fundamental importance for life. Even if this significance is not always seen, research on disease resistance in plants is as important as medical science. And also the mechanisms which lead to resistance in both kingdoms are similar complex and fascinating. Plants developed an elaborate defense system to engage a broad range of pathogen threats from various types including fungi, oomycota, bacteria, and virus. These pathogens face a defense system which is constructed of multiple layers (Thordal-Christensen, 2003; Jones and Dangl, 2006). Most simply, physical barriers prevent pathogens from entering the plant. Pathogens which breach these barriers face a moderate and non-specific response. If a pathogen successfully evades also this obstacle, the plant provides a pathogen specific immune response, which uses strong defense mechanisms. This pathogen specific response may be even harmful for the plant itself, but it efficiently defeats potent pathogens. This layered defense mirrors an evolutionary process, in which plants developed surveillance systems with increasing specificity to trigger immune responses with increasing intensity (Fig. 2-1), while the pathogen continuously evolves new mechanisms to perturb these defense mechanism (Jones and Dangl, 2006; Boller and He, 2009).

Below we will first discuss the plants repertoire of constitutive and inducible defense mechanism, the weaponry of the plant which is used during the different immune responses. Thereafter we will focus on the plants surveillance systems and the immune responses which are triggered through these.

2.1 The repertoire of plant defense mechanisms

Physical barriers play a major role in protecting plants from invading pathogens. These constitutive defense structures like the cutin layer and lignified cell walls restrain most microorganisms from entering and infecting the plants tissue (Thordal-Christensen, 2003).

Additionally infection is hindered by the deposition of antimicrobial enzymes, peptides, or toxic secondary metabolites, which provide a chemical obstruction (Heath, 2000; Dixon, 2001). To breach these barriers, pathogens can invade their host plant in a number of ways, e.g. by penetration of intact surfaces trough secretion of cell wall degrading enzymes or by entering through natural openings, such as stomata and hydathodes or alternatively through woundings (Dickinson and Lucas, 1977).

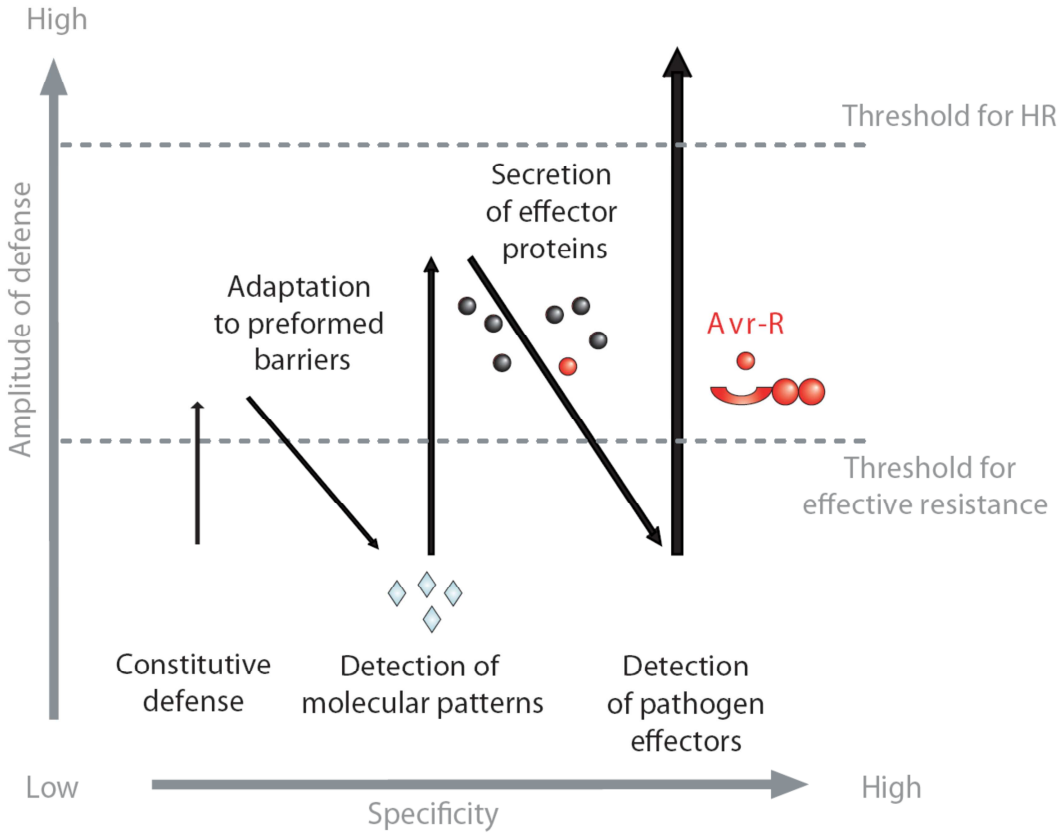


Fig. 2-1 A zigzag model explains the correlation of different response outputs and recognition events. Plants use constitutive mechanisms to restrict growth of the majority of pathogens. Adapted pathogens are able to overcome this barrier e.g. by secretion of lytic enzymes. Plants, in turn, recognizes molecular patterns (depicted as diamonds) which are common to a broad range of microbes, subsequently followed by the activation of a moderate defense response. This arms race proceeds by secreting effector proteins (depicted as beads) which interfere with the activation of plant immune responses. Detection of effector proteins by the plants surveillance system leads then to the activation of a strong defense reaction which often includes a cell death reaction. Adapted from Jones and Dangle, 2006.

As soon as bacterial pathogens are sensed, plants can close stomata openings to prevent entry of the microbes (Melotto *et al.*, 2006). In addition a plant cell can respond by *de novo* cell wall biosynthesis and by local deposition of the newly synthesized cell wall material and formation of papillae (Fig. 2-2). These papillae, callus structures beneath the

infection sites, present the ultimate barrier to stop invading microbes (Aist, 1976; Bestwick *et al.*, 1995; Soylu *et al.*, 2005). Plants which lack components of the secretion system are more susceptible to bacterial and fungal pathogens (Kalde *et al.*, 2007; Kwon *et al.*, 2008). The composition of papillae is highly variable but they commonly contain callose, phenolics, lignin, cellulose, pectin, suberin, chitin and lipids, but also proteins like hydroxyproline-rich glycoproteins or peroxidases (Schmelzer, 2002). The deposition of this material is accompanied by the generation of reactive oxygen species (ROS) which serves the function of papilla strengthening by driving cross-linking reactions, and direct intoxication of the pathogen (Lamb and Dixon, 1997). Interestingly, ROS functions also as second messenger in pathogen related signaling pathways as we will discuss more extensively later.

Plants also secret antimicrobial proteins and peptides or toxic compounds like phytoalexins (Darvill and Albersheim, 1984; van Loon and van Strien, 1999). Evidence has been provided in a number of studies that these *de novo* synthesized antimicrobial compounds not only accumulate at the site of infection, but do so quickly enough and in sufficiently high concentrations to inhibit the growth of fungi and bacteria (Darvill and Albersheim, 1984; Dixon, 2001; Meyer *et al.*, 2009). The unconventional myrosinase PEN2 for example produces toxic indole glucosinolate hydrolysis products which are believed to be transported into the apoplast by an ABC-transporter (Stein *et al.*, 2006b; Bednarek *et al.*, 2009). Lack of either myrosinase or ABC-transporter in *Arabidopsis thaliana* results in significantly increased susceptibility against the powdery mildew *Blumeria graminis* (Lipka *et al.,* 2005).

The most drastic reaction to an attempted invasion is rapid development of cell death at and immediately surrounding infection sites, called the Hypersensitive Response, or HR (Morel and Dangl, 1997; Mur *et al.*, 2008). HR occurs within a few hours and the biochemical and metabolic plant modifications are well conserved among different plant-microbe interactions. Besides isolating healthy tissue from the infected site, this reaction deprives biotic pathogens of their nutrition basis and provokes the release of toxic compounds (Morel and Dangl, 1997).

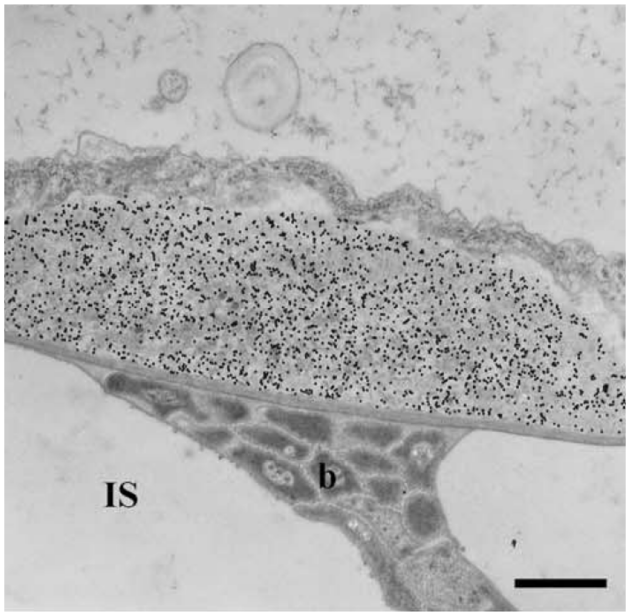


Fig. 2-2 Papilla formation during bacterial infections. Leaves of Arabidopsis wild-type plants were challenged with a non-host *Pseudomonas* strain (Pph RW60). The picture illustrates the deposition of layered papillae along the cytoplasmic face of a mesophyll cell next to a bacterial micro colony (b) 48 h after inoculation. Callose was localized by immunogold labeling with a monoclonal antibody. IS, intercellular space; bar = 1 mm. Adapted from Soylu *et al.* (2005).

2.2 A multitude of surveillance systems activates different immune responses

The different defense mechanisms which were stated in the proceeding paragraphs represent only tools which are employed by distinct immune responses. However, the prerequisite of a well adapted defense is the detection of pathogenic threats through the plants surveillance system and the coordinated activation of different defense mechanisms as part of an immune response. Here we want to discuss different surveillance systems, sensed elicitors, and the immune responses which are triggered by such.

2.2.1 Detection of microbial intruders via microbe associated molecular patterns (MAMPs)

Since in plants never an adaptive immune system evolved, they rely on preformed receptors for the detection of microbes. Considering the diversity and number of different plant pathogens this presents a difficult obligation. A possibility to face this challenge is the detection of microbe associated molecular patterns (MAMPs), historically also termed

PAMPs for pathogen associated molecular patterns. These strictly non-self molecular signatures can be found among a broad range of microbes (Medzhitov and Janeway, 2004; Boller, 2005; Boller and Felix, 2009). Perception of such widespread elicitors allows to sense multiple pathogens at once with only few receptors. A further characteristic of MAMPs is their invariance. Since they are in general essential to the microbial vitality, it is not easily possible for a pathogen to avoid recognition by elimination of its MAMP repertoire. Conversely, the chemical nature of these microbial 'non-self' elicitors can be very diverse. Some plants are able to recognize chitin molecules, found in fungal cell walls, other detected MAMPs are lipopolysaccharides, peptidoglycans, derived from the bacterial cell wall, or various conserved peptide epitopes within different important microbial proteins as illustrated in table 1-1 in a more complete manner (Silipo *et al.*, 2005; Gust *et al.*, 2007).

MAMPs/PAMPs activate a so called PAMP triggered immunity (PTI), which should be redefined as pattern triggered immunity as we will see later. In principle this immune response employs all response mechanisms which were discussed above, but their extent and intensity is low compared to pathogen specific immune responses. In particular the Hypersensitive Response is only rarely seen during PTI, hence this defense response is without harm to the cell. Together MAMP perception and PTI can be described as the recognition of a high number of pathogens on basis of wide spread molecules, which triggers a basic defense program.

Plants rely on an array of plasma membrane borne receptors to detect MAMPs and to activate PTI. These so called pattern recognition receptors (PRRs) differ strongly in their structure, but most of the known PRRs belong to the class of receptor-like kinases (RLKs). These type I transmembrane proteins are characterized by an extracellular ligand binding domain which is linked via a single transmembrane domain to a serine/threonine kinase in the cytoplasm (Morillo and Tax, 2006). The Arabidopsis genome codes for more than 600 of such RLKs and even twice as many are found in rice (Shiu and Bleecker, 2001; Shiu *et al.*, 2004). Several of these may function as PRRs, providing the genetic basis for the detection of diverse MAMPs. In addition, receptor like proteins, which lack a cytoplasmic kinase, and

membrane associated proteins in the apoplast contribute to pattern recognition (Albert *et al.*, 2010). Up to now only a few of these receptors are well characterized, which are discussed below.

2.2.1.1 FLS2 and flg22 the paradigm of PTI

The pattern recognition receptor Flagellin Sensing 2 (FLS2) and its ligand, the bacterial flagellin protein, represent probably one of the best studied models for pattern recognition (Felix *et al.*, 1999; Gómez-Gómez *et al.*, 1999). Obviously flagellin fulfills all requirements to be an excellent MAMP. Bacteria depend on its function to actively approach their host plant; it is present in ample amounts and not found in plants. FLS2 recognizes a 22 amino acid stretch at the N-terminus of the flagellin protein, which is highly conserved in eubacteria. That this recognition can be crucial for plant resistance was demonstrated by the examination of infections on Arabidopsis with the bacterial non-host strain *Pseudomonas syringae* pv. *tomato* DC3000 (Zipfel *et al.*, 2004). In wild-type plants this pathogen does not cause significant symptoms, but when sprayed on *fls2* deficient Arabidopsis mutants, plants were heavily affected by the pathogen (Zipfel *et al.*, 2004). Only few pathogens, e.g. *Agrobacterium tumefaciens,* bypass recognition by FLS2 through variations in the corresponding flg22 epitope (Felix *et al.*, 1999). Since FLS2 is localized in the plasma membrane of all analyzed tissues (roots, rosette leaves, stems, and flower petals) it may therefore confer resistance against bacteria in rhizosphere and phyllosphere (Robatzek *et al.*, 2006).

Structurally the FLS2 receptor belongs to the class of RLKs, and uses an extracellular LRR (leucine-rich repeat) domain with 28 LRR modules for ligand binding (Chinchilla *et al.*, 2006). According to the current understanding the flg22 elicitor binds to the LRR domain via its N-terminus, while the C-terminal part of the elicitor induces a conformational change, leading to the activation of the receptor. Flg22 derivates, lacking the two C-terminal amino acids, therefore still bind to the receptor, but fail to activate FLS2 mediated responses in Arabidopsis (Felix *et al.*, 1999; Chinchilla *et al.*, 2006).

A serine/threonine kinase domain, which descends from an ancestor of animal IRAK/PELLE like cytoplasmatic kinases, is located within the cytosolic portion of the FLS2 protein (Shiu and Bleecker, 2001). Absence of flg22 binding in Arabidopsis *fls2-17* mutants, coding for a kinase inactive FLS2 variant, led to the hypothesis that kinase activity of FLS2 is required for flagellin binding (Gómez-Gómez *et al.*, 2001). However, a subsequent study demonstrated lack of FLS2 protein accumulation in *fls2-17* plants, which fully explains the absence of flg22 binding (Chinchilla *et al.*, 2006). Conversely expression of the extracellular LRR domain is fully sufficient to bind the flg22 elicitor (Seraina Beeler *et al.*, unpublished).

The flagellin receptor is present in all lineages of seed plants (Boller and Felix, 2009; Albert *et al.*, 2010), indicating that this perception system was developed early in the evolution of plants. This conservation of FLS2 in many plants, but also the possibility to use chemically synthesized, highly pure peptides as ligand for this receptor, makes FLS2 an ideal model to study PTI.

2.2.1.2 Perception of the bacterial translation factor EF-Tu is similar as the perception of flg22 through the FLS2 receptor

Also the bacterial translation factor EF-Tu is a proteinaceous MAMP and similar to flagellin it is highly conserved and abundant in bacteria (Kunze *et al.*, 2004). The corresponding receptor EFR (EF-Tu receptor) recognizes an 18-26 amino acid long epitope (elf18/elf26) (Zipfel *et al.*, 2006). In contrast to agrobacterial flg22 the EF-Tu derived from *Agrobacterium tumefaciens* is detected by the Arabidopsis EFR receptor, which confers resistance to this pathogen (Kunze *et al.*, 2004; Zipfel *et al.*, 2006). Arabidopsis *efr* mutants, lacking a functional EFR protein, are more susceptible to *Agrobacterium,* hence the pathogen causes chlorosis development on *efr* plants and can transfer its T-DNA more successfully into *efr* plant than into Arabidopsis wild-type plants (Zipfel *et al.*, 2006).

Structurally EFR is also an LRR-RLK with an IRAK/Pelle-like serine/threonine kinase and belongs, like FLS2, to the subfamily LRR-XII of RLKs (Shiu *et al.*, 2004). Interestingly, the underlying signaling mechanism for both receptors seems to be conserved: *Nicotiana benthamiana* plants, which do not have an EFR receptor naturally, respond to the elf18 elicitor as soon as they express a transgenic EFR receptor (Zipfel *et al.*, 2006). But despite

MAMP	Description	Pathogen source(s)	Examples of perceiving plants	Reference
Cold shock protein	Constitutively expressed conserved bacterial protein	Bacteria	Tobacco	(Felix and Boller, 2003)
Elongation factor-Tu	Const. expressed conserved bacterial protein	Bacteria	Brassicaceae	(Kunze *et al.*, 2004)
Flagellin	Main component of the flagellum	Bacteria	Various plants	(Felix *et al.*, 1999; Meindl *et al.*, 2000; Albert *et al.*, 2010)
Lipopolysaccharides	Component of the bacterial cell wall	Bacteria	Arabidopsis, rice	(Newman *et al.*, 1997; Zeidler *et al.*, 2004; Silipo *et al.*, 2005; Desaki *et al.*, 2006)
Peptidoglycan	Component of the bacterial cell wall	Bacteria	Arabidopsis	(Erbs *et al.*, 2008)
Rhamnolipid	Glycolipidic biosurfactant	Bacteria	Grapevine	(Varnier *et al.*, 2009)
Superoxide dismutase	Major protein in the bacterial secretomes	Bacteria	Solanaceae	(Watt *et al.*, 2006)
Cerebrosid	Fungal sphingolipid	Fungi	Rice	(Koga *et al.*, 1998; Umemura *et al.*, 2000; Umemura *et al.*, 2002)
Chitin	Component of fungal cell walls	Fungi	Tomato, Arabidopsis, grapevine	(Felix *et al.*, 1993; Aziz *et al.*, 2006)
Ergosterol	Main fatty acid of basido- and ascomycetes	Fungi	Tomato, tobacco	(Granado *et al.*, 1995; Kasparovsky *et al.*, 2004)
Xylanase	Hemicelluloses degrading enzyme	Fungi	Tobacco, tomato	(Rotblat *et al.*, 2002)
β-glucan / oligosaccharides	Cell wall component	Oomycota, fungi	Fabaceae, rice, tobacco	(Sharp *et al.*, 1984; Cosio *et al.*, 1988; Klarzynski *et al.*, 2000; Yamaguchi *et al.*, 2000)
Arachidonic acid	Main fatty acid of Oomycota	Oomycota	Potato	(Preisig and Kuc, 1985)
Elicitin	Sterol-binding proteins	Oomycota	Tobacco	(Baillieul *et al.*, 2003)
Transglutaminase	Prominent protein in fungal cell walls	Oomycota	Parsley, potato	(Nürnberger *et al.*, 1994; Brunner *et al.*, 2002)

Table 2-1 Selected MAMPs perceived by different plant species.

this sum of parallels, differences between both systems exist: elf18/elf26 responsiveness is restricted to *Brassicaceae (Zipfel et al., 2006)*, indicating that EFR is a more recent development in plants. In addition FLS2 and EFR differ in their requirements for ER-quality control components (Lu *et al.*, 2009b; Nekrasov *et al.*, 2009). While plants which are defective in this system, which ensures the correct protein folding and glycosylation within the ER, do not accumulate functional EFR, flg22 perception and signaling is not influenced by such mutations (Lu *et al.*, 2009b; Nekrasov *et al.*, 2009).

2.2.1.3 Other receptors in MAMP perception

A close homologue of the Arabidopsis receptors FLS2 and EFR is the Xa21 protein in rice (Song *et al.*, 1995). It belongs to the same class of LRR-RLKs and contains a similar serine/threonine kinase in its cytoplasmic portion (Song *et al.*, 1995; Dardick and Ronald, 2006). The MAMP which is recognized through Xa21 is a sulfateted 17-amino acid epitope within Ax21 (Activator of Xa21), a protein derived from *Xanthomonas* bacteria with still unknown function (Lee *et al.*, 2009). This MAMP is highly conserved in different *Xanthomonas* strains and its perception confers immunity to most strains of the bacterium *Xanthomonas oryzae* pv. *oryzae (Song et al., 1995; Lee et al., 2009).*

Additional ligand/receptor couples (Fig. 2-3) are known in plants and several of these do not follow the paradigm of FLS2 and EFR (reviewed in Albert *et al.* 2010). The chitin oligosaccharide elicitor-binding protein CEBiP in rice detects chitin molecules derived from fungal pathogens, but it does not contain any serine/threonine kinase domain. Conversely, CERK1, which functions in chitin recognition in Arabidopsis plants, does include a kinase but direct interaction with the chitin ligand has not been shown yet (Kaku *et al.*, 2006; Miya *et al.*, 2007).

1,3-β-branched heptaglucoside, a MAMP present in cell walls of oomycetal pathogens, is recognized by the specific and high affinity binding site of the β-glucan-binding protein in *Fabaceae*, which in turn is part of a proposed receptor complex (Fliegmann *et al.*, 2004). Also EIX1 and EIX2, two tomato receptors for the fungal elicitor ethylene-inducing xylanase (EIX), function without kinase (Ron and Avni, 2004). This raises the question, how

important kinases are in PRRs. Do receptor-like proteins, which function as pattern binding sites, recruit additional kinases to transmit a signal or do they use kinase independent mechanisms?

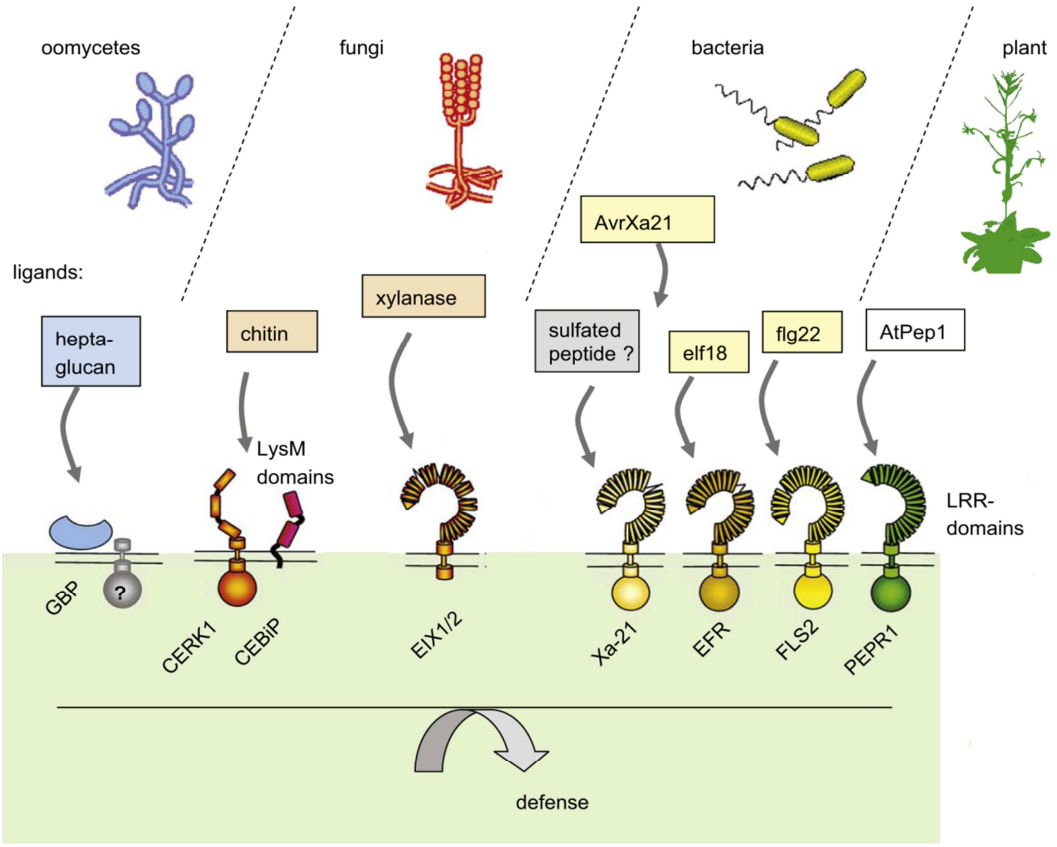


Fig. 2-3 Ligand/receptor pairs involved in pattern recognition by plants. The scheme depicts selected MAMP/DAMPs and their corresponding receptors. The glucan-binding protein (GBP) (Umemoto *et al.*, 1997; Fliegmann *et al.*, 2004) does not contain a trans membrane domain; CERK1 (Felix *et al.*, 1993; Miya *et al.*, 2007) from Arabidopsis and CEBiP (Kaku *et al.*, 2006) from rice represent essential components for perception of chitin fragments. EIX1/2 (Rotblat *et al.*, 2002; Ron and Avni, 2004) represent examples for LRR-receptors lacking a cytoplasmic kinase domain. Xa21 (Song *et al.*, 1995; Lee *et al.*, 2009) from rice, detects a bacterial signal specifically modified by the AvrXa21 protein of bacteria. FLS2 (Felix *et al.*, 1999; Gómez-Gómez and Boller, 2000) and EFR (Kunze *et al.*, 2004; Zipfel *et al.*, 2004) perceive proteinaceous MAMPs from bacteria and *At*PEPR1 (Huffaker *et al.*, 2006; Yamaguchi *et al.*, 2006), a DAMP receptor, detects endogenous peptide ligands. Adapted from Albert *et al.* (2010).

2.2.1.4 Pattern recognition receptors in animals

During vertebrate evolution animals developed an adaptive immune system which is characterized by its ability to adapt specifically to new pathogens. However, this antibody based immune-system reacts slowly; hence also mammals rely on MAMP/PAMP based detection systems to monitor and defend invading pathogens. The assortment of detected MAMPs includes dsRNA, ssRNA or unmethylated CpG motives in DNA, which are not

known to act as MAMPs in plants, but also MAMPs like β–glucans, LPS or bacterial flagellin (table 2-2), which are very similar to MAMPs detected by the plant surveillance systems (Zipfel and Felix, 2005; Mogensen, 2009).

The corresponding PRRs, responsible for their detection, are diverse in their structure and localization. However, the most prominent group is represented by the membrane localized toll-like receptors (TLRs). These type I transmembrane receptors, are characterized by an extracellular LRR domain and an intercellular Toll/IL-1 receptor (TIR) domain (Medzhitov and Janeway, 2000). In particular their ligand binding domain is similar to the LRR-RLKs found in plant immunity, and especially the human flagellin receptor TLR5, provokes comparisons with the LRR-RLK FLS2, because of overlap in both, ligand and receptor (Ausubel, 2005; Zipfel and Felix, 2005). However, the epitope on the flagellin protein recognized by TLR5 differs from flg22 which is detected by FLS2 (Fig. 2-4) and both receptor systems developed independently (Felix *et al.*, 1999; Smith *et al.*, 2003; Boller and Felix, 2009).

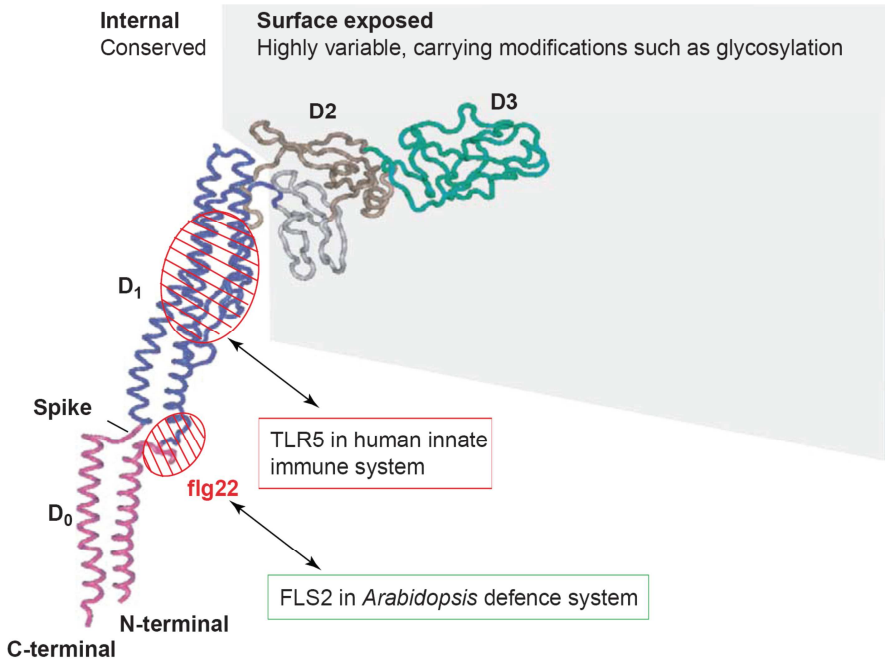


Fig. 2-4 Flagellin derived MAMPs in mammals and plants. Structure of a flagellin monomer from *Salmonella typhimurium*, red-shaded areas indicate domains which are recognized by mammalian TLR5 or Arabidopsis FLS2. Adapted from Zipfel and Felix (2005).

Receptor	Cellular location	Ligand(s)	Origin(s)
Toll like receptors			
TLR1/TLR2	Cell surface	Triacyl lipopeptides	Bacteria
TLR2/TLR6	Cell surface	Diacyl lipopeptides	Mycoplasma
		Lipoteichoic acid	Gram-positive bacteria
TLR2	Cell surface	Lipoproteins	Viruses
		Peptidoglycan	Bacteria
		Lipoarabinomannan	Mycobacteria
		Porins	*Neisseria*
		Envelope glycoproteins	Viruses
		GPI-mucin	Protozoa
		Phospholipomannan	Candida
		Zymosan	Fungi
TLR3	Cell surface endosomes	dsRNA	Viruses
TLR4	Cell surface	LPS	Gram-negative bacteria, viruses
		Mannan	Fungi
		HSP70	Host
TLR5	Cell surface	Flagellin	Bacteria
TLR7/8	Endosome	ssRNA RNA	Viruses
TLR9	Endosome	CpG DNA	Viruses, bacteria, protozoa
Retinoid acid-inducible gene I like receptors			
RIG-I	Cytoplasm	dsRNA (short)	Viruses
MDA5	cytoplasm	dsRNA (long)	Viruses
Nucleotide-binding oligomerization domain like receptors			
NOD1	Cytoplasm	Diaminopimelic acid	Gram-negative bacteria
NOD2	Cytoplasm	Muranyl dipeptide	Gram-positive and -negative bacteria
NALP1	Cytoplasm	Muranyl dipeptide	Gram-positive and -negative bacteria
NALP3	Cytoplasm	ATP, uric acid crystals, RNA, DNA	Viruses, bacteria, and host
Miscellaneous			
DAI	Cytoplasm	DNA	Viruses, intracellular bacteria
AIM2	Cytoplasm	DNA	Viruses
PKR	Cytoplasm	dsRNA, 5-triphosphate	RNA Viruses

Table 1-2 Recognition of microbial components by mammalian PRRs. Overview of mammalian receptors and their corresponding MAMP ligands. Adapted from Mogensen *et al.* (2009)

Interestingly, instead of high numbers of receptors, the mammalian system has optimized the present PRRs for the binding of different ligands. In parts this is also achieved by combinations of different receptors e.g. TLR2 can bind different ligands as homodimer or as heterodimer together with TLR1 or TLR6 (Mogensen, 2009).

2.2.2 Damage sensing as indirect mechanism to monitor pathogens

In contrast to MAMPs, which are absent from the host plant, the damage associated molecular patterns (DAMPs) arise from the plant itself trough degradation of various components in the host plant and serve as endogenous signals (Boller and Felix, 2009). These endogenous elicitors appear during infection e.g. by degradation of the cell wall or are released from dead or injured cells (Darvill and Albersheim, 1984; Matzinger, 2002). DAMP perception is therefore a surveillance system which does not detect the pathogen directly, but rather monitors events which occur during an infection.

Examples for such plant derived elicitors are oligogalacturonides, released from the plant cell wall, hydroxyproline containing glycopeptides (HypSys) and rapid alkalinization inducing factor (RALF), peptides derived from degradation of cell wall located proteins, but up to know a receptor system for these ligands is unknown (Nothnagel *et al.*, 1983; Doares *et al.*, 1995; Pearce *et al.*, 2001a; Pearce *et al.*, 2001b).

Interestingly, DAMP perception is not exclusive to plant-pathogen interaction, but the appearance of plant derived elicitor molecules is found also upon wounding during predator attack. For several years systemin, a herbivory associated 18-aa peptide in tomato, derived from a 200-aa precursor protein and the corresponding LRR-RLK, SR160/BRI1 (Systemin receptor 160kDa/brassinosteroid insensitive 1), represented the first DAMP/RPP couple known (Pearce *et al.*, 1991; Scheer and Ryan, 2002). However, *sr160/bri1* mutant plants are still capable of conferring a systemin induced defense response, indicating that SR160/BRI1 is not the systemin receptor (Holton *et al.*, 2007; Lanfermeijer *et al.*, 2008).

Another group of DAMPs is represented by *At*Pep peptides, which are, similar to systemin, short proteinaceous elicitors, derived from small cytoplasmic precursor proteins

PROPEP1-7 (Huffaker *et al.*, 2006). The receptor of the *At*Pep DAMPs appears to be an LRR-RLK similar to FLS2 and EFR, referred as PEPR1 (Yamaguchi *et al.*, 2006). But so far, only gain of function experiments were accomplished and the analysis of *pepr1* mutants is lacking.

To the current understanding endogenous and microbial derived elicitors contribute both to PTI. If and to what extent these two systems differ is still under investigation. However, there are additional functions proposed for DAMPs e.g. as amplifier of defense response or as far distance signal, but these considerations are not supported by significant evidence yet (Huffaker and Ryan, 2007; Vlot *et al.*, 2008). Further experiments need to show whether MAMP and a DAMP induced immunity represent independent immune responses or if both can be summed to pattern triggered immunity.

2.2.3 Effector triggered immunity - a pathogen specific defense response

Many plant pathogens manipulate their host through the secretion so called effector proteins into apoplast or cytoplasm of the host cell (Staskawicz *et al.*, 1984; van Kan *et al.*, 1991; de Wit *et al.*, 2009). A major object for these effector proteins is to interfere with the plants immune system (Studholme *et al.*, 2009).

This evolutionary advancement of the pathogen is a prerequisite to breach PTI and to allow a successful infection. An example of a bacterial effector is the *Pseudomonas syringae* effector AvrPtoB which is injected into the plant cell via a type three secretion system, where it targets FLS2, CERK1 and possibly other PRRs for degradation as well as it prevents their oligomerization with other proteins to impede plant immunity (Göhre *et al.*, 2008; Shan *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009).

But effector proteins are a double-edged sword, since they perturb PTI and pave the way for infection, but they also represent an ideal target for a pathogen specific surveillance system (Fig. 2-1). The plant recognizes such pathogen-secreted effector proteins, which is subsequently followed by the activation of the so called effector triggered immunity (ETI). This immune response is an accelerated and amplified PTI

response, which uses a similar set of defense mechanisms but includes usually HR. However, it is difficult to define ETI specific defense mechanisms, since ETI does not occur isolated and PTI is always a part of ETI (Jones and Dangl, 2006).

For the detection of effector proteins the plant uses so called R-gen (resistance gen) products. The two major types of R-gene products are plasma membrane born receptor like proteins, which detect effectors secreted into the apoplast, and intracellular NBS-LRR proteins (nucleotide binding site–leucine-rich repeat proteins) for detection of effectors secreted into the cytoplasm (Tör *et al.*, 2009).

Interestingly, only a few R-gene products function as receptors recognizing effectors through a direct interaction and the most R-gene products use indirect mechanism. A guarding-mechanism, which monitors the integrity of effector targets, allows to sense the activity of effectors instead of their presence (Dangl and Jones, 2001). An even more elaborate approach used by the plant to detect effector proteins is the use of molecular decoys, which resemble the natural target of effector proteins (van der Hoorn and Kamoun, 2008). Instead of guarding the natural effector target the integrity of such decoys is monitored. As soon an effector modifies or degrades these proteins, the R-gene product may sense this event which is followed by the activation of ETI. The tomato NBS-LRR protein Prf for example can sense the effectors AvrPto and AvrPtoB indirectly through modifications on Pto, a second host protein (Gutierrez *et al.*, 2010). To the current understanding bacteria secret AvrPto and AvrPtoB to target the kinase domain of PRRs. Pto, which is structurally strongly related to the kinases of FLS2 and other PRRs, may function as decoy to attract these effectors (van der Biezen and Jones, 1998; van der Hoorn and Kamoun, 2008). This, in turn, is sensed by the R-gene product Prf resulting in the activation of ETI (Gutierrez *et al.*, 2010). Of course the recognition of AvrPtoB perception through Pto is only one out of many examples, since pathogens developed a broad range of effectors which target very different processes in the plant cell and the detection of these effectors is similar divers accordingly.

2.3 Activation and regulation of receptors in pattern recognition

Recognition of pathogens and defense mechanisms, like callose deposition or stomata closure, are just two aspects of plant immunity. But clearly they need to be connected through a signaling system which propagates the information of pathogen presence, integrates it with other information received by the plant cell and finally triggers the optimal response.

The signaling events of plant immunity are deciphered to limited degree only. Known components include various proteinaceous factors, second messengers and plant hormones. These signaling elements represent only puzzle pieces and for the most of them it is unclear how they are connected to each other, but the increasing knowledge in PTI provides a basic understanding how immune signaling proceeds during plant immunity.

2.3.1 The function of BAK1 in the activation of pattern recognition receptors

Oligomerization of transmembrane proteins is likely to be one of the first steps in the signal transduction of pattern recognition. The flagellin perception through the pattern recognition receptor FLS2 provides an example how such an oligomerization process can proceed upon pathogen recognition. Ligand binding induces a hetero-oligomerization of FLS2 with a second RLK known as BAK1 (BRI1 associated kinase 1), and it is hypothesized that both proteins function together, to transduce the information of pathogen attack (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). This is supported by genetic and biochemical evidence: in *bak1* deficient plants the flg22 dependent generation of ROS is strongly delayed and the activation of MAP-kinases is absent (Chinchilla *et al.*, 2007). In addition a formation of a FLS2/BAK1 complex can be found as fast as 1 second after flg22 treatment and peaks after 15 seconds (Schulze *et al.*, 2010).

BAK1, also referred as SERK3 (somatic embryogenesis receptor-like kinase 3), was originally found to function during embryogenic cell formation (Hecht *et al.*, 2001). This RLK, with its short extracellular 4 LRR-repeat domain, is involved in several processes in the

plant cell, but so far it was never found to function as receptor itself. BAK1 dimerizes *in vivo* and *in vitro* with the brassinosteroid receptor (BRI1) and is required for full signaling in response to the hormone ligand brassinosteroid (Li and Chory, 1997; Li *et al.*, 2002a; Nam and Li, 2002; Wang *et al.*, 2008). A possible function of BAK1 in the regulation of photomorphogenesis may be also connected to this process (Whippo and Hangarter, 2005). In addition, mutations in the *BAK1* gene cause spreading necrosis after an infection with hemibiotroph bacteria and necrotrophic fungi, which points to an additional function in cell death regulation (He *et al.*, 2007; Kemmerling *et al.*, 2007). In Arabidopsis the five SERK-family members are highly homologues to each other and in particular the C-terminus of these proteins shows a striking conservation (Boller and Felix, 2009). To what extent different SERK-family members overlap in their functions is still under investigation, but lethality of several SERK double and triple mutants hampers a detailed analysis of this gene family (He *et al.*, 2007; Albrecht *et al.*, 2008).

Interestingly, also other LRR-RLKs which function as pattern recognition receptors were shown to function through BAK1. *In vivo* phospho-labeling experiments indicate, that EFR and PEPR1 are phosphorylated upon elicitor treatment and co-purifiy together with BAK1 (Schulze *et al.*, 2010). *bak1* mutants show also reduced sensitivity to elf18 and to *At*Pep1 (Chinchilla *et al.*, 2007; Krol *et al.*, 2010) and in addition an interaction between the cytoplasmic domains of PEPR1 and BAK1 was seen in a yeast (Postel *et al.*, 2009).

However, not all pattern recognition receptors function through BAK1. An example is provided by the perception of the fungal MAMP chitin by the Arabidopsis receptor like kinase CERK1, which is independent of BAK1 or related molecules (Gimenez-Ibanez *et al.*, 2009). Interestingly, *cerk1* mutants are not only impaired in fungal defense but are also more susceptible to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Gimenez-Ibanez *et al.*, 2009). Since also direct binding of chitin molecules to CERK1 was not shown, it is well possible that CERK1 may have a similar role as BAK1 as associated kinase in different receptor complexes.

2.3.2 Similarities and differences in flagellin and brassinosteroid perception

The finding that BAK1 functions together with the pattern recognition receptors FLS2, EFR, and PEPR1, but also with the brassinosteroid receptor BRI1, indicates common mechanisms for theses different signaling pathways. Conversely, multiple differences are found in the perception of brassinosteroids and flagellin. BRI1 forms homodimers, which are stabilized in response to ligand binding (Russinovaa *et al.*, 2004; Wang *et al.*, 2005c). In contrast, homo-dimerization is found for FLS2 neither in absence nor in presence of its ligand (Ali *et al.*, 2007). Also the interaction of FLS2 and BRI1 with the BAK1 protein follows very different kinetics, since oligomerization of BRI1 and BAK1 was shown after 90 minutes only (Wang *et al.*, 2005b; Wang *et al.*, 2008). In both systems the perception of the ligand results in phosphorylation of receptor and complex partner, but phosphorylation of the FLS2/BAK1 complex is seen already 15 seconds after elicitor application (Schulze *et al.*, 2010), while the earliest time point reported for phosphorylation of BRI1 is 10 minutes after brassinosteroid treatment (Wang *et al.*, 2005c). This phosphorylation is a prerequisite for the formation of the BRI1/BAK1 complex, since kinase inactive BRI1 is unable for hetero-dimerization with BAK1 (Wang *et al.*, 2008). Interestingly, a treatment with the kinase inhibitor K-252a can interfere with phosphorylation of FLS2 and BAK1, but does not perturb the FLS2/BAK1 complex formation (Chinchilla *et al.*, 2007; Schulze *et al.*, 2010). Taken together it seems that these receptor complexes may share common aspects, but exhibit also strong differences. Therefore recent insight in the activation of BRI1 cannot be transferred one to one on the perception of flagellin through FLS2. How exactly the activation of FLS2 and other PRRs is regulated is therefore an important object of research.

2.3.3 Non-RD kinases are found in pattern recognition receptors in plant and animal immunity

There is an additional noteworthy difference between RLKs as BRI1 compared to most of the known PRRs. EFR, Xa21 and FLS2, as well as several other receptors which are known to be involved in pathogen defense, can be grouped into a kinase-class referred to as non-RD (Dardick and Ronald, 2006). Non-RD kinases lack a conserved arginine (R) in kinase

subdomain VI. The correlation between the function in immunity and non-RD in plants promoted the speculation that this non-RD motive may be a part of an underlying mechanism which regulates defense signaling and this mechanism may differ from the activation of other known plant receptors like BRI1 (Dardick and Ronald, 2006).

Pattern recognition receptors in mammals do not contain kinase domains. After dimerization TLRs recruit a set of adapter proteins via their TIR domain (reviewed in Mogensen, 2009). The adapter proteins, in turn, attract combinations of IRAK (interleucine receptor 1 associated kinase) and RIP (receptor-interacting protein) kinases to the TLR complex. Strikingly, the IRAK and RIP kinases belong to the group of IRAK/Pelle like kinases which are monophyletic with respect to kinase domains when compared to plant RLKs, indicating that these kinases are derived from a common ancestor (Shiu and Bleecker, 2001). After the similarities of ligand binding domains in plant and animal PRRs, this is a second analogy of both systems. It seems that plants and animals use similar kinases for the activation of immune responses, only that the plant PRRs have these kinases incorporated into the receptor protein and PRRs in mammals need to recruit them. And also in the animal immune system these kinases can be differentiated into two subclasses: RD (IRAK-4, and RIP3) and non-RD type kinases (IRAK1, IRAK2, IRAK-M and RIP1) (Meylan and Tschopp, 2005). The current understanding is that RD kinases function as upstream kinases, responsible for the activation of non-RD type kinases (Meylan and Tschopp, 2005).

2.3.4 Other protein components may regulate the activity of pattern recognition receptors

As discussed at least a substantial number of PRRs is regulated through the associated kinase BAK1. But other proteins were found to interact with pattern recognition receptors and may play an important role in the regulation of their downstream signaling response.

The first protein which was found to interact with the pattern recognition receptor FLS2 in a yeast two hybrid analysis, is the kinase associated protein phosphatase (KAPP) (Stone *et al.*, 1994; Braun *et al.*, 1997; Trotochaud *et al.*, 1999; van der Knaap *et al.*, 1999; Gómez-

Gómez *et al.*, 2001; Shah *et al.*, 2002; Rienties *et al.*, 2005). The relevance of KAPP/FLS2 interaction for the downstream signaling has been not investigated.

Another protein which was found to interact independently with FLS2 and BAK1 in protoplasts is the botrytis-induced kinase (BIK1) (Veronese *et al.*, 2006; Lu *et al.*, 2009a). BIK1, originally identified as a component in plant defense against necrotrophic fungal pathogens, is strongly transcriptionally induced upon flg22 treatments. Interestingly, elicitor stimulation results in BIK1 phosphorylation and activation (Lu *et al.*, 2009a). Also growth inhibition effect, caused by flg22 treatment of seedlings, is strongly reduced in *bik1* mutant plants, which verifies its relevance for FLS2 related immune responses (Lu *et al.*, 2009a). How BIK1 contributes to the PRR signaling is an important question which remains to be answered.

BIR1 is an LRR-RLK which interacts with BAK1, but not with FLS2*, in vivo* (Lu *et al.*, 2009a). The analysis of *bir1* mutants indicates a function for this protein as negative regulator of immune signaling. Lack of BIR1 causes a constitutive immune response which also results in resistance to *Hyaloperonospora parasitica* Noco2, a pathogenic oomycete, but also causes constitutive cell death (Lu *et al.*, 2009a). In addition an flg22 induced activation of the MAP-kinase 4, a negative regulator of immune signaling, is absent in the *bir1* mutants. The mechanism which is underlying this down regulation of immune signaling is unknown.

2.3.5 Endocytosis in pattern recognition

Another interesting aspect of PTI is the endocytosis of the pattern recognition receptors. Within 30-60 min after flg22 perception GFP labeled FLS2 is translocated from the plasma membrane to vesicle structures and finally degraded (Robatzek *et al.*, 2006). A putative PEST domain, a peptide sequence known to be important for ubiquitination, seems to be involved in this process (Salomon and Robatzek, 2009). An amino acid substitution within this motif completely abolishes flg22 dependent FLS2 endocytosis (Salomon and Robatzek, 2009).

The ligand dependent ubiquitination and endocytosis of the mammalian pattern recognition receptor TLR4 seems to be part of a down-regulation mechanism, since inhibition of endocytosis increases the sensitivity to the TLR4 ligand LPS (Husebye *et al.*, 2006). In contrast the hormone receptor BRI1 was shown to be continuously endocytosed (Russinovaa *et al.*, 2004) and this endocytosis seems to increase the activation of the downstream signaling of brassinosteroid perception (Geldner *et al.*, 2007). The function of the ligand induced FLS2 endocytosis is unknown up to now and it remains to be answered whether FLS2 endocytosis is activating downstream signaling as it is seen for BRI1; if FLS2 internalization attenuates the flagellin signaling as demonstrated for LPS perception in mammals; or if FLS2 endocytosis serves a very different function in pattern recognition.

Strikingly also other pattern recognition receptors contain an endocytosis motif. EIX1 and EIX2 as well as EFR contain a Yxxϕ signal (Y = Tyr, x = any amino acid, ϕ = hydrophobic residue) and at least for EIX2 a mutational analysis showed that this motif is relevant for the activation of downstream signaling (Ron and Avni, 2004; Zipfel *et al.*, 2006). However, whether endocytosis is of general relevance for pattern recognition in plants needs to be examined in future.

2.3.6 Early events in pattern recognition

How information of a pathogen presence is propagated within the cell is still unclear, but several elements of the signaling are known. Ion fluxes such as K^+/H^+ exchange, Cl^- effluxes and Ca^{2+}-influx, are generally observed very early upon elicitor treatment (Boller, 1995; Trewavas and Malho, 1998; Jeworutzki *et al.*, 2010). Among these ion fluxes, Ca^{2+}-influx is regarded as one of the most significant events, since Ca^{2+} is a key second messenger for diverse physiological changes and cellular processes (reviewed by Trewas and Malho, 1998).

Ca^{2+}-dependent protein kinases 4, 5, 6, and 11 (CDPKs) were identified as sensor for this Ca^{2+}-influx during MAMP perception (Boudsocq *et al.*, 2010). The Ca^{2+}-dependent activation of these kinases, is crucial for the activation of ROS production, possibly through direct phosphorylation of NADPH oxidases (Kobayashi *et al.*, 2007; Boudsocq *et al.*, 2010).

In particular the NADPH oxidase *At*RbohD was shown to be the source of the apoplastic ROS in *Arabidopsis thaliana* (de Torres *et al.*, 2002; Kroj *et al.*, 2003; Nühse *et al.*, 2007). This oxidase is phosphorylated on a regulatory side upon flg22 treatment and plants mutated in the *RbohD* coding gene do not longer produce ROS in response to MAMPs (Nühse *et al.*, 2007). The function of ROS in immunity is complex and it is not understood where in the signal transduction its taking action (de Torres *et al.*, 2006b; Pogany *et al.*, 2009; Boudsocq *et al.*, 2010). Interestingly, since several oxygen species can cross plant membranes easily, it may function also in intercellular communication suppressing HR in surrounding cell (de Torres *et al.*, 2005). As discussed ROS may directly act also as antimicrobial compound and is involved in the reinforcement of the plants cell wall.

The downstream signaling of PTI includes also the activation of so called mitogen activated protein kinases (MAP-kinases) (Nühse *et al.*, 2000). In Arabidopsis a cascade of the MAP-kinase kinases MKK4 and 5, and the MAP-kinases MPK3 and 6 is found downstream of the FLS2 receptor, which is connected to the regulation of transcriptional processes via the transcription factors WRKY22 and 29 (Asai *et al.*, 2002). Additional MAP-kinase elements involved are the MAP-kinase kinase kinase MEKK1, MKK1 and 2 and MPK4, which function in the fine tuning of the signaling by down regulation of different immune related genes (Suarez-Rodriguez *et al.*, 2007; Gao *et al.*, 2008; Qiu *et al.*, 2008a; Qiu *et al.*, 2008b). Still, this is a simplified view of the role of MAP-kinases in immunity, since various different MAP-kinase modules function in the regulation of MAMP induced resistance (Pitzschke *et al.*, 2009). Interestingly MAP-kinases and Ca^{2+}-dependent protein kinases seem to function independently and the transcriptional reprogramming, which results from MAP-kinase activity, differs from the modulations achieved through CDPK activity (Boudsocq *et al.*, 2010).

2.3.7 Transcriptional regulation during defense

Pattern recognition results in a massive transcriptional reprogramming. A considerable number of the upregulated genes encode signaling components, including receptor like kinases as FLS2, EFR, BAK1 or PEPR1, MAP-kinase cascade components or transcription regulatory factors like WRKY transcription factors (Ramonell *et al.*, 2002; Navarro *et al.*,

2004; Zipfel *et al.*, 2004; Moscatiello *et al.*, 2006; Zipfel *et al.*, 2006; Denoux *et al.*, 2008). Furthermore, some of the genes differentially regulated are involved in secondary metabolism or in synthesis of cell wall components (Maleck *et al.*, 2000; Lu *et al.*, 2005). Consistently, genes involved in vesicle trafficking and secretion are also up-regulated (Thilmony *et al.*, 2006).

Importantly, there is a striking overlap of genes induced by different MAMPs, DAMPs and genes which are differential regulated during ETI. This indicates a convergence in the corresponding signaling (Navarro *et al.*, 2004; Zipfel *et al.*, 2006). Still, transcriptional regulation can differ not only in timing and amplitude, but several groups of genes are e.g. activated during flg22 elicitation and not during a DAMP response (Denoux *et al.*, 2008). Thus, plants may use conserved and specific signaling mechanisms to achieve transcriptional responses which are similar for different recognition events, but have also unique aspects depending on the pathogen which is sensed.

2.3.8 Plant hormones in defense

The second messenger salicylic acid (SA) is not only induced during HR but also accumulates strongly upon MAMP stimulation and is a major component of the MAMP-triggered signaling (Morel and Dangl, 1997; Mishina and Zeier, 2007; Tsuda *et al.*, 2008b). Accumulation of this plant hormone is involved in local defense responses but also in the activation of defense in not infected distal leaves during the activation of the so called systemic acquired resistance (SAR). Hence, SA was also believed to function as long distance signal, however, grafting experiments with plants expressing salicylic acid degrading salicylate hydroxylase have dismissed SA as the systemic signal in SAR (Vernooij *et al.*, 1994). Still, the SA derivate methyl salicylate is discussed as component of long distance signaling (Shah, 2009). SA modulates major parts of the transcriptional response during pattern recognition (Tsuda *et al.*, 2008a). However, it is not acting isolated, but rather in a complex network of interacting pathways.

The antagonistic function of SA and the plant hormones jasmonic acid (JA) and ethylene (ET) is widely accepted, but also synergistic effects for these hormones are observed

(Glazebrook *et al.*, 2003; Glazebrook, 2005; Leon-Reyes *et al.*, 2009). JA and ET are usually associated with resistance to necrotrophic pathogens and are also central players in induced systemic resistance (ISR), an immune response induced through soil born microbes (Loake and Grant, 2007; Pieterse *et al.*, 2009). But, the accumulation of the gaseous hormone ethylene is also strongly induced upon MAMP and DAMP treatment and an involvement of JA was proposed for flg22 signaling (Felix *et al.*, 1991; Boller *et al.*, 1995; Zipfel *et al.*, 2004; Halim *et al.*, 2009). This crosstalk provides a regulatory basis for activating multiple resistance mechanisms in varying combinations (reviewed in Pieterse *et al.,* 2009). Pathogens, in turn, try to perturb this balance, by secretion of hormone analogues. Coronatine for example is a phytotoxin produced by the plant pathogen *Pseudomonas syringae*, which acts as a molecular mimic of JA (Mitchell, 1982; Weiler *et al.*, 1994; Melotto *et al.*, 2006).

The phytohormone abscisic acid (ABA) does not only regulate developmental processes and responses to abiotic stress, but also plays an ambivalent role in defense responses to pathogens (Mauch-Mani and Mauch, 2005; Asselbergh *et al.*, 2008). An example is the closure of stomata upon infection with *Pseudomonas syringae* which requires a functional ABA signaling pathway (Melotto *et al.*, 2006); conversely other defense responses are suppressed upon ABA application (de Torres-Zabala *et al.*, 2007).

Similarly the growth hormone auxin promotes virulence during biotrophic interactions. Various pathogens synthesizes high levels of indole-3-acetic acid (Glickmann *et al.*, 1998) and also the exogenous application of the auxin analog 2,4-dichlorophenoxyacetic acid enhances disease symptoms to the bacterial pathogen *Pseudomonas syringae* pv*. tomato* DC3000 (Navarro *et al.*, 2006). Plants counteract auxin related immune suppression through downregulation of auxin-signaling related genes via miRNA regulated mechanisms during PTI (Navarro *et al.*, 2006). Whether or not this crosstalk causes the growth inhibiting effect which is observed for seedlings grown in the continuous presence of MAMPs was not examined yet.

Other plant hormones may shape pathogen defense. The plant hormone gibberellic acid, which is also secreted by the "foolish seedling" disease pathogen *Gibberella fujikuroi,*

was found to function as negative regulator of JA signaling (Grant and Jones, 2009). Also a cross-talk between brassinosteroids and MAMPs is well possible, since they share BAK1 as a common signaling partner (Chinchilla *et al.*, 2009).

How this multitude of plant hormones, second messengers and protein components functions together, how they are activated by different pathogen surveillance systems and how they trigger the optimal defense mechanisms upon different pathogenic threats remains to be answered. We have to identify still missing puzzle pieces and we need to reveal linkages and functions of known components in plant immunity to understand how these elements concert the activation of an optimal defense against the multitude of different plant pathogens.

2.4 Aims of this thesis

The barrier for the majority of potential pathogens is defined by the plants basal innate immune system. This defense is based on an array of membrane localized receptors, which detect pathogen or plant derived danger signals. At the beginning of my thesis numerous of such signals/elicitors, referred as MAMPs and DAMPs, were known and the identification of the MAMP-receptors FLS2 and EFR provided the basis to study the corresponding signaling processes in the model plant *Arabidopsis thaliana*. A comparable background was missing for the investigation of DAMP signaling. The characterization of *At*Pep1 perception through PEPR1 should provide this fundament. Hence, an analysis of *At*Pep1 induced responses in wild-type and *pepr1* deficient mutants was initiated, to validate PEPR1 as the receptor and, in parallel, to compare the *At*Pep1 stimulated DAMP-signaling to the responses which are triggered upon MAMP elicitation.

Of particular interest in this study was the function and regulation of the FLS2 and EFR kinase domains. The parallel identification of the associated kinase BAK1 as signaling partner of FLS2 and EFR expanded this topic accordingly (Chinchilla *et al.*, 2007). Up to now it is not clear how the kinase domains are activated and how they are involved into the MAMP signaling. An *in vitro* and *in vivo* characterization of FLS2-kinase and EFR-kinase was initiated to study the catalytic properties of PRR kinases; to analyze the function of

phosphorylation events during ligand perception and scrutinize the relevance of PRR kinases for immune signaling in general.

The downstream signaling of PRRs is still a black box. To get insights into such, it is necessary to provide a framework with protein factors which take part in this process. This requires the identification of additional signaling components. Hence, a survey for FLS2 interaction partners, based on co-immunopurification was started, to fill some of these gaps in the PRR signal transduction and to understand how defense is regulated during pattern recognition.

3 Perception of the danger signal *At*Pep1 in *Arabidopsis thaliana* involves two pattern recognition receptors, PEPR1 and PEPR2

3.1 Abstract

The relevance of MAMP perception for plant immunity is well documented and widly accepted. In contrast not much is known about how plants perceive endogenous danger signals. Here we examined the perception of the DAMP *At*Pep1, an endogenous peptide of Arabidopsis identified earlier and shown to be perceived by the leucine-rich repeat receptor kinase (LRR-RK) PEPR1. Using seedling growth inhibition, elicitation of an oxidative burst and induction of ethylene biosynthesis, we show that wild-type plants and the *pepr1* and *pepr2* mutants, affected in PEPR1 and in its homologue PEPR2, are sensitive to *At*Pep1, while the double mutant *pepr1/pepr2* is completely insensitive. Our findings provide a basic framework to study the biological role of *At*Pep1-related danger signals and their cognate receptors.

3.2 Introduction

While the perception of MAMPs is well studied, only little is known about the function and impact of another class of surveillance system recognizing plant derived molecules known as DAMPs (damage-associated molecular patterns): DAMPs are released as degradation products from the cell during a pathogen attack or emerge from cleavage of larger precursor proteins as small peptides within the cytoplasm (Boller and He, 2009). *At*Pep1 and its six homologues represent such a group of plant derived, intracellular elicitors which can stimulate defense responses when being present in the apoplast (Huffaker *et al.*, 2006). According to the current understanding *At*Pep peptides are released from cytosolic precursor proteins, so called PROPEPs, through proteolytic cleavage. Either through an active transported or alternatively by the destruction of the cell during wounding, the *At*Pep may reach the apoplast.

A receptor for *At*Pep1 was purified after photoaffinity labeling with its radioactive marked ligand and subsequently cloned (Yamaguchi *et al.*, 2006), providing the first known

DAMP/PRR couple in *Arabidopsis*. This receptor, termed PEPR1 (Pep-receptor 1), like FLS2 and EFR, belongs to the group of LRR receptor kinases. Interestingly, the Arabidopsis genome encodes a close homologue of PEPR1 called PEPR2 (Ryan *et al.*, 2007) and at least 6 genes distantly related to the *At*Pep1 precursor *PROPEP1* (Yamaguchi *et al.*, 2006); synthetic peptides representing the C-terminus of these homologues, called *At*Pep2-*At*Pep7, were found to cause medium alkalinization like *At*Pep1, and all except *At*Pep4 competed with *At*Pep1 binding to the PEPR1 receptor (Huffaker *et al.*, 2006).

The function of *At*PEP signaling is still elusive. Since the destruction of the cell would represent a simple mechanism for *At*Pep release and the PROPEP1 expression is upregulated during wound response, a role in anti-herbivore defense may be proposed (Huffaker *et al.*, 2006). Interestingly, PROPEP1 and PEPR1 are transcriptionally induced upon MAMP perception and the *At*Pep1 signaling causes in turn a similar transcriptional response as the MAMPs their self. This positive feedback loop, supports a model in which *At*Pep peptides function as amplifier of immunity (Huffaker and Ryan, 2007). The observation that overexpression of the precursor PROPEP1 reduces the susceptibility to root pathogen *Pythium irregular* supports this theory (Huffaker *et al.*, 2006).

Here we examine the nature of DAMP signaling and its interrelation to the MAMP system. We focus on plasma membrane delimited responses induced by the activation of the PRRs *At*PEPR1, identified by Huffaker *et al.* (2006). We demonstrate that *At*Pep1 initiates the accumulation of ethylene and ROS production. Thus, together with growth inhibition, the overall response initiated by the DAMP signal *At*Pep1 and the MAMP flg22 exhibit strong similarity.

Plant response to *At*Pep1 is reminiscent to the response to MAMPs such as flg22 or elf26, although differing in kinetics and amplitude. However, we found *pepr1* mutants affected but not insensitive to the elicitor. Our analysis identified PEPR2 the closest homologue of the known Pep-receptor to function also in the Pep-signaling. Mutant plants lacking both receptor were fully insensitive for *At*Pep1, showing that two receptors function in the perception of these DAMP signals in a mostly redundant manner.

3.3 Results

3.3.1 The Pep-receptor mutant is not insensitive to *At*Pep1

Characteristically, exogenously applied MAMP signals such as flg22 and elf26 cause a strong inhibition of seedling growth (Zipfel, 2009). In contrast, Arabidopsis plants expressing the *At*Pep1 precursor PROPEP1 exhibited increased root and aerial growth compared to wild-type plants (Huffaker *et al.*, 2006). In order to compare the MAMP and DAMP *At*Pep1 response directly, we incubated Arabidopsis Col-0 seedlings for 10 days in presence of *At*Pep1 or flg22.

Compared to untreated control plants, seedlings grown in the presence of either flg22 or *At*Pep1 exhibited pronounced growth retardation (Fig. 3-1). With respect to fresh weight, flg22 had a stronger effect than *At*Pep1, while *At*Pep1 inhibited root growth more strongly than flg22 (Fig. 3-1).

In gain of function experiments involving heterologous expression in *Nicotiana benthamiana*, the LRR receptor kinase protein *At*PEPR1 was clearly demonstrated to act as a receptor for *At*Pep1 (Yamaguchi *et al.*, 2006). However, *pepr1* T-DNA insertion mutant plants remained responsive to *At*Pep1 (Fig. 3-1). Like WT, *pepr1* mutants exhibited a reduction in seedling fresh weight of about 25% when grown in presence of *At*Pep1. The reduction of root length was substantial as well, but less pronounced than in WT plants; it was similar to the effect seen in response to flg22 (Fig. 3-1). These results indicate that Arabidopsis plants have at least one additional *At*Pep1-receptor.

3.3.2 Both PEPR1 and its homologue PEPR2 are involved in *At*Pep1 perception

The Arabidopsis genome harbors a close homologue of the PEPR1 which exhibits 72% similarity at the amino acid level (Ryan *et al.*, 2007). To test whether this putative Pep-receptor 2 (PEPR2) is involved in *At*Pep1 signaling, we analyzed the corresponding T-DNA insertion mutant. *At*Pep1 caused a significant growth inhibition in *pepr2* plants, albeit to a somewhat lesser degree than in wt Col-0 and *pepr1* mutants (Fig. 3-2 A and C). While root growth in Col-0 and *pepr1* mutants was inhibited by *At*Pep1 to the same extent, the roots

of *pepr2* mutants appeared to be completely insensitive (Fig. 3-2 B and C), indicating that PEPR2 has a crucial role as a receptor for *At*Pep1 in the roots. To test whether PEPR1 and PEPR2 show functional redundancy we created the double mutant *pepr1/pepr2* and examined its response to *At*Pep1. The double mutant was completely insensitive to *At*Pep1 with respect to overall growth and root length (Fig. 3-2 A and C). Together, these results show that both, PEPR1 and PEPR2, function redundantly as receptors for *At*Pep1.

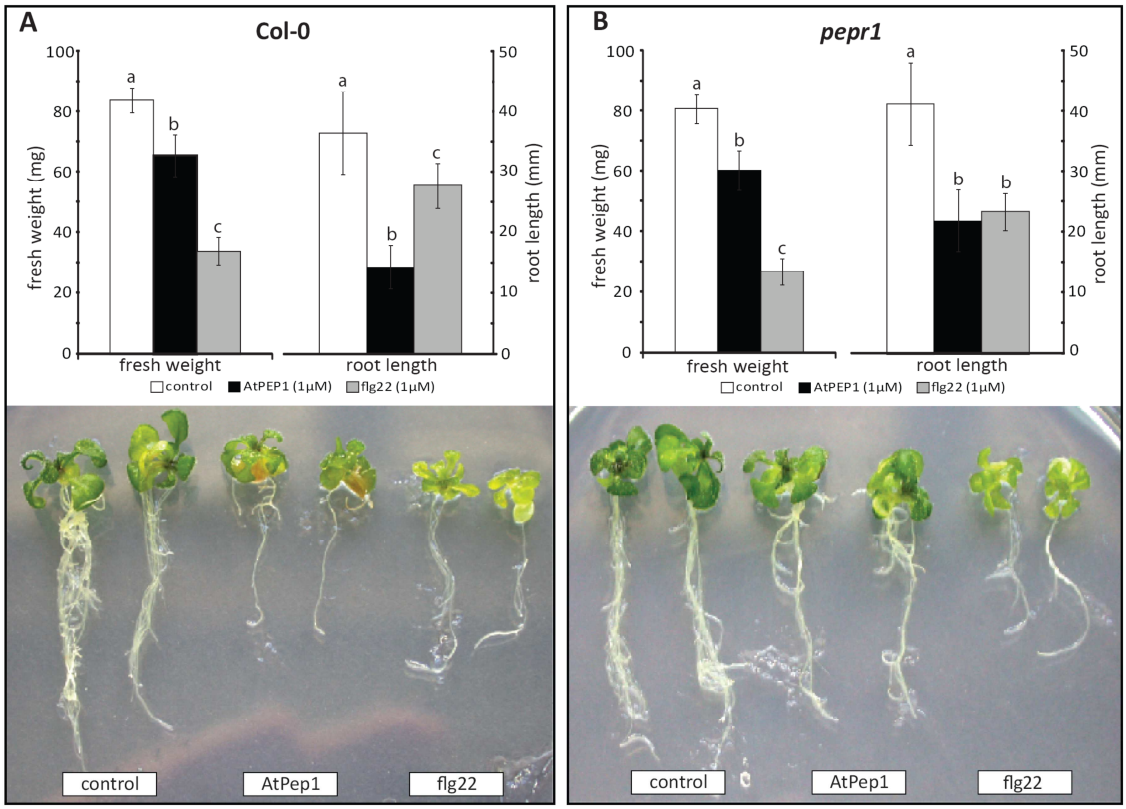


Fig. 3-1 Effect of AtPep1 and flg22 peptide on wild-type seedlings and pepr1 mutants. Seedlings (5 day old) of Arabidopsis Col-0 wild-type (A) and *pepr1* mutants (B) were incubated for 10 days in MS medium in presence of *At*Pep1 (1 µM), flg22 (1 µM) or in absence of elicitors. Growth was quantified by determining total fresh weight per seedling and length of the longest root per seedling. The experiment was repeated three times with similar results. Shown are means ± standard error (fresh weight: n=6; root length: n=12). Means shown with the same letters were not significantly different based on least significant difference test ($p < 0.05$). Representative seedlings were photographed.

In roots, growth inhibition caused via the *At*Pep1 perception of PEPR2 appears to be dominant. This correlates to a certain extent with published microarray data on gene expression of untreated wild type Arabidopsis plants: As analyzed by Genvestigator, PEPR2 is particularly high expressed in the radicle (Fig. 3-3); however, PEPR1 appears to be expressed in roots and radicles as well (Zimmermann *et al.*, 2004).

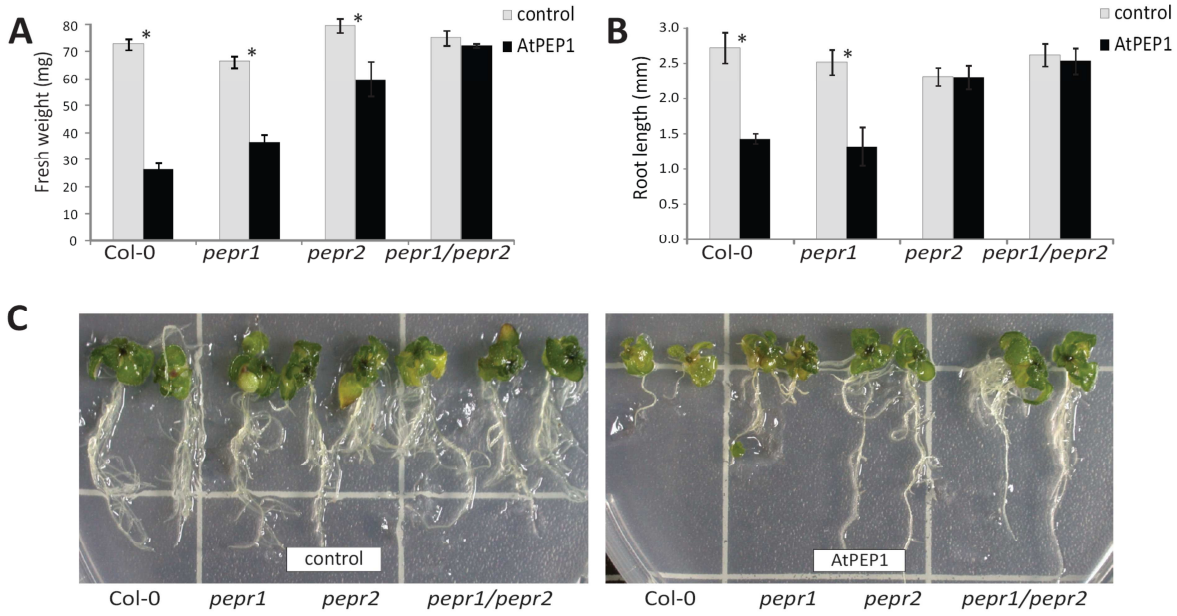


Fig. 3-2 Growth response of wild-type plants (Col-0), pepr1 and pepr2 single mutants and pepr1/pepr2. Growth was quantified by determining total fresh weight per seedling (A) and length of the longest root per seedling (B) after 10 days of growth in the absence or presence of *At*Pep1 (1 µM). Representative seedlings were photographed (C). Open bars represent untreated controls, filled bars represent *At*Pep1 treatments. Error bars represent standard error (n ≥ 6). Asterisks (*) indicate a significant difference based on t-test analysis ($p < 0.05$).

One hallmark of the defense response elicited by both MAMPs and DAMPS is the rapid production of ROS (Huffaker *et al.*, 2006). When *At*Pep1 was applied to leaf sections derived from WT Col-0 plants, it elicited ROS generation in a similar way as flg22, although the signal was about ten times smaller (Fig. 3-4 A and B). While the *pepr2* mutant behaved like the wild-type after stimulation with *At*Pep1, the *pepr1* mutant showed a slightly delayed increase in ROS production; however, peak production of ROS was similar for both mutants (Fig. 3-4 A). Importantly, upon *At*Pep1 stimulation, a ROS signal was not observed with the *pepr1/pepr2* double mutant (Fig. 3-4A). As a control, we examined the response of the plant lines to flg22 (Fig. 3-4 B). Beside a strong variation between different experiments in the maximal amplitude of ROS generation, the wild-type, the single *pepr1,* and *pepr2* mutants, as well as the *pepr1/pepr2* double mutant all showed a strong increase in ROS production after a lag phase of about 2 minutes. As expected, the *fls2* mutant was completely insensitive to flg22 (Fig. 3-4 B).

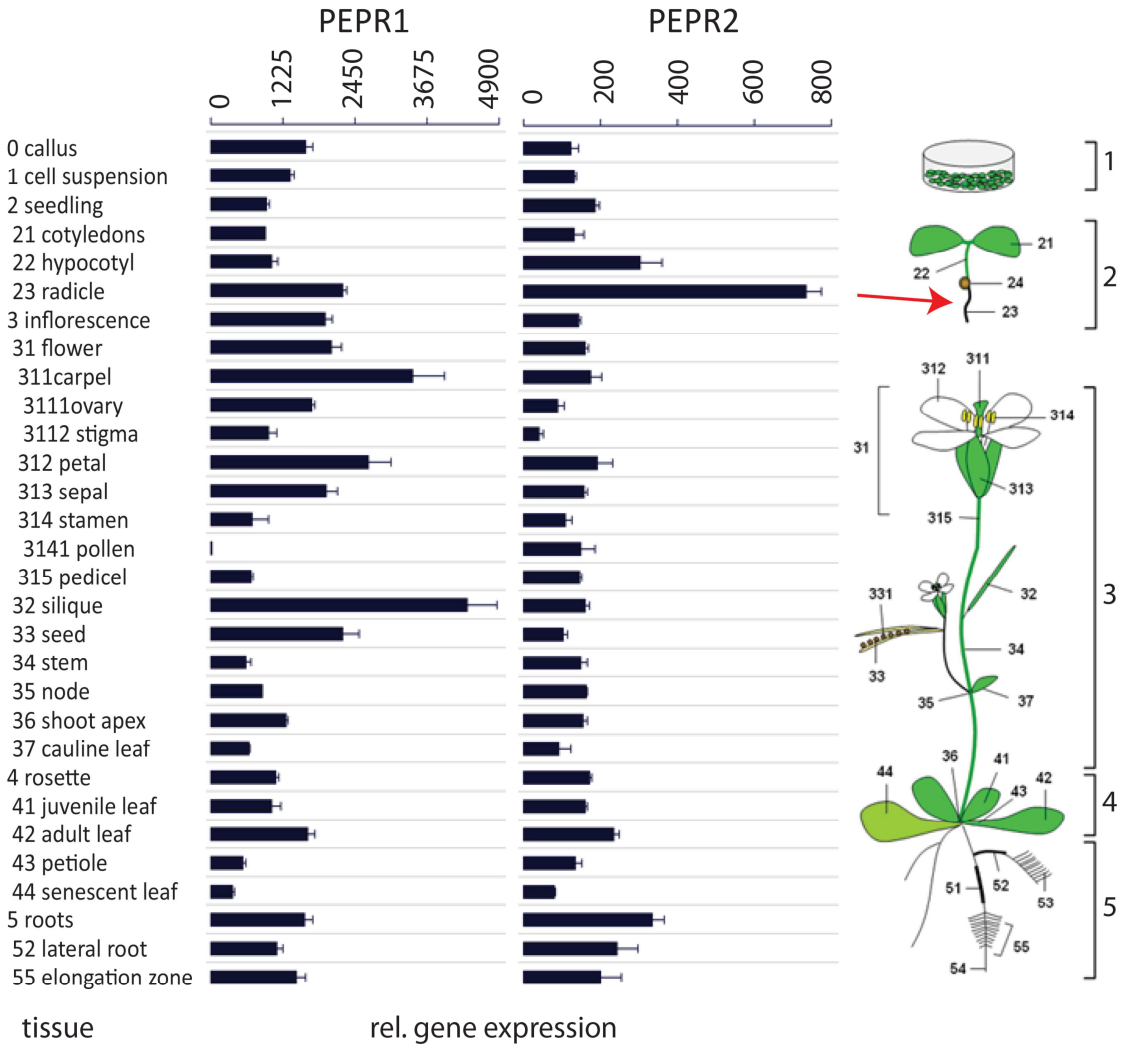


Fig. 3-3 Transcription levels of PEPR1 and PEPR2 genes in different Arabidopsis organ tissues. The transcription level of PEPR1 and PEPR2 was analyzed based on published Affimetrix chip data using the digital northern tool of the Genvestigator database (Zimmermann *et al.*, 2004). Both genes show a basal expression in all plant organs as well as an increased transcriptional level in siliques and carpels for PEPR1 and higher levels in the radicle of seedlings for PEPR2.

In previous work with MAMPs, the production of ethylene was frequently assayed, because enhanced production of this volatile stress hormone serves a highly reproducible and robust marker for an ongoing defense process. Leaf material of both single *pepr* mutants fully responded to *At*Pep1 treatment to the same extent as wild-type plants (Fig. 3-5). In the *pepr1/pepr2* double mutant, however, ethylene production was not enhanced in response to *At*Pep1, confirming that the double mutant was unable to perceive the DAMP.

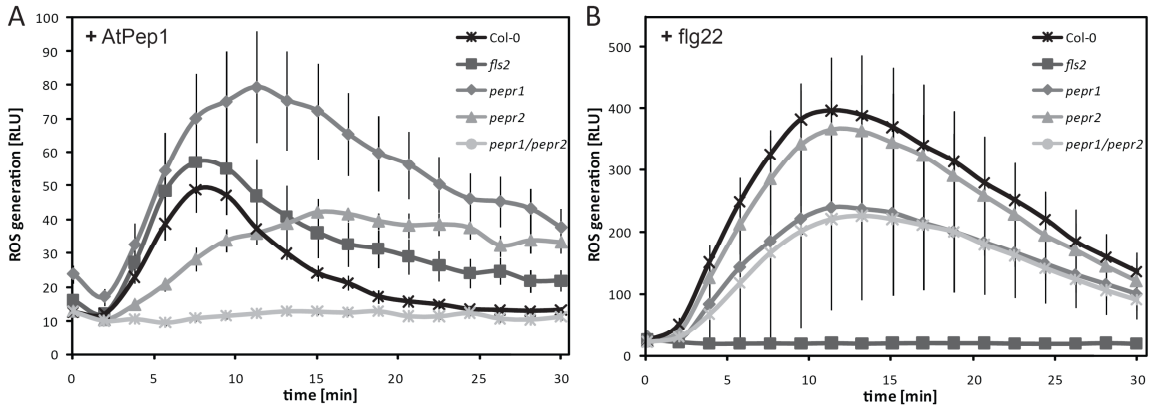


Fig. 3-4 Time course of ROS production and in various Arabidopsis lines stimulated with AtPep1 or flg22. Wild-type plants (Col-0), *fls2, pepr1, pepr2* single mutants, and *pepr1/pepr2* double mutants were analyzed continuously for ROS production after induction with 1 µM *At*Pep1 (A) or 1 µM flg22 (B). Curves represent the average of 6 independent measurements ± standard error.

3.3.3 The analysis of SERK-family mutants does not indicate their contribution to *At*PEP signaling

Several Arabidopsis LRR-RKs were shown to signal through the complex formation with members of somatic embryogenesis receptor-like kinase (SERK) family. Hence we tested T-DNA mutants of SERK1, 2, 3, 4 and 5 for their capacity to generate reactive oxygen species. All lines exhibited a similar kinetic of ROS biogenesis upon *At*Pep1 treatment, which was not significantly different to wild-type plants (Fig. 3-6A). The divergences in amplitude of ROS generation are neither clearly significant nor reproducible between different experiments. Thus, absence of single SERK receptor kinases does not affect the *At*Pep1 signaling. In contrast, *serk3/bak1* T-DNA mutants respond with a clearly delayed generation of ROS upon flg22 stimulation, compared to the wild-type control (Fig. 3-6B).

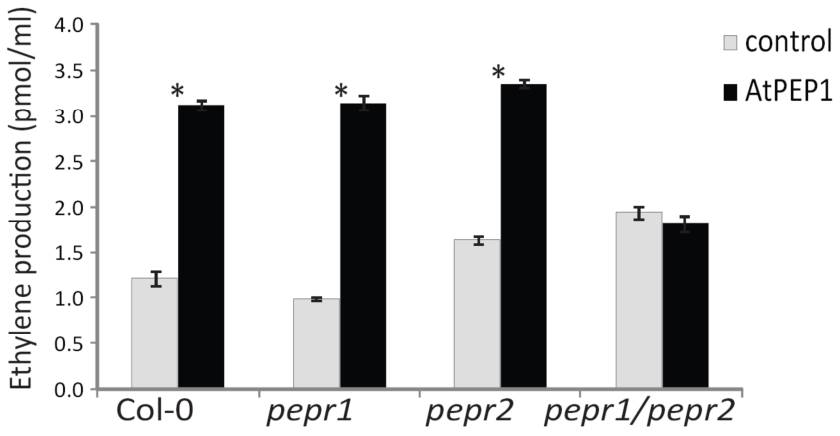


Fig. 3-5 Ethylene accumulation in response to AtPep1 treatment. *Total ethylene biosynthesis of wild-type plants (Col-0), fls2, pepr1, pepr2 single mutants, and pepr1/pepr2 double mutants were measured 3 h after stimulation with BSA (control) or flg22 (1 µM). Results shown are a mean of six replicates ± standard error.*

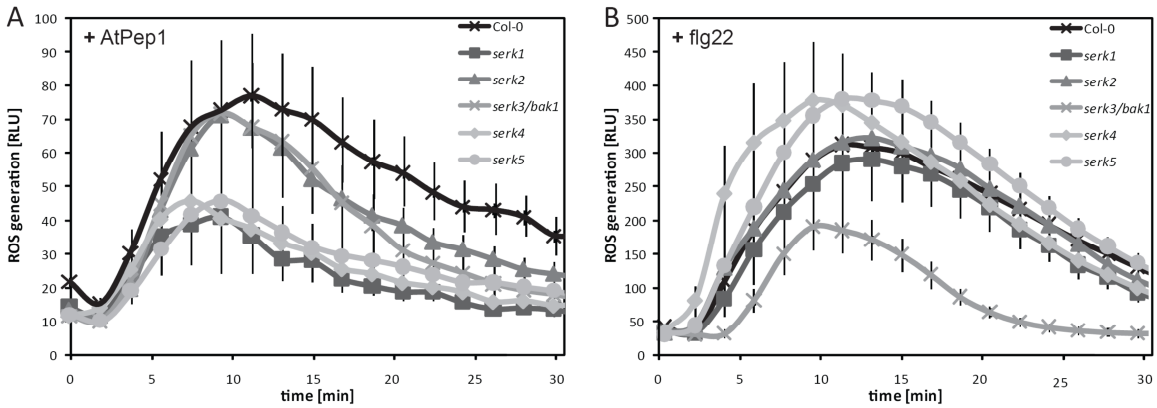


Fig. 3-6 Time course of ROS production in wild-type plants and different serk-mutants. Wild-type plants (Col-0) and single mutants of the five SERK family members were analyzed for ROS production after induction with 1 µM *At*Pep1 (A) or 1 µM flg22 (B). Curves represent the average of 6 independent measurements ± standard error.

3.4 Discussion

Our work highlights the strong parallels between MAMP and DAMP perception in plants. The ROS and ethylene generation, early responses seen after stimulation with the MAMP flg22 and the DAMP *At*Pep1, were very similar, and both flg22 and *At*Pep1 caused a comparable seedling growth inhibition.

Earlier studies observed an increase root and aerial growth in plant expressing constitutively the *At*Pep1 precursor PROPEP1, which seems to be in contrast to the effect of *At*Pep1 on Arabidopsis seedling observed here (Huffaker *et al.*, 2006). However, this contradiction results rather from different experimental approaches, than from differences between MAMPs and DAMPs. While in this study peptides were supplied in a form which can be easily perceived by membrane receptors, it is unclear how PROPEP1 is processed and how the *At*Pep1 is transported to the apoplast after this processing. Therefore PROPEP1 overexpression may induce a very mild type of resistance, at low costs, providing an advantage over control plants in a non sterile environment (van Wees *et al.*, 2008).

Interestingly, while Arabidopsis possesses only single receptors for the MAMPs flg22 and elf26, so that *fls2* or *efr* mutations are completely insensitive to the respective MAMP, there is a certain, limited redundancy in the case of DAMPs: *At*Pep1 perception is mediated by a pair of receptors, PEPR1 and PEPR2, and only the double mutant *pepr1/pepr2* is

completely insensitive to *At*Pep1. Despite of this overlap between PEPR1 and PEPR2, obvious differences appear with regard to the *At*Pep1 dependent growth inhibition. While *At*Pep1 treatment results in a strong inhibition of root growth in *pepr1* single mutants, only a minor effect is seen in *pepr2* mutants. The public available expression data show a predominantly expression of PEPR2 in the radicle of the roots, indicating that this difference may reflect distinct expression patterns of the two receptors (Zimmermann *et al.*, 2004). But also PEPR1 is well expressed in the radicle, hence the observed difference in root growth inhibition between *pepr1* and *pepr2* mutants cannot be fully explained by transcriptional regulation only. Possibly an analysis of differences in protein accumulation may explain the observed differences better. Interestingly, a recent analysis of MAMP perception in Arabidopsis roots demonstrated that different perception systems are active in localized tissue areas only (Yves *et al.*, 2010). Strikingly these zones differ for distinct MAMPs despite of the expression of the corresponding receptors in the full root (Yves *et al.*, 2010). This observation demonstrates that other explanations than differences in receptor expression may explain the distinct root phenotype in *pepr1* and *pepr2* mutants. We cannot exclude that, the two Pep-receptors differ in their subsequent downstream signaling. This observation points out that, beside their high homology and their common ligands, PEPR1 and PEPR2 may have partially diverging functions.

Redundancy is also seen on side of the ligand, since not only one but seven different *At*Pep peptides are found in *Arabidopsis thaliana*. However, the question remains, whether all seven peptides have an identical function? The number of different ligands and different regulation of the cognate precursor genes indicates a more complex relationship. The existence of two receptors may allow a differential binding to the two PEPRs, followed by individual outputs. Thus, the pair of receptors provides the basis for the seven *At*Peps to trigger different outputs. However, the specificity of PEPR1 and PEPR2 for *At*Pep1-7, and a more refined analysis of the PEPR1 / PEPR2 downstream signaling, will be an important task for future experiments. A parallel study of Yamaguchi *et al.* (2010), which verified PEPR2 as second receptor for *At*Pep1, indicate that PEPR1 binds all *At*Pep peptides, but has

a significantly reduced affinity to *At*Pep4. Conversely PEPR2 seems to exhibit strongly increased affinity to *At*Pep1 and *At*Pep2, compared to *At*Pep3-6.

Our experiments, using single T-DNA for different SERK proteins, did not indicate a function of these receptor kinases in *At*Pep signaling. This seems to be in contrast to the recent findings of Schulze and colleagues (2010) and Postel and colleagues (2009). Both teams observed an interaction of the LRR-receptor like kinase BAK1 with PRPR1 and PRPR2, either in a directed yeast two hybrid approach or *in vivo* based on an immunopurification approach. Furthermore BAK1 and a second signal corresponding to PEPR1 and PEPR2 are phosphorylated *in vivo* in response to *At*Pep1 stimulation (Schulze *et al.*, 2010). This indicates that BAK1, which is known to be required for the signal transduction of FLS2, mediates both, MAMP and DAMP signaling. Strikingly, an electrophysiological analysis revealed a reduced *At*Pep1 sensitivity in *bak1* mutants. This corroborates the hypothesis that BAK1 is involved in both, MAMP and DAMP signaling (Krol *et al.*, 2010).

Our finding that single *serk* mutants were not significantly affected in the generation of ROS is likely due to functional redundancy of different SERK paralogues. Already earlier studies showed that SERK-family members can function in a redundant manner, and in particular the two highly homologue proteins BAK1 and SERK4/BKK1 can substitute each other partially (He *et al.*, 2007; He *et al.*, 2008). To investigate the relevance of SERK proteins for *At*Pep-signaling more deeply, an analysis with mutants which are affected in multiple SERK genes must follow. Such an analysis is difficult, since the combination of T-DNA mutations for BAK1 and BKK1 in a *bak1/bkk1* double mutant is lethal (He *et al.*, 2007). A new EMS mutant of BAK1*, bak1-5,* which is affected in the signaling of flg22 and elf26 but not in brassinosteroid signaling allows the creation of a weak *bak1-5/bkk1* double mutant which is viable (Schwessinger *et al.*, unpublished). This double mutant may provide the possibility to analyze the role of BAK1/BKK1 for *At*Pep1 signaling.

Of particular interest is the question how DAMPs may contribute to MAMP-induced pathogen resistance. While an induced resistance through *At*Pep1 perception was reported earlier, signaling of flg22 and the endogenous DAMP signal oligogalacturonan interfere with each other (Aslam *et al.*, 2009; Yamaguchi *et al.*, 2010). In addition an

electrophysiological analysis of *pepr1/pepr2* single and double mutants demonstrated a significant increase in flg22 and elf26 sensitivity (Krol *et al.*, 2010). This indicates that there is possibly trade-off, between DAMP and PAMP signaling. Alternatively one may speculate that increased MAMP sensitivity in *pepr1/pepr2* mutants results from overexpression of other PRRs as compensation for the missing DAMP receptors. Taken together, this demonstrates that the role of DAMPs in plant immunity is not fully understood. The identification of PEPR2 lays the foundation for further studies on the biology of DAMPs of the *At*Pep1 type and their function for pathogen resistance.

4 The pattern recognition receptor FLS2 is a substrate for the associated receptor like kinase BAK1 *in vitro* and is phosphorylated on its kinase domain *in vivo*

4.1 Abstract

Effective defense requires a fast, but also stringently regulated, recognition of pathogens. Pattern recognition receptors, like FLS2, are key components of this surveillance, but how these receptors are controlled, how they are activated, is completely elusive. To understand the mechanism of PRR activation we performed a characterization of the serine/threonine kinase of FLS2 and its interaction partner BAK1. This analysis showed that FLS2 is a kinase substrate for BAK1 *in vitro*, demonstrating that BAK1 dependent phosphorylation for FLS2 is a possible mechanism of PRR activation. Furthermore, immunoprecipitation of FLS2 from *Arabidopsis thaliana* cell cultures followed by liquid chromatography–tandem mass spectrometry identified S-1084 and S-1078, two amino acids within the potential PEST domain of FLS2, as putative *in vivo* phosphorylation sites. S-1078 was found to be phosphorylated in an elicitor dependent manner, but a functional analysis in plants demonstrated that it is not required for several elicitor dependent defense responses.

4.2 Introduction

Pathogens are recognized by the plants surveillance system trough the detection of conserved signatures originally termed pathogen associated molecular patterns (Boller and Felix, 2009). The change to the currently used term microbe associated molecular patterns is not only more precise, it also indicates a challenge which needs to be faced by the plants surveillance system: MAMPs are conserved through whole classes of microbes and are therefore also present in non pathogenic microorganisms (Bittel and Robatzek, 2007; Boller and Felix, 2009). Consequently, these elicitors are likely to be continuously present in phyllosphere and rhizosphere. Pattern recognition receptors like FLS2 or EFR must be able to detect any arising pathogen attack, but must be controlled stringently enough to avoid continuous signaling in presence of non pathogenic microorganisms (Bittel and Robatzek, 2007).

The underlying mechanisms of this regulation are still not understood, but the first steps of this process are known. FLS2 and EFR form a heteromeric complex with the receptor like kinase BAK1 in response to ligand binding to mediate the activation of a signal cascade within the cell (Heese *et al*., 2007; Chinchilla *et al*., 2007; Schulze *et al*., 2010; chapter 3 of this thesis). The two PRRs and BAK1 exhibit a very similar structure: a leucine-rich repeat domain facing the apoplast which is linked via a transmembrane domain to an IRAK/PELLE like serine/threonine cytoplasmic kinase. Despite these similarities between FLS2, EFR and BAK1, there is a noteworthy difference. While the kinase of BAK1 harbors all critical residues of serine/threonine kinases, FLS2 and EFR kinase are classified as non-RD type, defined by the absence of an arginine-aspartic acid (RD) motif in the catalytic region. So far it is poorly understood to what extent non-RD kinases are functional and how their activity is controlled (Johnson *et al.*, 1996). Importantly, most of the known MAMP receptors in plants contain such a non-RD type kinase (Dardick and Ronald, 2006) and mammalian MAMP perception involves non-RD kinases as well: Toll-like receptors (TLR) and the IL-1 (interleukin like 1) receptors, recruit sets of non-RD and RD type IRAK and RIP kinases, which then together orchestrate the downstream steps of basal immunity inflammatory response (Janssens and Beyaert, 2003; Meylan and Tschopp, 2005). These similarities suggest a common mechanism in the regulation of IRAK like kinases in animal and plant immunity and the activation mechanism of these kinases may also provide the key for the regulation of PRRs in plants.

To study the regulation of plant PRRs we initiated a characterization of the FLS2 and EFR kinases and the kinase of their associated receptor BAK1. An *in vitro* analysis of heterologously expressed kinases demonstrated, that the cytoplasmic portions of FLS2 and EFR are indeed functional as kinases, but have a very low activity compared to BAK1 and other LRR-RLK derived serine/threonine kinases. A mutational analysis of the FLS2-kinase indicates that classical mechanisms, which regulate the activity of various kinases, including phosphorylations in the juxta-membrane domain or activation loop, are not sufficient to control FLS2 activation. This suggests that FLS2 is regulated via a rather exclusive mechanism, which possibly correlates with the absence of the RD signature from the PRR kinases.

However, the activation of the receptor complex is likely to involve phosphorylation events, since BAK1-kinase can phosphorylate the FLS2-kinase *in vitro*. In addition, we show that the FLS2/BAK1 complex, purified from plant cells, gains kinase activity after flg22 stimulation, resulting in phosphorylation of both proteins *in vitro*. The subsequent mass spectrometric analysis of the FLS2 phosphorylation pattern revealed two potential phosphorylation sites on FLS2, and at least one of these is elicitor stimulus dependent. A further analysis of these sites may reveal new aspects of the FLS2 activation and the regulation of PRRs in general.

4.3 Results

4.3.1 FLS2 intracellular domain encodes a functional kinase

In vitro analysis of proteins reduces the complexity of a system and allows to focus on specific relations ignoring the bulk of processes which run in parallel in living cells. To use these advantages we heterologously expressed the FLS2 cytosolic domain and studied its kinase activity *in vitro*. The protein expressed in *Escherichia coli* (*E. coli*) bacteria contained the full cytoplasmic domain of FLS2 including the juxta-membrane domain (JM), the serine/threonine kinase domain itself and the very C-terminal part of FLS2 fused at its N-terminus to a GST-tag, which was used for purification (Fig. 4-1D). In parallel similar constructs for the BAK1 C-terminus were prepared and expressed in *E. coli*.

In presence of γ-p^{32} ATP the FLS2-kinase is able to auto-phosphorylate and to phosphorylate the artificial substrate myeline basic protein (MBP) (Fig. 4-1A). This verifies earlier studies and demonstrates that the kinase of FLS2 is catalytically active harboring all residues necessary to function as kinase in the plant cell (Gómez-Gómez *et al.*, 2001; Zhou and Chai, 2008). However, the amount of radioactivity incorporated into the kinase of HAESA, a RD-clade LRR-RLK involved in floral organ abscission (Horn and Walker, 1994; Dardick and Ronald, 2006), is of several magnitudes higher and also the phosphorylation of MBP by the HAESA kinase exceeds the activity of FLS2 by far (Fig. 4-1A). Also for other artificial substrates (protamine, histone, and casein) a similarly low transphosphorylation by the FLS2-kinase was observed (not shown).

4.3.2 Amino acid substitutions in JM-domain, activation loop, or within the RD-motif are not sufficient for an activation of the FLS2-kinase *in vitro*

Serine/threonine kinases are frequently regulated via phosphorylation of the so called activation loop, an internal loop which lies close to the catalytic center of the kinase domain (Johnson *et al.*, 1996; Nolen *et al.*, 2004). In these cases the negative charge of an attached phosphate can induce changes in the kinase structure, followed by the release of an auto-inhibitory regulation (Johnson *et al.*, 1996). Substitutions of serine/threonine residues with negatively charged aspartate or glutamate can mimic a phosphorylated state and induce such a conformational change and activation of the catalytic activity (Yoshida and Parniske, 2005). If the low activity displayed by the FLS2-kinase is due to auto-inhibitory regulation, this mutational approach might lead to catalytic activation *in vitro*.

To test this hypothesis two mutated FLS2-kinase versions were created, by introducing either two glutamate substitutions close to the conserved DFG motif which marks the start of the activation loop (FLS2-kin T1031E/S1035E) or by substituting a well conserved serine residue further downstream in the activation domain (FLS2-kin T1040E) (Johnson *et al.*, 1996; Iglesias *et al.*, 1998; Gong *et al.*, 2002; Cartlidge *et al.*, 2005). However, FLS2-kinase variants carrying such substitutions within the activation loop, show similarly low activity as the wild type form of FLS2 (Fig. 4-1B).

The juxta-membrane domain links the serine/threonine kinase domain with the transmembrane domain and has no catalytic function. However, such putative spacer regions often participate in the regulation of the kinase itself (Johnson *et al.*, 1996; Chen *et al.*, 2010). Furthermore, published phospho-proteome analysis of plant membrane proteins indicated a surprisingly high number of phosphorylation sites to be on the JM-domain of RLKs rather than the kinase itself (Nühse *et al.*, 2004). It is therefore likely that the JM-domain has an important role in the regulation of many RLKs. Similar to the modifications which were introduced within the activation loop, the effect of phosphorylation mimicking substitutions in the JM-domain of the soluble FLS2-kinase construct was tested. Also these mutations, as well as a combination of mutations in JM-domain and activation loop, did not

lead to an increased activity of FLS2-kinase *in vitro* in respect to their autophosphorylation activity or the activity on artificial substrates (Fig. 4-1B).

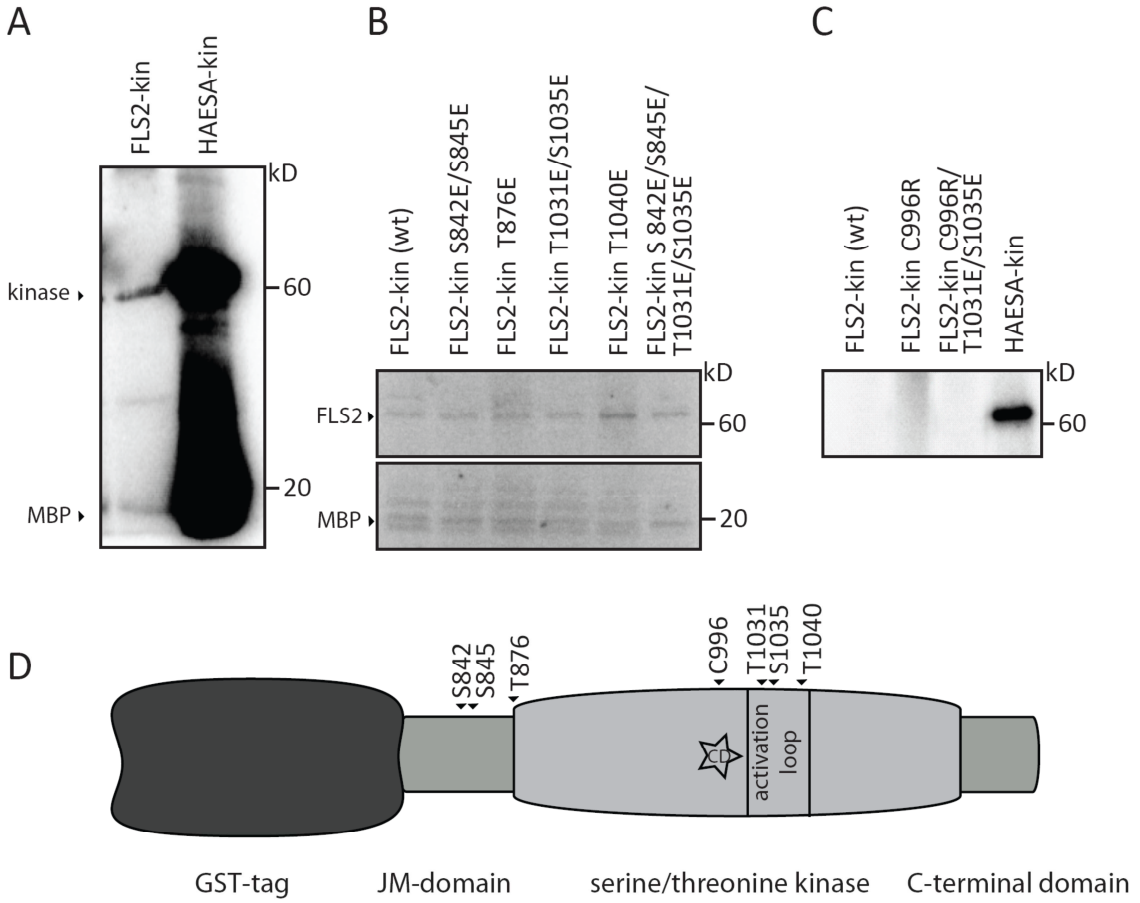


Fig. 4-1 Characterization and mutational analysis of the FLS2-kinase activity in vitro. Affinity-purified recombinant GST-FLS2-kin (1 µg) or GST-HAESA-kin (100 ng) were subjected to a kinase reaction together with the artificial substrate MBP (1 µg). The incorporation of radiolabeled phosphate into the different proteins was monitored by separation on SDS-PAGE and detection using autoradiography. A: Comparison of the wt-form of FLS2-kinase with HAESA-kinase B: Analysis of different FLS2 mutant-forms carrying phosphorylation mimicking substitutions in JM-domain or activation loop. C: FLS2-kinases carrying an artificially introduced RD motif or an introduced RD-motif in combination with phosphorylation mimicking mutations in the activation loop. D: Scheme of the recombinant GST-FLS2-kin construct and the introduced amino acid substitution analyzed in B and C.

A low *in vitro* activity is not restricted to FLS2, but was found also for other non-RD LRR-RLKs, which were examined with similar approaches (Wesche *et al.*, 1997; Shane *et al.*, 2006; Stein *et al.*, 2006a). To test whether the low activity correlates with the lack an RD-motif, the non-RD kinase FLS2 was converted into a RD kinase by mutating the cysteine at position 996 to arginine. However, this change from CD to RD was not linked to a gain of

higher *in vitro* auto-phosphorylation activity. Also a combination of the RD version of the FLS2-kinase together with phosphorylation mimicking mutations in the activation loop did not create a constitutive active kinase (Fig. 4-1C). This demonstrates that the difference between RD and non-RD kinases is not restricted to the presence or absence of an arginine within the catalytic center of the kinase domain, but might include additional components which take part in its regulation.

4.3.3 Characterization of the BAK1-kinase *in vitro*

Since a mutational analysis of FLS2 did not reveal the activation mechanism controlling this receptor, we introduced an additional factor into the analysis. The fast dimerization of the LRR-RLK BAK1 with FLS2 upon flg22 stimulation (Chinchilla *et al.*, 2007) and the fast phosphorylation of both proteins (Schulze *et al.*, 2010) strongly indicate that the BAK1-kinase is involved in the process of FLS2 activation.

To test this we cloned the cytoplasmic domain of BAK1 (BAK1-kin), containing the JM-domain, kinase domain, and the very C-terminal domain of BAK1, in a fusion with a N-terminal GST-tag. The BAK1-kin fusion protein shows autophosphorylation activity when incubated with γ-p^{32} ATP, but a strongly increased autophosphorylation activity is observed when the kinase was incubated in presence of artificial substrates like MBP, histone, caseine, or protamine, indicating that also the BAK1-kinase is by default in a rather inactive stage when heterologously expressed as fusion protein (Fig. 4-2A). The small basic protein used in this experiment might interact with the BAK1-kinase leading to an activation of the protein. Other additives like the non ionic detergent TritonX-100 or the divalent detergent 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) also induced activity of the BAK1-kinase, but not to the same degree as observed in presence of basic proteins (not shown). Changes in ion concentrations or pH in the kinase buffers or the addition of BSA did not cause a significant increase in BAK1-kinase activity (not shown). Therefore MBP was used in the kinase buffer for the subsequent analysis of the BAK1-kinase.

4.3.4 The conserved C-terminus of BAK1 has no relevance for the BAK1-kinase activity *in vitro*

The C-termini of BAK1 and other SERK-family members show a striking degree of conservation (Boller and Felix 2009). In particular they all have the same four amino acids motif (SGPR) at the C-terminus, as do most of the members of the LRR II family. To investigate whether this motif has a regulatory function for BAK1-kinase activity, we expressed a deletion mutant (BAK1-kin ΔSGPR), deleted on the SGPR amino acids, and tested its kinase activity *in vitro*. Independent from the presence of MBP, BAK1-kin ΔSGPR showed an autophosphorylation activity which was comparable to the wild-type BAK1-kinase (Fig. 4-2B). Also the phosphorylation activity on MBP was identical for BAK1-kin (WT) and for BAK1-kin ΔSGPR. In contrast, the kinase inactive BAK1-kin K317R, which harbors a mutation in the ATP binding site, did not show auto- nor transphosphorylation activity, demonstrating that the observed phosphorylation signals resulted from the BAK1-kinase itself and not from a co-purified *E. coli* kinase (Wang *et al.*, 2008). The experiments were repeated several times, showing that differences in the intensity of the phosphorylation signal clearly correlated with the amount of protein used in the kinase assay only.

4.3.5 FLS2 is a kinase substrate for the BAK1-kinase

For animal receptor kinases and the Arabidopsis brassinosteroid receptor BRI1 the activation mechanism includes the sequential trans-phosphorylation of both receptors (Schlessinger, 2002; Rahimi and Leof, 2007; Wang *et al.*, 2008). Assuming a similar mechanism for the FLS2/BAK1 system, the FLS2-kinase should be able to phosphorylate the BAK1-kinase and vice versa. To test this hypothesis we incubated both kinases together in a kinase assay and monitored their phosphorylation. The joint incubation of FLS2- and BAK1-kinase resulted in the phosphorylation of both proteins, indicating that *in vitro* the proximity of both proteins is sufficient for FLS2 and BAK1 phosphorylation (Fig. 4-2C).

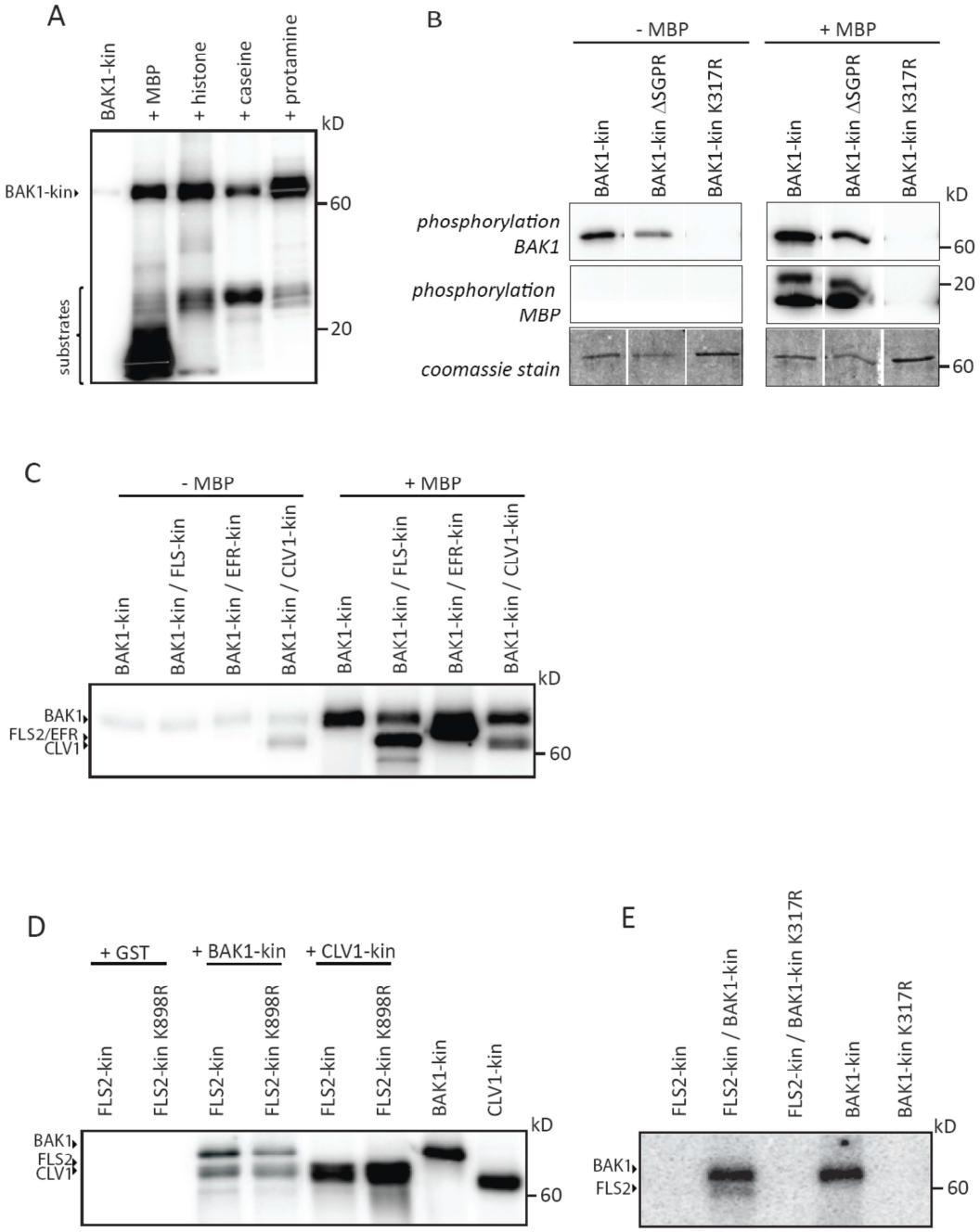


Fig. 4-2 Characterization of BAK1 and FLS2 auto- and transphosphorylation activity. Affinity-purified recombinant kinases (100 ng) were incubated in kinase assays together with artificial substrates (1 µg) and/or in combination with a second kinase. The incorporation of radiolabeled phosphate into the different proteins was monitored by separation on SDS-PAGE and detection using autoradiography. A: Kinase activity of the BAK1-kinase in presence of different artificial substrates. B: Kinase activity of BAK1 kinase deleted on the C-terminal SPGR motif (BAK-ΔSPGR), compared to wild-type (BAK1-kin) and kinase inactive variant (BAK1-kin K317R). C: Combination of the BAK1-kinase with either FLS2-, EFR- or in presence and absence of MBP. D: Phosphorylation activity of the BAK1-kinase or the kinase of the unrelated receptor CLV1 (CLV1-kin) with wild-type (FLS2-kin) and kinase inactive (FLS2-kin K898R) FLS2 kinases. E: Phosphorylation activity of FLS2-kinase on wild-type (BAK1-kin) and kinase inactive (BAK1-kin K317R) BAK1 kinase.

However, this phosphorylation was observed only in presence of MBP, which activates the BAK1-kinase in the used conditions. Also a strong phosphorylation of the EFR-kinase and phosphorylation of the unrelated kinase of CLAVATA1 (CLV1) (Clark *et al.*, 1993) was observed (Fig. 4-2C), when incubated together with the BAK1-kinase, indicating a low substrate specificity for kinases in such an *in vitro* analysis.

To discriminate between auto- and transphosphorylation of different kinases we created also a catalytically inactive version of the FLS2 kinase (FLS2-kin K898R) using a substitution of the critical lysine residue with arginine, similar to the mutations introduced in earlier studies into BAK1 and FLS2 kinases (Asai *et al.*, 2002; Wang *et al.*, 2008). Using 100 ng of heterologously expressed protein, we did not observe any phosphorylation for the wild-type FLS2 or the kinase inactive variant FLS2-kin K898R, when they were incubated in kinase buffer without other kinases (Fig. 4-2D). Adding 100 ng of wild-type BAK1 kinase resulted in phosphorylation of FLS2-kin or FLS2-kin K898R and the BAK1 kinase (Fig. 4-2D). This phosphorylation was similarly strong in both kinase assays regardless whether the kinase reaction contained a wild-type or a catalytic impaired FLS2 kinase. This demonstrates clearly, that BAK1 is able to trans-phosphorylation FLS2 *in vitro* and FLS2 kinase activity is not required for this process. It should be noted that a similar result was obtained when incubating wild-type or inactive FLS2-kinase with the cytoplasmic domain of CLV1 (Fig. 4-2D), again pointing to a low substrate specificity in such kinase assays.

In a reciprocal setup, using the catalytic inactive BAK1-kin K317R variant together with the wild-type form of the FLS2 kinase, no phosphorylation is observed (Fig. 4-2E). This indicates that FLS2 was not capable of BAK1 trans-phosphorylation *in vitro.* Hence, phosphorylation of heterologously expressed BAK1-kinase and FLS2-kinase are due to BAK1 auto- and trans-phosphorylation activity and not to the kinase activity of FLS2.

4.3.6 Immunoprecipitated FLS2 complex is active *in vitro*

To exclude that the low activity of the FLS2-kinase *in vitro* is a result of heterologous expression only, we purified the full length FLS2 or BAK1 from wild-type Arabidopsis cells using anti-FLS2 or anti-BAK1 antibodies coupled to proteinA-sepharose (Chinchilla *et al.*,

2006; Schulze *et al.*, 2010). Also for full length FLS2, purified in absence of the flg22 elicitor, no phosphorylation of the protein was observed in an *in vitro* kinase assay (Fig. 4-3, FLS2 IP –flg22). Immunoprecipitated BAK1 showed, similar to the *in vitro* experiments with heterologously expressed kinase, low incorporation of labeled ATP, possibly by auto-phosphorylation (Fig. 4-3, BAK1 IP –flg22).

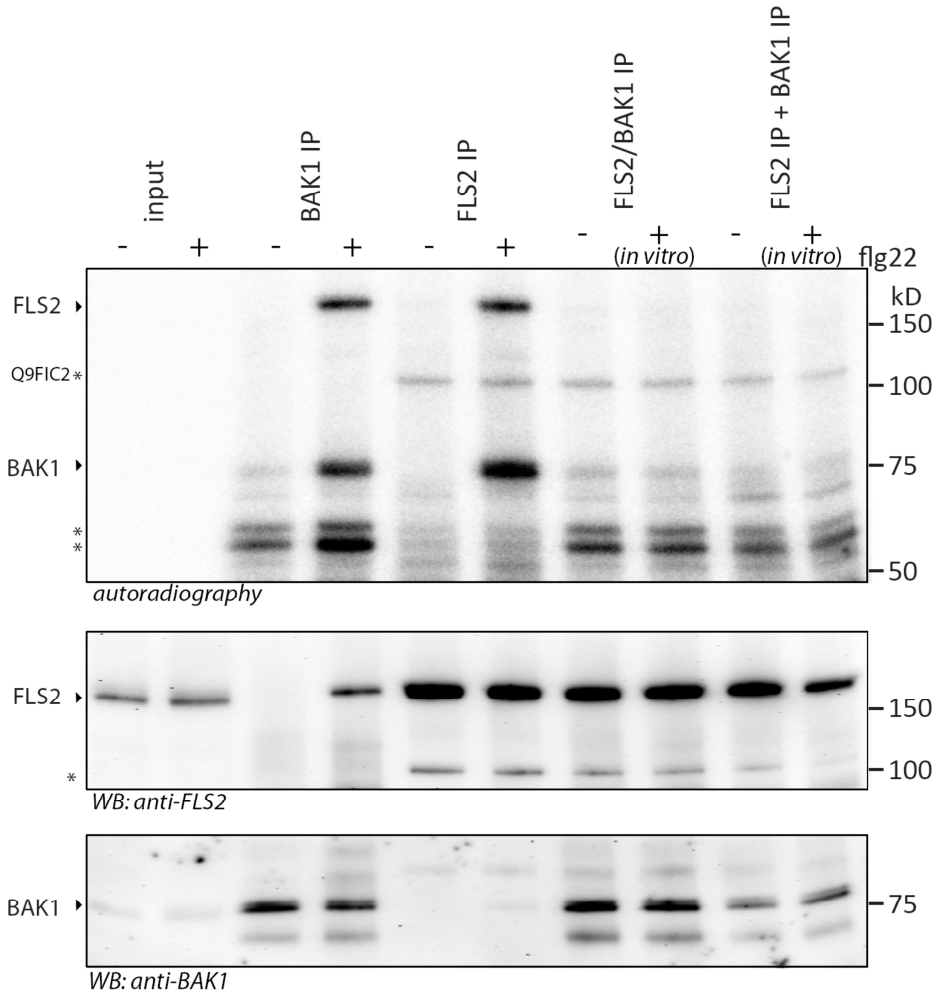


Fig. 4-3 Phosphorylation of full length FLS2 and BAK1 in vitro. Immunoprecipitations with anti-FLS2 and anti-BAK1 were performed from flg22-treated or untreated Arabidopsis cells. Precipitates were subjected to a kinase assay solely, in combination, or FLS2 and BAK1 were precipitated together on the same proteinA-sepharose beads and analyzed in a kinase assay. FLS2 and BAK1 were strongly phosphorylated when immunoprecipitated from flg22-treated cells, but not when FLS2 and BAK1 were simultaneously immunoprecipitated from untreated cells and flg22 was added after the IP into the kinase assay (*in vitro*). The protein Q9FIC2 which is additionally recognized by FLS2 antibodies and signals from unknown proteins are marked by asterisks (Chinchilla *et al.*, 2006).

Surprisingly, when cells were treated with flg22 prior to purification, we found FLS2 to be phosphorylated additionally to a second signal corresponding by size to BAK1 (Fig. 4-3, FLS2 IP +flg22). Also the immunoprecipitation of the FLS2/BAK1 complex from flg22 treated cells, using anti-BAK1 antibodies, resulted in strong phosphorylation of both proteins (Fig. 4-3, BAK1 IP +flg22).

To study this surprising result further, we mixed BAK1 and FLS2 protein from untreated cells using two approaches: first by pooling the proteinA-sepharose beads of separate FLS2 and BAK1 IPs (Fig. 4-3 FLS2-IP+BAK1-IP) or second by coating proteinA-sepharose with both, anti-FLS2 and anti-BAK1, antibodies to purify both proteins in parallel (Fig. 4-3; FLS2/BAK1-IP). As a control flg22 was added into the kinase assay after the IP *in vitro*. In either case, the kinase assays using a FLS2/BAK1 mixture, purified from un-stimulated cells, did not result in phosphorylation of FLS2 and BAK1 (Fig. 4-3). Hence, it is not simply common presence of immune-purified BAK1 and FLS2 proteins per se which leads to phosphorylation of these proteins, but rather an additional process which cannot be mimicked by simple mixing of BAK1 and FLS2. This might involve the correct orientation of both membrane proteins in a micelle like structure or additional unknown protein factors which interact with the forming FLS2/BAK1 complex during elicitation and are co-purified during the IP after flg22 treatment.

4.3.7 Identification of FLS2 phosphorylation sites *in vivo*

The observed FLS2 phosphorylation in *in vitro* assays, which is found only for precipitates from flg22 treated samples, points out that phosphorylation of FLS2 is indeed involved in the activation of the receptor. This is in accordance with the findings of Schulze and colleagues (2010) who observed *in vivo* a very rapid phosphorylation of FLS2 and BAK1 in response to flg22 treatment. Both results indicate, that phosphorylation of the FLS2 receptor is part of the signal transduction process.

Therefore a mass spectrometric approach was applied to identify specific FLS2 phosphorylation sites *in vivo*. Using an antibody recognizing the C-terminus of FLS2 (Chinchilla *et al.*, 2006) we purified 5 µg of FLS2 protein and subjected it, in collaboration

with the laboratory of Prof. S. Clouse (NC State University), to various mass spectrometric analysis procedures including the separation on SDS-PAGE followed by tryptic in gel digests and MALDI-LC/MS/MS, but also electro spray injection (ESI) of samples, which were not separated by SDS-PAGE. To identify phosphorylation sites, FLS2 peptides were identified by searching the MS/MS spectra against a database containing the FLS2 protein sequence, followed by an analysis for mass modifications on serine and threonine residues corresponding to the additional mass of HPO_3 (Wang *et al.*, 2005b). To obtain the necessary amount of FLS2 protein for this analysis we used about 30 µg of antibodies and 100 g of wild type Arabidopsis cells. In order to get insights into the process of FLS2 activation, we proceeded with this analysis on two types of samples: one from plant cells elicited with the flg22 for 5 min and one sample derived from untreated cells.

In spite of high reproducibility of the mass spectrometric results from run to run and high coverage of analyzed FLS2 (appendix A.2) only one site, S-1084, was reproducibly found to be phosphorylated in all three performed experiments. This site appeared to be phosphorylated independent of the elicitor treatment. Since the applied mass spectrometric approaches do not allow a quantitative interpretation, it is not possible to determine the ratio of receptors which are phosphorylated before or after the flg22 treatment. A second site on FLS2, S-1078, identified in one out of three experiments, was found to be phosphorylated after flg22 treatment only.

4.3.8 Phosphorylation of S-1078 and S-1084 is not required for ethylene accumulation, ROS generation, or the growth inhibiting effect of flg22

The functional significance of identified FLS2 phosphorylation sites was assessed *in planta* by substituting either S-1078 and S-1084 to alanine, to eliminate phosphorylation at these sites, or to aspartate, which can mimic constitutive phosphorylation. The modified FLS2 variants were stably expressed, as FLS2-YFP-HA fusion protein, in *fls2* mutant background and analyzed for their ability to trigger immune responses after flg22 elicitation. Despite very high variations in transgene expression we selected lines

accumulating similar levels of fusion protein (Fig. 4-4A, lower panel). No obvious differences in development were observed for these plants.

In *fls2* mutants expressing the wild-type form of FLS2 flg22 treatment induced accumulation of ethylene, which was exceeding the amount which was found for Col-0 plants (Fig. 4-4A), possibly due to an overexpression of the transgene under the control of a 35S promoter. A similar strong accumulation of ethylene was found for plants expressing the modified FLS2 receptors FLS2-S1078A, FLS2-S1084A, and FLS2-S1084D (Fig. 4-4A). Only plants expressing FLS2-S1078D accumulated lower amounts of ethylene upon flg22 treatment. This can be explained by lower expression of the transgene (Fig. 4-4A). The amount of ethylene, which was accumulated in absence of elicitor stimulus, was similar in all tested plants. Therefore we conclude that FLS2 receptor variants carrying aspartate or alanine at position 1078 or 1084 trigger an ethylene response which is identical to the response triggered by wild type FLS2. A similar picture was obtained with the analysis of reactive oxygen species generation in response to flg22 (Fig. 4-4B). *fls2* mutant plants were fully insensitive to flg22 and hence did not synthesize ROS in response to flg22 elicitation. In contrast transgenic lines expressing the FLS2 fusion protein in its wild-type or mutant form responded clearly to a treatment with flg22.

The continuous presence of the MAMPs flg22 and elf26 in the growth medium causes a strong inhibition of seedling growth. We used this effect to verify the observations received from the analysis of ROS generation and ethylene accumulation. In absence of any elicitor all seedlings, including seedlings expressing the phosphorylation mimicking variants of FLS2 (FLS2-S1078D and FLS2-S1084D), showed a comparable fresh weight. Compared to untreated seedlings, all lines showed a similar reduction of growth (70-85%), when treated with flg22, indicating that receptors harboring aspartate or alanine mutations at position S-1078 and S-1084 function similar as wild type receptors. Again, the observed variations are likely to result from differences in the protein accumulation levels in different plant lines (Fig. 4-4C).

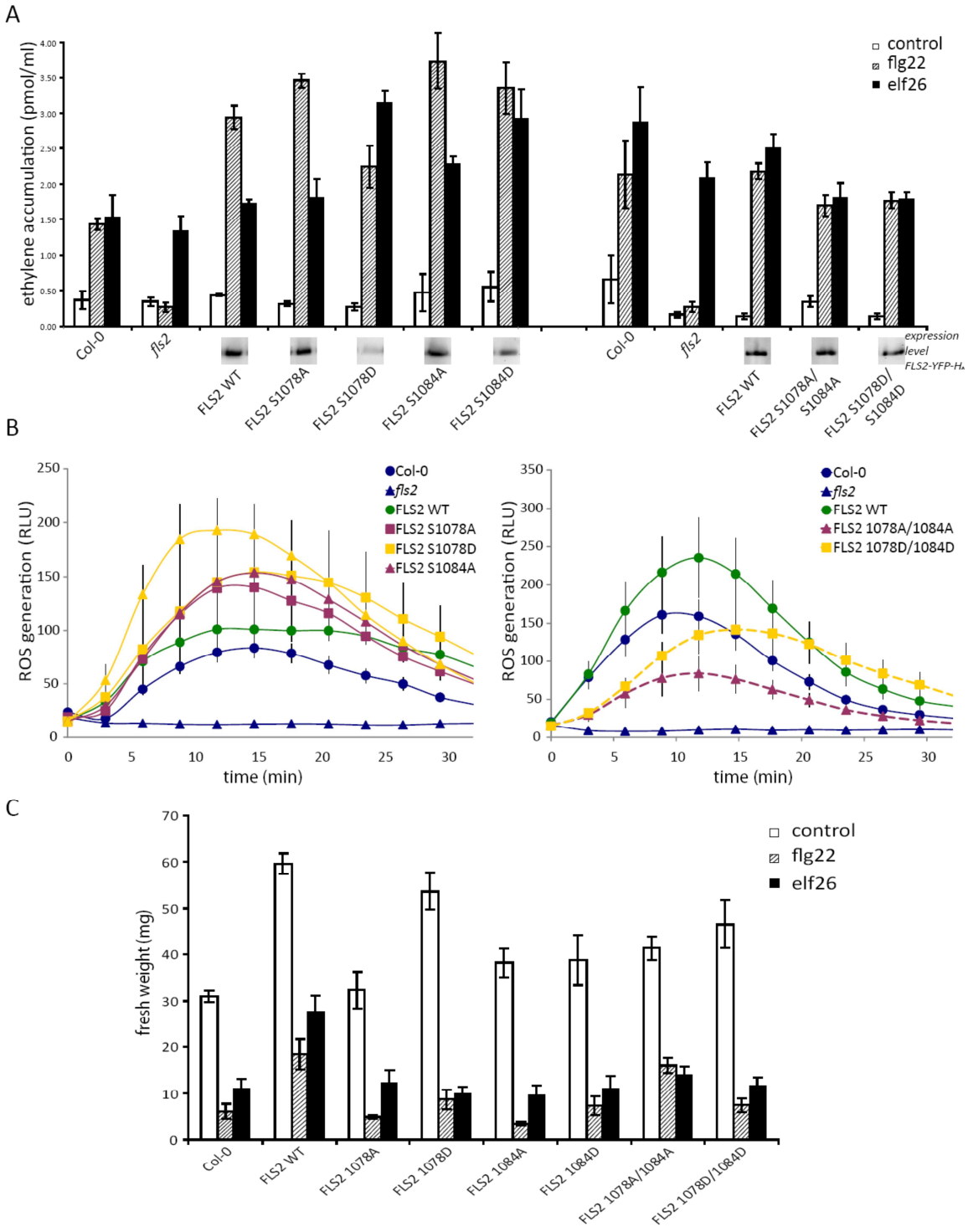


Fig. 4-4 Functional analysis of FLS2 phosphorylation sites in planta. Lines expressing a 35S-FLS2-HA transgene with alanine or aspartate substitutions at position S-1078 and S-1084 in *fls2* mutant background are not altered with regard to flg22 induced ethylene accumulation (A), generation of ROS (B) or growth inhibition of seedlings (C), compared to plants overexpressing the WT-form of FLS2-HA. Expression level of the transgene was monitored by Western blot analysis using anti-HA antibodies (A, lower panel).

To exclude, that these two phosphorylation sites are not functionally redundant, we created double mutants, FLS2-S1078A/S1084A and FLS2-S1078D/S1084D. With regard to ethylene accumulation no differences were seen for FLS2-S1078A/S1084A and FLS2-S1078D/S1084D lines and also ROS accumulation is not significantly altered (Fig. 4-4). Both lines are strongly inhibited in growth, when treated with flg22 or elf18, whereas the flg22 effect on FLS2-S1078A/S1084A, which exhibited a reduction of seedlings fresh weight by 60% is less distinct, when compared to FLS2 wt (70% reduction) or Col-0 (80% reduction). However, such moderate variations are likely due to differences in transgene expression rather than to the mutation itself (Fig. 4-A). In conclusion the analysis of S-1078 and S-1084 mutants showed that the identified phosphorylation sites are not key sites responsible for the transmission of the flg22 signal via ethylene or ROS generation. Substitution of serine 1078 and serine 1084 with alanine or aspartate does not result in a constitutive active nor inactive FLS2 receptor. However, other aspects of FLS2 mediated signaling remain to be tested.

4.4 Discussion

4.4.1 The non-RD kinase of FLS2 and the RD kinase of BAK1 are regulated by unknown mechanisms

A peculiarity of kinases involved in MAMP recognition is the frequent absence of a conserved arginine adjacent to catalytic active aspartate in the in domain VIb of their kinase (Dardick and Ronald, 2006). Most of the identified plant RLKs which function as PRRs, including FLS2, EFR and the Xa21 protein in rice (activator of Xa21-mediated immunity) are assigned to this non-RD class of kinases. In this analysis differences between the non-RD and RD kinases with regard to their basal kinase activity became apparent. While RD kinases e.g. from the CLAVATA1 or the HAESA RLKs strongly auto-phosphorylate and also phosphorylate artificial substrates, the non-RD kinases FLS2 and EFR show only a very low activity. The same observation holds true for other non-RD kinases which were

tested in independent studies (Wesche *et al.*, 1997; Shane *et al.*, 2006; Stein *et al.*, 2006a). This indicates crucial differences in the regulation of these distinct kinase clades.

The basis for the regulation of RD kinases is usually a phosphorylation event within the activation loop. The negative charge of the attached phosphate interacts and neutralizes the positive charge of the arginine residue and induces activation of the kinase. Such a mechanism is not possible for non-RD kinases. Correspondingly, a phosphorylation of the activation loop of non-RD kinase was not identified so far (Johnson *et al.*, 1996). Also in our analysis we did not observe an activation of modified FLS2-kinase versions, which carry a phosphorylation mimicking substitution within their activation loop. Also the introduction of the RD signature into the FLS2-kinase did not result into an activation of the kinase. This artificial conversion from non-RD to RD might rather introduce an additional control mechanism, than releasing the still unknown mechanism which is employed by the kinases in PRRs. However, it should be considered, that mutational approaches are often limited. There are many ways to alter proteins function and entire surface patches of residues may play critical roles in the function of the kinase (Peck, 2006). Thus, the changes we introduced into the FLS2-kinase may not be sufficient for an activation of the kinase.

Similarly to FLS2 and EFR, the BAK1 kinase has a low auto-phosphorylation activity *in vitro.* This is in contrast to other tested RD kinases. But different from FLS2, the addition of basic proteins or low concentrations of detergents activates the kinase. How these additives induce changes in kinase activity, and whether this process corresponds to a mechanism which is also operated *in vivo*, remains to be answered. Taken together the analysis of FLS2- and BAK1-kinase demonstrates clear differences of kinases in the activation mechanism involved in plant defense, compared to the CLV1-kinase or HAESA-kinase which regulated developmental processes. This difference may reflect the requirement of more stringently regulated signaling pathways in immunity.

4.4.2 Phosphorylation events on the FLS2/BAK1 complex

BAK1 was originally identified as the associated kinase of the brassinosteroid receptor BRI1, where binding of the hormone ligand to BRI1 triggers hetero-oligomerization with

BAK1 (Li *et al.*, 2002a; Nam and Li, 2002). This interaction induces an activation of BAK1-kinase activity by an initial BRI1-dependent trans-phosphorylation. Subsequently, this is followed by BAK1 dependent BRI1 phosphorylation, which fully activates the BRI1/BAK1 complex (Wang *et al.*, 2008).

Also upon flg22 perception the receptor, FLS2, dimerizes with the associated kinase BAK1. A phosphorylation analysis, using an *in vivo* phospho-labeling approach in Arabidopsis cell cultures, demonstrated a rapid *de novo* phosphorylation FLS2 and BAK1 (Schulze *et al.*, 2010). This phosphorylation was visible already 15 seconds after elicitor application and reached its maximum after 30-60 seconds (Schulze *et al.*, 2010).

Interestingly, these phosphorylation events seem to proceed in a different order as it is observed for brassinosteroid perception, since we did not find the corresponding FLS2 dependent phosphorylation of BAK1 in our *in vitro* kinase assays. Conversely, the cytoplasmic domain of FLS2 is clearly a kinase substrate for BAK1-kinase. The impact of the BAK1 dependent FLS2 phosphorylation on the FLS2-kinase activity is not fully investigated yet and more refined *in vitro* and *in vivo* analysis would be necessary to approach this point. However, a characterization of *bak1* mutants expressing a kinase inactive variant of BAK1, could already demonstrate the importance of BAK1 kinase activity for the FLS2 downstream signaling (Schulze *et al.*, 2010).

It should be noted that an *in vitro* kinase assay, using heterologously expressed cytoplasmic domains, represents a simplified experiment which does not reflect the *in vivo* situation in all cases. Already the use of plant derived proteins results in different observations: mixing full length FLS2 and BAK1, after their purification from non stimulated cells, in an *in vitro* kinase assay, does not result in a signal for phosphorylated FLS2 and only a weak signal resulting from the phosphorylated BAK1. Also simultaneous BAK1/FLS2 purification with proteinA-sepharose beads loaded with anti-FLS2 and anti-BAK antibodies, and a subsequent kinase assay with such beads did result in an increased phosphorylation of the two proteins. Importantly, if both proteins are co-purified as complex after elicitation with flg22 and subsequently used in a kinase assay, strong phosphorylation of FLS2 and BAK1 was observed. This clearly demonstrates, that common incubation of BAK1

and FLS2 is not sufficient to trigger a trans-phosphorylation of both proteins. Possibly the spatial arrangement, which might be preserved in the FLS2/BAK1 complex during solubilization of the lipid membrane, is the crucial factor, as it is observed for other receptor like kinases like the epidermal growth factor receptor in mammals (Zhang *et al.*, 2006). Alternatively other factors within the FLS2/BAK1 complex, eventually also kinases, may contribute to BAK1 and FLS2 phosphorylation.

4.4.3 A comparison of plant and mammal MAMP perception reveals a high number of analogies

Comparisons between mammalian and plant systems for pattern recognition focus usually on different MAMPs and the receptors which recognize these molecular signatures. It is astonishing, to what extent MAMPs from the two independent kingdoms resemble each other (Ausubel, 2005). In both systems chitin, lipopolysaccharides (LPS) and flagellin are recognized as elicitors, and in the case of flagellin and LPS perception both systems use a LRR domain to bind the pathogen derived ligand (Ausubel, 2005; Zipfel and Felix, 2005; Reese *et al.*, 2007). However, it is even more astonishing that the analogies of MAMP perception exceed this level clearly. The pendant of plant PRRs in mammals are the Toll-like receptors (TLRs), type-I transmembrane receptors, without any kinase in the cytoplasmic domain. Instead they utilize a so called TIR domain and a set of adapter proteins to attract IRAK and RIP (receptor interacting protein) kinases to the membrane. This recruitment combines usually a non-RD type kinase like IRAK-2, IRAK-M, or RIP1 with RD kinases (IRAK4 and RIP3). In any case it seems that the combination of non-RD kinases and a RD-kinase is a key step in the activation signaling of TLRs, e.g. the non-RD kinase RIP1 functions through the RD-type kinase RIP3. IRAK1, 2 and M, again non RD kinases, function together with IRAK4 which contains the RD-motif (Li *et al.*, 2002b; Meylan and Tschopp, 2005).

Similar to BAK1 in our analysis, the RD-kinase IRAK4 is actively auto-phosphorylating and can trans-phosphorylate other IRAK kinases *in vitro.* Reciprocally, IRAK-1 cannot phosphorylate IRAK4 *in vitro*, just like in our analysis where we observed no phosphorylation of BAK1 by the FLS2-kinase. Hence it is believed that IRAK4 is an upstream

kinase, responsible for phosphorylation and activation of other kinases in the TLR complex, and our results indicate a similar function for BAK1 in the FLS2/BAK1 complex (Li *et al.*, 2002b; Janssens and Beyaert, 2003). Possibly this combination of non-RD kinases with a RD kinase holds the key for the activation MAMP signaling.

4.4.4 FLS2 phosphorylation events during flg22 signaling *in vivo*

Similar as the *in vivo* analysis of Schulze *et al.* (2010), our analysis of the FLS2 phosphorylation status revealed one site, S-1078, which is potentially phosphorylated in response to flg22 treatment. This site was identified in one out of three replicates only. Hence, on the basis of the mass spectrometric data this site cannot yet be categorized as a bona fide phosphorylation site, which is targeted during flg22 signaling. It would be important to know whether the *de novo* phosphorylation which was observed using *in vivo* phospho-labeling (Schulze *et al.*, 2010) results from phosphorylation of the here identified sites S-1078 or S-1084. A similar *in vivo* phospho-labeling experiment, using mutant plants expressing FLS2-S1078A/S1084A, may answer this question, but an efficient protocol for *in vivo* phospho-labeling in full plants or protoplasts is not available up to now.

The functional analysis of S-1078, as well as the analysis of the constitutively phosphorylated S-1084, did not evidence their participation in flg22 signaling. Plants expressing FLS2-S1078A/D, FLS2-S1084A/D, or FLS2 constructs with both mutations could generate ROS and ethylene upon flg22 treatment and seedlings were similarly reduced in growth as plants expressing wild-type FLS2, when grown in presence of flg22. However, both sites are situated within the potential PEST domain, a proline, aspartate, serine and threonine enriched stretch, usually targeted by E3 ligases for ubiquitination. While mono-ubiquitination can regulate endocytosis processes, poly-ubiquitinations can mark proteins for the degradation via the proteasome (Rechsteiner and Rogers, 1996). Both processes seem to be of relevance for the FLS2 receptor and both are flg22 dependent (Robatzek *et al.*, 2006; Göhre *et al.*, 2008). It was hypothesized that endocytosis functions in the removal of used FLS2 during a pathogen infection, but the importance of endocytosis for resistance is not clear yet (Robatzek *et al.*, 2006). Interestingly, it was demonstrated, that the endocytosis of FLS2 depends on the putative PEST motif (Salomon and Robatzek, 2009).

Thus it is likely that phosphorylation events within the putative PEST domain will influence the FLS2 internalization. The Arabidopsis lines used in this study express YFP-tagged variants of FLS2 modified. Hence, they will be a useful tool for a microscopic analysis, to elucidate the potential impact of PEST site phosphorylations on FLS2 endocytosis.

5 Kinase-independent signaling: a catalytically impaired EF-Tu receptor can trigger downstream events in a diverging signaling network

5.1 Abstract

The activation of the so called basal immunity or PTI via pattern recognition receptors is a key element in plant defense. However, surprisingly little is known about the architecture of the signaling network underlying basal immunity and the mechanisms which activate these signaling pathways. Here we show that EFR requires the activity of its cytoplasmic serine/threonine kinase only for some of the defense responses normally activated upon EF-Tu perception. Catalytically impaired EFR induces accumulation of the plant hormone ethylene in response to the elicitor elf26 and confers elicitor dependent growth inhibition and resistance to *Agrobacterium tumefaciens*. These responses do not depend on the activation of MAP-kinases and the generation of reactive oxygen species, which are abolished in this mutant. These results demonstrate that basal immunity uses network like rather than linear transduction pathways.

5.2 Introduction

The perception of pathogen associated molecular patterns allows an unspecific but fast and efficient response to defeat a huge number of pathogens. Most of the corresponding pattern recognition receptors identified up now are plasma membrane localized receptor-like kinases, characterized by extracellular ligand binding domains, linked to cytoplasmic serine/threonine kinases (Morillo and Tax, 2006; Boller and He, 2009). Surprisingly, only very little is known about how such RLKs, like EFR and FLS2, activate defense (Gómez-Gómez *et al.*, 1999; Zipfel *et al.*, 2006).

For the structurally related hormone receptor BRI1, it is known that its activation involves oligomerization with the BRI1 associated kinase (BAK1) and a sequential transphosphorylation process (Li and Chory, 1997; Li *et al.*, 2002a; Nam and Li, 2002; Wang *et al.*, 2008). During brassinosteroid perception the kinase activity of the receptor BRI1 is essential for receptor complex formation, for the initiation of transphosphorylation, and

for activation of downstream responses (Wang *et al.*, 2008). Biochemical and genetic evidence showed that also FLS2 and EFR oligomerize with BAK1 in response to ligand perception, which is necessary to achieve full activation of downstream responses (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Schulze *et al.*, 2010). This complex formation is followed by phosphorylation of both proteins, BAK1 and the PRRs (Schulze *et al.*, 2010). Hence it seems that activation of RLKs in plant immunity follows the paradigm of receptor activation through oligomerization and transphosphorylation known from BRI1 and animal RLKs (Wang *et al.*, 2008). But importantly both systems differ also in various points: The complex formation between FLS2 and BAK1 proceeds within seconds (Schulze *et al.*, 2010), while the formation of the BRI1/BAK1 complex was shown only 90 min after stimulation with brassinosteroids (Wang *et al.*, 2005b) and experiments using the kinase inhibitor K-252a indicate that for this rapid complex formation no kinase activity is required (Chinchilla *et al.*, 2007).

Interestingly an increasing number of receptor kinases appear to be functional in the absence of kinase activity. The RLK STRUBBELIG plays an essential role in Arabidopsis organ development but lacks any detectable kinase activity (Chevalier *et al.*, 2005). Other RLKs including CRINKLY4, FEI1 and 2 contain functional kinases in wild type plants, but catalytically impaired mutants are still functional (Gifford *et al.*, 2005; Castells and Casacuberta, 2007; Xu *et al.*, 2008). The PRR Xa21, which confers resistance to all *Xanthomonas* strains carrying the Ax21 protein, depends on kinase activity for full resistance, but kinase impaired mutants still display a partial resistance to *Xanthomonas* (Song *et al.*, 1995; Andaya and Ronald, 2003; Lee *et al.*, 2009). This finding is up to now the only indication that PRR signaling can take place, at least partially, in the absence of kinase activity.

With regard to these examples, which do not depend on intrinsic kinase activity, it is far from clear that the kinase domains of PRRs in *Arabidopsis* require catalytic activity for function. Do PRRs follow the classical scheme of activation through dimerization trans-phosphorylation as it was shown for the brassinosteroid receptor or does the mechanism which activates the receptor and its downstream signaling differ in immunity signaling?

To address this question the EF-Tu receptor was chosen as model. A catalytically impaired EFR receptor was expressed in Arabidopsis *efr* mutant background (Zipfel *et al.*, 2006), followed by a detailed analysis of EFR mediated defense responses. The kinase-dead EFR forms heteromers with BAK1 in response to elf26 treatment, but is unable to trigger activation of the MAP-kinase cascades, ROS generation or defense gene expression. Interestingly, the accumulation of elf26-triggered ethylene is observed even in absence of kinase activity. Finally a partial pathogen resistance is observed, demonstrated by reduced susceptibility to *Agrobacterium tumefaciens* of plants expressing kinase inactive EFR compared to *efr* mutants. This finding indicates that the recognition of pathogens by PRRs triggers multiple pathways: some, like ROS generation or MAP-kinase activation, are PRR kinase dependent, while others do not require signaling via the PRR-kinase. Obviously plants have evolved a complex system to signal the danger of a pathogen attack, which might be more appropriate for fine tuning the disease immune responses. This may also reflect a strategy of plants to counteract the interference by pathogen derived effectors.

5.3 Results

5.3.1 Stable expression of a catalytic impaired EFR in Arabidopsis *efr* mutant plants

The critical lysine residue within the ATP binding domain is an absolutely invariant part of a core structure shared by all members of the protein kinase superfamily and modifications at this site disable the catalytic activity of protein kinases (Scheeff and Bourne, 2005). Therefore we used a lysine to alanine substitution at this critical lysine position of the EFR receptor (EFR-K741A) to fully inactivate its kinase activity. The modified receptor variant, as well as wild-type EFR, were expressed under the control of the constitutive active 35S promoter as YFP-HA tagged fusion proteins in Arabidopsis *efr* mutant background to test their functionality. For subsequent analysis we selected two independent lines, one with low (EFR WT-1 and EFR K741A-1) and one with high (EFR WT-2 and EFR K741A-2) expression levels of the transgene, for each construct (Fig. 5-1A).

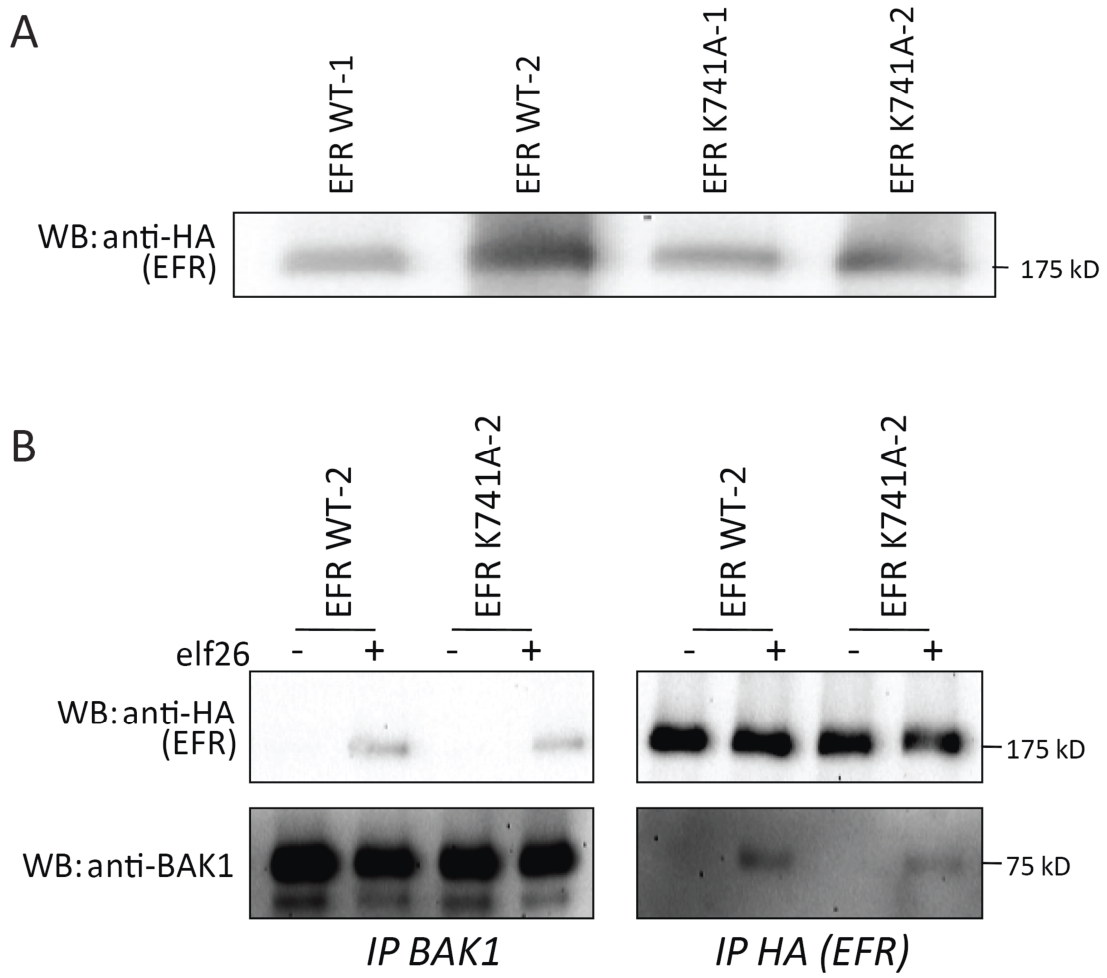


Fig. 5-1 elf26 induced formation of the EFR/BAK1 complex is not dependent on EFR kinase activity.
A: Expression of the EFR-YFP-HA transgene in independent lines. Full protein extracts, derived from leaf material of 3 week old plants, were separated by SDS-PAGE and analyzed by Western blot using anti-HA antibodies. B: Leaf material, treated for 5 min, or not, with elf26 (1 µM). After extraction, solubilized membrane proteins were immunoprecipitated with anti-BAK1 (left panel) or anti-HA (right panel). Resulting precipitates were separated by SDS-PAGE and probed with anti-HA, to reveal transgenic EFR, and anti-BAK1 antibodies.

5.3.2 An EFR/BAK1 complex is formed in absence of EFR kinase activity in a ligand dependent manner

To analyze the relevance of the kinase activity of EFR for the EFR/BAK1 complex formation we immunopurified EFR-WT or EFR-K741A from leaf material, treated or not with elf26, using antibodies directed against the C-terminal HA-tag of the transgenic EFR, and probed the resulting precipitates with anti-BAK1 antibodies to test co-purification of the two proteins. In untreated controls we revealed BAK1 neither in samples from WT-EFR lines nor in immunoprecipitates from EFR-K741A plants (Fig. 5-1B). In contrast, in

immunoprecipitates treated with elf26 for 5 min BAK1 co-immunoprecipitated with both, the EFR-WT and the EFR-K741A, to similar levels, demonstrating that BAK1 interacts with EFR in a ligand inducible fashion, independent of the presence of EFR kinase activity (Fig. 5-1B). In the reciprocal experiment, using anti-BAK1 antibodies for the immunoprecipitation, EFR was detected in precipitates from elf26 treated plant material expressing EFR-WT and EFR-K741A, confirming that the oligomerization of EFR with BAK1 does not require the kinase activity of EFR (Fig. 5-1B).

5.3.3 Induction of ROS generation, MAP-kinase activation and transcriptional regulation by elf26 requires EFR kinase activity

To investigate if the kinase inactive EFR, which is able to form a complex with BAK1, triggers downstream signaling cascades, we monitored a broad set of early cellular responses induced by MAMPs, including generation of ROS, MAP-kinase activation and changes in gene expression, which proceed all within minutes after elicitor perception (Felix *et al.*, 1999; Nühse *et al.*, 2000; Zipfel *et al.*, 2004).

The generation of ROS was measured in leaf pieces by the H_2O_2-dependent luminescence of luminol. Leaf material from Col-0 or EFR-WT complemented *efr* plants respond with a ROS generation starting about 2-3 min after application of elf26 (Fig. 5-2A). In contrast, plants transformed with the kinase inactive EFR did not respond with a significant generation of ROS. All lines were responsive to flg22 treatment in a similar way as wild type plants, demonstrating that the tested plants were vital and MAMP signaling was not generally perturbed in the transgenic lines (Fig. 5-2A).

To analyze the activation of MAP-kinases we used antibodies which detect the fully phosphorylated form of MAP-kinases. In plants transformed with EFR-WT the elicitor elf26 triggered a strong phosphorylation of the MAP-kinases MPK3 and MPK6, to the same extent as it is observed in wild-type Col-0 plants (Fig. 5-2B). MAP-kinase phosphorylation was absent in plants expressing EFR-K741A.

The perception of MAMPs induces an up- or downregulation of more than 1000 genes (Zipfel *et al.*, 2006). We picked the *FRK1* gene (Flg22 induced receptor like kinase 1), which

is strongly transcriptionally upregulated upon MAMP treatments, to monitor the ability of kinase inactive EFR to trigger transcriptional changes (Asai *et al.*, 2002; Kunze *et al.*, 2004; Thilmony *et al.*, 2006; Tsuda *et al.*, 2008b). While wild-type plants and *efr* plants transformed with EFR-WT show a clear induction of *FRK1* expression after 4 h, no change of expression is seen in plants which do not contain a catalytically active EFR receptor (Fig. 5-2C).

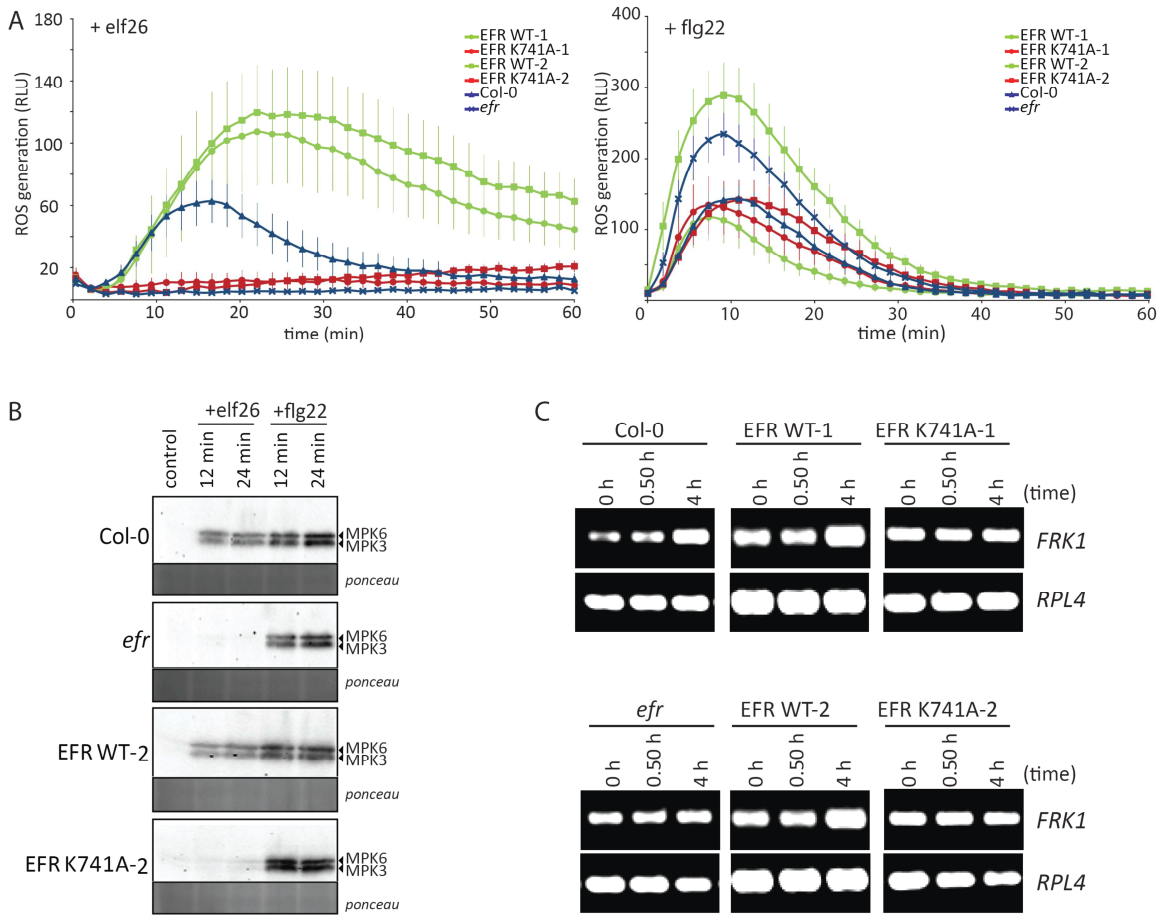


Fig. 5-2 elf26 dependent activation of ROS generation, MAP-kinase activation and marker gene expression requires EFR kinase activity. A: Oxidative burst induced by 1 µM elf26 (left graph) and 1 µM flg22 (right graph), measured in relative light units (RLU) in leaves of wild-type (Col-0), *efr* or *efr* mutants transformed with EFR-WT or EFR-K741A. Elf26 induces a significantly increase ROS accumulation in Col-0 plants and *efr* transformed with EFR-WT only. Flg22 causes clear accumulation of ROS in all tested lines. Results shown are means of eight replicates ± standard error. B: MAP-kinase activation in wild-type (Col-0), *efr* and EFR-WT / EFR-K41A expressing seedlings were treated for 12 min or 24 min. C: Transcriptional expression of the MAMP induced gene *FRK1* (At2g19190) in wild-type (Col-0), *efr* and EFR-WT / EFR-K41A expressing plants. Leaf stripes were treated for 0.5 h or 4 h with elf26 (1 µM). Transcription of the constitutive expressed *RPL4* gene (ribosomal protein L4) was monitored to ensure equal treatment of the different samples.

5.3.4 Ethylene accumulation is induced independently from other known cellular responses

Taken together the analysis of ROS, MAP-kinase activation and the gene expression analysis indicate, that the EFR kinase activity is required to initiate cellular responses. We extended this analysis to the accumulation of ethylene, which takes place during a range of a few hours after elicitor treatment. To monitor ethylene biosynthesis leaf pieces of different plants with or without elicitors were incubated in sealed glass tubes. All tested plants showed only little accumulation of ethylene in absence of any elicitor and accumulated comparable amounts of ethylene when incubated with the unrelated MAMP flg22 (Fig. 5-3).

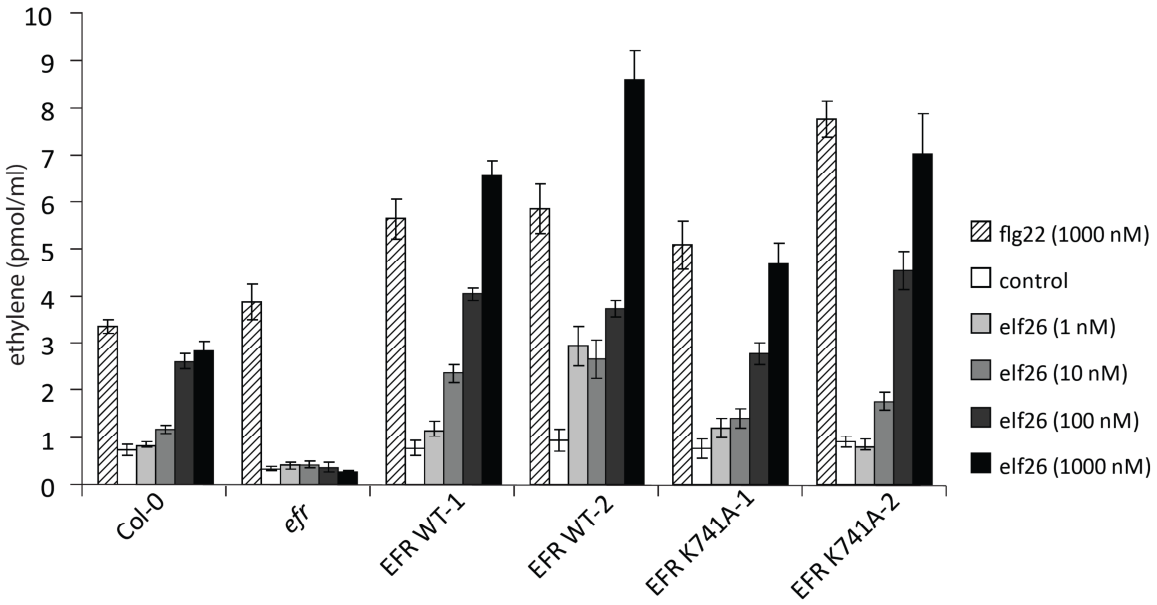


Fig. 5-3 EFR-kinase independent accumulation of ethylene after elf26 treatment. Total ethylene biosynthesis of wild-type plants (Col-0), *efr*, and *efr* mutants transformed with EFR-WT or EFR-K741A were measured 3 h after stimulation with flg22 (1 µM), BSA (control) or different concentrations of elf26 (1 nM – 1000 nM). Plants expressing kinase inactive EFR show a similar accumulation of ethylene as control plants expressing the wild-type form of EFR. Results shown are means of six replicates ± standard error.

In both EFR-WT expressing tested lines and in one out of two EFR-K741A lines ethylene accumulation was observed already in presence of 1 nM elf26 and the ethylene accumulation increased with higher elf26 concentrations. Also the amount of accumulated ethylene at high elicitor concentrations was similar in all transgenic lines (EFR-WT and

EFR-K741A) and the variations in ethylene accumulation correlated with the amount of transgene expression in these different transgenic lines (Fig. 5-1A). This ethylene response of EFR-K741A lines clearly demonstrates that ethylene accumulation upon elf26 stimulation does not require receptor kinase activity. But even more strikingly, since we did not find ROS generation, MAP-kinase phosphorylation and defense gene expression, this result is the first evidence that ethylene biosynthesis can be induced independently of these cellular responses.

It should be noted that for wild-type Col-0 plants ethylene accumulation was observed upon a minimal elf26 concentration of 10 nM only. Also the ethylene accumulation measured for Col-0 plants upon high elf26 concentrations did not reach a level as found for EFR-WT or EFR-K741A plants. This indicates that the EFR receptor is over expressed in the transgenic lines, resulting in increased elf26 sensitivity compared to Col-0 plants.

5.3.5 Late responses including elf26 induced growth inhibition and resistance are partially observed in absence of receptor kinase activity

MAMP perception results in an increased resistance to pathogens, but also a reduced seedling growth can be observed when seedlings are grown in continuous presence of the elicitor (Gómez-Gómez *et al.*, 1999; Zipfel *et al.*, 2004; Zipfel *et al.*, 2006). Since the kinase inactive EFR triggers a part of the downstream signaling, we analyzed these late responses to get insight not only in the signaling cascade itself, but also into the relevance of the different events for immunity.

A late response which is frequently used to analyze the effect of MAMPs is the growth inhibiting effect which is caused in seedlings upon elicitor application (Gómez-Gómez *et al.*, 1999; Zipfel *et al.*, 2006; Krol *et al.*, 2010). The application of the unrelated MAMP flg22 to Col-0 plants, *efr* mutants, EFR-WT lines or EFR-K741A lines causes in all cases a similar strong reduction of the seedlings growth. Interestingly, elf26 caused a growth reduction not only in Col-0 plants or *efr* plants complemented with the EFR-WT, but also inhibited growth of plants expressing the EFR-K741A transgene (Fig. 5-4 A and B). Quantification of

this effect by measuring the fresh weight of the seedlings showed that plants missing EFR kinase activity were less sensitive to elf26 than plants expressing wild-type EFR (Fig. 5-4 A and B). However, a residual response was clearly visible and correlated as well to the expression level of the transgene (Fig. 5-1A). Therefore growth inhibition, caused by elf26, depends only partially on EFR kinase activity.

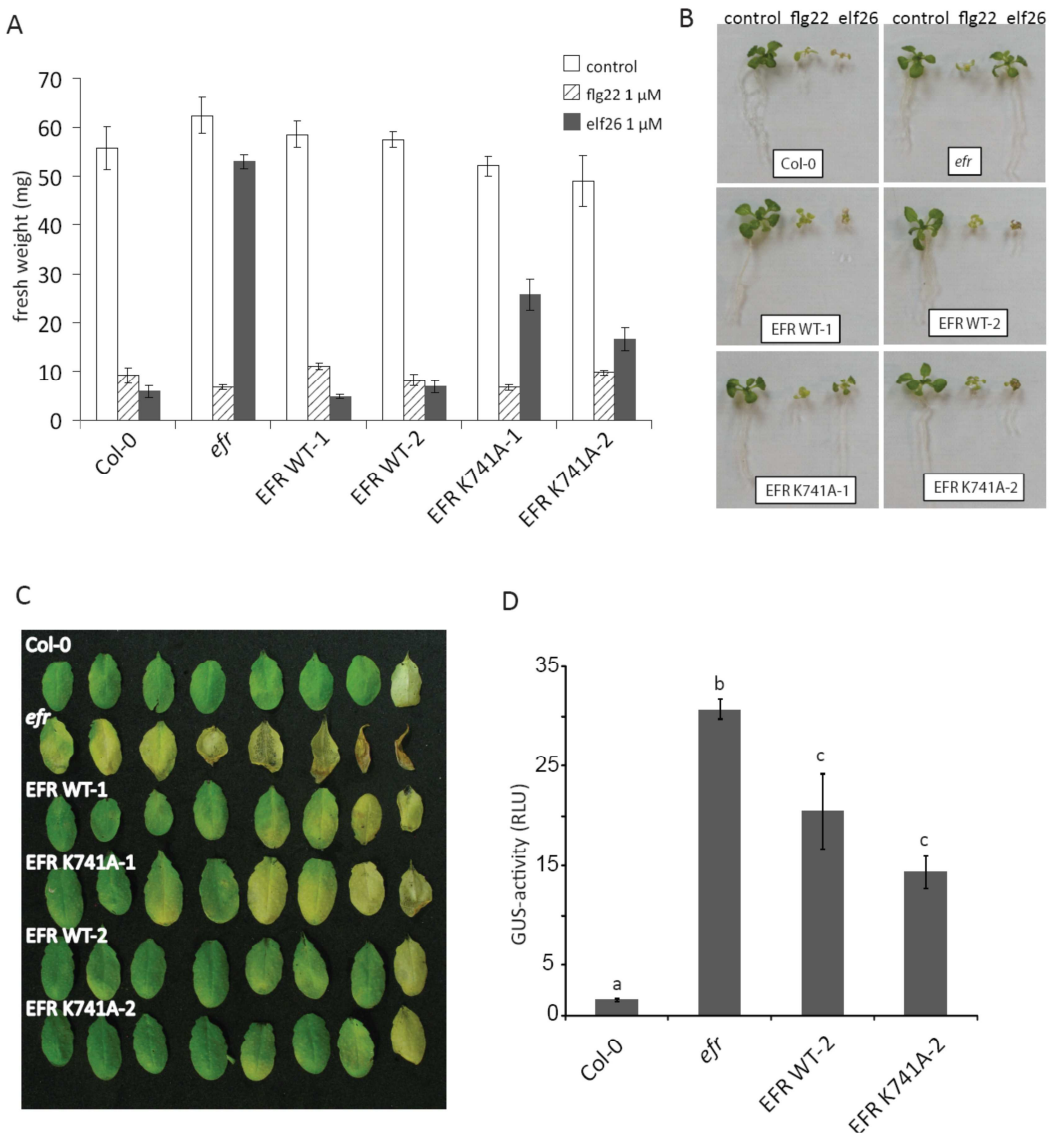


Fig. 5-4 Kinase inactive EFR confers seedling growth inhibition and resistance to Agrobacterium tumefaciens.
A: Seedling growth of wild-type plants (Col-0), *efr,* and *efr* mutants transformed with EFR-WT or EFR-K741A in presence of BSA (control), flg22 (1 µM) or elf26 (1 µM). Results shown are means of six replicates ± standard error. Representative seedlings were photographed (B). C: Symptom development seven days after infiltration of leaves with *Agrobacterium tumefaciens*. All infiltrated leaves were photographed. D: Resistance against *Agrobacterium tumefaciens* was monitored by transformation efficiency using a quantitative MUG assay with extracts from leaves of wild-type, *efr* mutant and EFR-WT or EFR-K741A expressing seedlings, four days after inoculation with *Agrobacterium* carrying pBIN19-35S::GUS (Zipfel *et al.*, 2006). Results shown are means of six replicates (± standard error) of GUS-enzyme activity in relative units (RU). Means shown with same letters were not significantly different based on a least significant difference test ($p < 0.05$).

To test if EFR confers bacterial resistance in absence of its kinase activity, we employed an infection assay using *Agrobacterium tumefaciens*. Resistance to *Agrobacterium tumefaciens* clearly depends on the presence of EFR resulting in higher transformation rates and more pronounced disease symptoms in *efr* mutants (Zipfel *et al.*, 2006). Seven days post infection with the *Agrobacterium* strain GV3101 *efr* plants displayed a strong chlorosis phenotype as well as, in a few examples, necrotic lesions. Col-0 plants developed only very moderate symptoms, such as chlorosis, in a low number of leaves (Fig. 5-4C). Importantly, *efr* lines which were transformed with EFR-WT or EFR-K741A exhibited leaf chlorosis, which was intermediate between Col-0 plants and *efr* mutants, but only little development of necrotic lesion was observed in these plants (Fig. 5-4C). The increased chlorosis development in EFR WT-1 and EFR K741A-1 compared to EFR WT-2 and EFR K741A-2 correlates with the transgene expression in these lines (Fig 5-1A). The result indicates that the kinase inactive EFR confers resistance similar to the active wt-control.

To quantify the susceptibility of host plants to *Agrobacterium* the efficiency of T-DNA transfer to plant cells can serve as reliable readout (Zipfel *et al.*, 2006). To monitor the transformation efficiency we used an *Agrobacterium* GV3101 strain harboring a 35S::GUS construct on its T-DNA (Zipfel *et al.*, 2006). Seedlings growing on solid MS-medium were covered with an *Agrobacterium* solution, incubated for 5 days under standard growth conditions, and were then analyzed for their glucuronidase activity. Enzyme extracts derived from infected Col-0 plants contained only very little glucuronidase activity, compared to *efr* mutants (Fig. 5-4D), which demonstrates the reduced T-DNA transformation efficiency due to an increased resistance to *Agrobacterium* in presence of a functional EFR. Plants expressing the EFR-WT construct were not fully complemented, as demonstrated by a transformation rate which is intermediate between *efr* and Col-0 plants (Fig. 5-4D). This may be a result of miss-expression, due to the 35S promoter, or a consequence of the YFP-HA tag. More importantly, lines expressing EFR-K741A exhibited glucuronidase activity which is similar as the wt-control (Fig. 5-4D). This clearly demonstrates that kinase inactive EFR can confer at least a partial resistance to *Agrobacterium tumefaciens.*

5.4 Discussion

The presence of kinase domains in several pattern recognition receptors, like EFR and FLS2, indicates a function of receptor kinase activity in the activation of immune responses. However, up to now the relevance of receptor kinase activity was never clearly investigated. In this study we addressed this point by a characterization of a kinase impaired EFR receptor expressed in *efr* mutant background. This analysis showed clearly that one set of immune responses strictly depends on the catalytic activity of the EF-Tu receptor, while a second set of responses does not or only partially depend on EFR kinase activity. Strikingly, this observation not only demonstrates the significance of kinase activity for receptor function it also reveals independent pathways in the immune signaling downstream of this pattern recognition receptor. While one branch of the EFR downstream signaling, which comprise the accumulation of the gaseous plant hormone ethylene, is intact in EFR-K741A expressing plants, other pathways, including the activation of MAP-kinases or the generation of ROS, are not activated upon elf26 stimulation in absence of EFR kinase activity.

But how are these two pathways linked to each other, is the accumulation of ethylene absolutely independent of ROS generation and MAP-kinase pathway? Several earlier studies which analyzed the linkage of ethylene accumulation and MAP-kinase activation came to contrasting conclusions. Yoo and coworkers (2008) observed the involvement of a MAP-kinase cascade, constituted of CTR1 (a MAPKKK), MKK9 and MPK6, suggesting that it connects the ethylene signaling downstream of the ethylene receptor 1 (ETR1) to transcriptional regulation of ethylene responsive genes (Ouaked *et al.*, 2003). Other authors contradict this observation since ethylene treatments or application of the ethylene precursor ACC does not induce the activation of MAP-kinases (Bethke *et al.*, 2009). Similarly, our analysis did not reveal the phosphorylation of MPK6, despite a strong ethylene accumulation in EFR-K741A expressing plants. In a study of Lu and Zhang (2004) it was shown that the bacterial MAMP flg22 induces the activation of MPK6 which directly phosphorylates and stabilizes ACC-synthase 6 (ACS6), a key enzyme in the ethylene synthesis. This observation would indicate that MPK6 resides upstream of ethylene

accumulation. However, *mpk6* mutants lacking this kinase, still produce ethylene in response to flg22 and also our analysis indicates that a MAP-kinase independent pathway is responsible for the observed ethylene accumulation. These contradictions support our observation, that MAP-kinases and ethylene accumulation function in distinct pathways, but they also show that such pathways are likely to be interconnected at different points.

Interestingly, the generation of ROS during plant defense seems to be regulated through Ca^{2+} dependent kinases (CDPK), which also function independent from MAP-kinase activation (Boudsocq *et al.*, 2010). Our finding that ethylene accumulates independent of both responses, MAP-kinase activation and ROS generation, indicates the existence of at least three independent signaling branches. How these pathways are connected and whether they function additive, synergistic or independent from each other remains to be answered.

Our analysis showed that in absence of EFR kinase activity the MAMP inducible gene *FRK1* is not transcriptionally upregulated upon elf26 stimulation, demonstrating that beside a partial activation of the signaling cascade no full gene induction is obtained. However, a comparison of transcriptional regulation in response to expression of constitutive active MAP-kinases and constitutive active CDPK variants demonstrated, that distinct sets of genes are differentially regulated by these two pathways (Boudsocq *et al.*, 2010). Also *FRK1* is transcriptional upregulated through active MAP-kinases, but is not induced upon CDPK-activity (Boudsocq *et al.*, 2010). This differential transcriptional regulation indicates that a more extended analysis of genetic regulation is required to visualize all processes which proceed during elf26 elicitation in absence of EFR kinase activity. It is well possible that *FRK1* expression is regulated through MAP-kinase activity specifically, and therefore requires the kinase activity of EFR, while other genes are upregulated upon elf26 stimulation in absence of EFR-kinase activity.

Providing the existence of multiple signaling pathways downstream of EFR one may ask, whether individual pathways are able to mediate pathogen resistance. *efr* mutants expressing the kinase impaired variant of EFR show a susceptibility to *Agrobacterium* which is intermediate between Col-0 plants and *efr*, demonstrating that at least a partial

pathogen defense can be activated in absence of EFR kinase activity. This partial resistance in absence of ROS and MAP-kinase signaling shows, that plant defense can be activated through individual pathways in the signaling network downstream of EFR. This observation is in agreement with an analysis of the pattern recognition receptor Xa21 (Song *et al.*, 1995; Andaya and Ronald, 2003). Transgenic lines expressing a kinase inactive Xa21-K736E variant in rice, carrying a similar mutation of the critical lysine as the kinase impaired EFR-K741A, display partial resistance to *Xanthomonas oryzae* pv *oryzae (Andaya and Ronald, 2003)*. This strong parallels indicate that the here observed mechanism is not specific for EFR and may be true also for other plants PRRs.

It should be noted that also the expression of EFR WT could not fully complement the lack of the receptor in *efr* mutants, and such plants exhibited a resistance to *Agrobacterium* which was comparable to plants expressing kinase inactive EFR only. Interference by the C-terminal YFP-HA tag or missing transcriptional regulation of the EFR gene, due to the constitutive 35S promoter, may explain this observation. An analysis using the native EFR promoter in absence of a fusion tag, may help to answer this question. Also an analysis of EFR mediated resistance with different pathogens like the coronatine mutant of *Pseudomonas syringae* pv. *tomato* DC3000*,* which is restricted by EFR mediated defense (Nekrasov *et al.*, 2009), would possibly shed more light on the role of EFR kinase activity and the different downstream pathways in resistance to bacterial pathogens.

It remains to be answered how the kinase impaired EF-Tu receptor activates downstream responses. A sequence analysis of the Arabidopsis kinome revealed that 13% of all kinase genes and 20% of the RLKs miss invariant amino acids within kinase catalytic domains, and are thus putatively kinase-defective (Castells and Casacuberta, 2007). Hence, signaling through catalytically impaired kinases seems to be common for receptor like kinases in plants (Castells and Casacuberta, 2007). For EFR the recruitment of additional components, like the associated kinase BAK1, represents a well possible mechanism to activate downstream responses, since EFR kinase activity itself is not required for complex formation and the associated kinase can be recruited. Hence, BAK1 may be responsible for the initiation of the active signaling branches in EFR-K741A expressing plants. A

combination of kinase inactive BAK1 with kinase inactive EFR in a *bak1* and *bak1/efr* mutant background may answer this question. However, other phosphorylation independent mechanisms, as well as the recruitment of additional kinases are also possible.

AvrPto an effector protein from *Pseudomonas syringae* pv. *tomato* DC3000, which is secreted into the plant cytoplasm to block plant immunity (de Torres *et al.*, 2006a; Hann and Rathjen, 2007), was shown in function as kinase inhibitor of EFR (Xiang *et al.*, 2008). The possibility to signal in absence of catalytic activity secures the plants surveillance system at least partially against such interference. An analogous conclusion can be drawn for the downstream signaling of EFR. Blocking of bifurcate pathways, with several alternative possibilities to transmit information, requires interference at multiple points and such network like systems are therefore clearly more stable against the bacterial effectors. This finding may also explain the high number and variety of different effector proteins found in many plant pathogens (de Wit *et al.*, 2009; Studholme *et al.*, 2009). In addition a complex network of cellular events is the basis for an integration of different information within the cell. Hence, the increasing complexity of immunity signaling in plants, observed in this study, is likely to be a key for an efficient and well adapted immune response.

6 Co-immunoprecipitation of FLS2 interacting proteins reveals new aspects in MAMP signaling

6.1 Abstract

Perception of microbe associated molecular patterns, such as the bacterial flagellin, provides the basis for non-host resistance in plants. To understand how this resistance is activated and how pathogens are defeated during this response, we need to know the involved components. Here we report the identification of yet unknown putative intracellular binding partners of FLS2, by immunoprecipitation followed by mass spectrometry. The group of identified proteins includes candidates known to be involved in signal-transduction and proteins of unknown function. In addition a surprisingly high number of proteins involved in vesicle trafficking, including different RAB-GTPases, SNARE-proteins, and ARF-GTPases, were identified. This indicates that FLS2 not only triggers the activation of downstream pathways, but also is involved in targeting vesicles directly to the infection site.

6.2 Introduction

The identification of several MAMPs and their cognate pattern recognition receptors (PRRs) advanced the understanding of how microbes are perceived in plants. Especially the recognition of the bacterial protein flagellin, recognized by FLS2, became a paradigm for this type of pathogen surveillance (Gómez-Gómez *et al.*, 1999; Gómez-Gómez and Boller, 2000). For full signaling, FLS2 requires its binding partner BAK1, which is also involved in multiple other processes such as brassinosteroid signaling or morphogenesis (Li *et al.*, 2002a; Whippo and Hangarter, 2005; Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Schulze *et al.*, 2010 and chapter 5 of this thesis). The complex formation with BAK1, which is also a receptor-like kinase (RLK) like FLS2 explains partially how signals may be transferred into the inside of the cell. The recent finding that BIK1 (Botrytis-Induced Kinase 1), a receptor like-cytoplasmic kinase, interacts with FLS2 and BAK1, added another piece to the signaling cascade induced by FLS2 (Veronese *et al.*, 2006; Lu *et al.*, 2009a). However, it is still not understood how receptor activation leads to the induction of innate immune responses

including cell wall reinforcement, stomata closure or secretion of antimicrobial components (Gómez-Gómez *et al.*, 1999; Melotto *et al.*, 2006; Kwon *et al.*, 2008). To decipher the immunity signaling in plants we clearly need to identify additional factors which receive, transmit and possibly integrate the information from the different PRRs. Hence we initiated a survey to identify interacting proteins of FLS2, using an immunopurification approach. This allows to study the *in vivo* interaction-partners of the FLS2/BAK1 receptor complex and it also enables the comparison of plant material with and without elicitor stimulation, to reveal proteins which bind specifically to the activated receptor complex. Indeed we indentified several novel putative FLS2 interactors. Among them we found several proteins, which were previously shown to be involved in plant defense, such as SERK family members and 14-3-3 proteins (Chinchilla *et al.*, 2007; Yang *et al.*, 2009). Their presence in a FLS2 complex allows to position these factors within the signaling cascade and to assign possible functions. A remarkably high number of our candidate proteins functions in protein trafficking, including RAB-GTPases and SNARE proteins, which provide specificity for membrane fusion events, or ARF-GTPases and dynamine like proteins, which function in formation of vesicles (Chavrier and Goud, 1999; Vernoud *et al.*, 2003). But we also found putative interactors without any known role in plant immunity. Overall, these proteins will help to increase our understanding of the first steps of receptor mediated signal transduction.

6.3 Results

6.3.1 Identification of FLS2 interacting proteins by co-immunopurification

Using antibodies directed against the C-terminus of FLS2 (Chinchilla *et al.*, 2006), the FLS2 protein was purified from detergent solubilized protein extracts. The cell cultures used are highly sensitive to flg22 treatments, and the accessibility of the single cells for applied peptide allows a homogenous and synchronized stimulation. We applied such a purification in parallel on cells treated for 5 min with flg22 (1 µM) and untreated cells. This allows to identify proteins which specifically bind to FLS2 or are released from the receptor in response to elicitation.

After immunoprecipitation (IP) FLS2 and co-purified proteins were separated via SDS-PAGE, stained with colloidal coomassie, and subsequently analyzed via LC-MS/MS (in collaboration with Steven Clouse and Mike Gosh, North Carolina State University). To identify also low abundant proteins, which cannot be detected by total protein stains in the gel, the full gel was analyzed applying an automated protein identification technique. This method quantifies each present tryptic peptide followed by MS/MS fractionation to reveal the amino acid sequence of the fragment. Each determined peptide is subsequently eliminated from the analysis. Proceeding along these routine the amino acid sequence of all present fragments was stepwise determined, which allows the identification of proteins by the presence of one or few high quality peptide spectra (Mc Hugh and Arthur, 2008). The results of the analysis are summarized in appendix A.1.

The high sensitivity of this system introduces a danger of detecting unspecific co-purified proteins. The observation that the majority of the identified proteins functions in protein translation and folding or represent structural constituents of the cytoskeleton, like tubuline, supports this assumption (appendix A.1). In addition, the FLS2 antibody used for this experiment recognizes not only the C-terminus of FLS2, but purifies also an unrelated protein (Q9FIC2) (Chinchilla *et al.*, 2006). To reduce the number of false positives, we focus on proteins which were found only in the flg22 elicited samples.

6.3.2 Different types of LRR-RLKs interact with FLS2 after flg22 stimulation

As expected, we found the FLS2 interaction-partner BAK1 in the flg22 treated samples and no peptides of the BAK1 protein were present in samples from untreated cells. The identification of BAK1 in the IP of flg22 treated cells confirms first, that the FLS2 protein perceived the applied elicitor and got activated to form a complex with its cognate interaction partner, second, it proofs that the applied technique is sensitive enough to identify interacting partners of FLS2.

BAK1 belongs to the SERK family composed of five different members. Importantly peptides specific for each member of the family were identified in the flg22 stimulated

FLS2-IP. Thus, FLS2 forms not only a complex with BAK1/SERK3, but also interacts directly or indirectly with SERK1, 2, 4, and 5.

Beside SERK family members, an additional LRR-RLK protein was found to be associated with the flg22 stimulated FLS2 receptor in two independent experiments. This protein (At2g01210), from now on referred to as 'FLS2 interacting LRR-RLK' (FIL1), contains a short extracellular domain consisting of only three LRRs. To verify a role of FIL1 in flagellin signaling, we analyzed a *fil1* T-DNA insertion line (SALK_021338C), which bears an insertion within the *FIL1* coding region. Typical cellular responses upon MAMP treatments are the accumulation of reactive oxygen species, accumulation of the plant hormone ethylene and also a growth reduction, when seedlings are grown in continuous presence of an active elicitor. All three responses were analyzed using different concentrations of flg22 as well as elf26. However, in none of these responses we observed a significant difference between the *fil1* mutant and wild-type Arabidopsis Col-0 plants (Fig. 6-1). An analysis of the Arabidopsis genome offers a likely explanation for the absence of a visible phenotype in *fil1* mutant lines. A second RLK coding gene (At1g25320) shares 62% identity on DNA and a similarity of 72% on the protein level (Fig. 6-2) with the *FIL1* coding or amino acid sequence, respectively. Creation of a double mutant line, lacking the genes for *FIL1* and its paralog are needed to elucidate a potential role for FIL1 in FLS2 mediated signaling.

Several components which take part in the MAMP signaling are transcriptionally upregulated upon elicitor stimulation (Zipfel *et al.*, 2004) and the analysis of this feedback regulation aided in many cases the identification of proteins, which contribute to MAMP signal perception and transmission, including the EF-Tu receptor EFR and BAK1 (Zipfel *et al.*, 2006; Chinchilla *et al.*, 2007). To investigate if an elicitor dependent gene induction is found also for the *FIL1* gene, we analyzed its expression, based on public microarray data (Zimmermann *et al.*, 2004). 1 h after elicitor treatment, *FIL1* is expressed 0.23 fold (flg22), 0.58 fold (elf 26), 0.35 fold (LPS) or 0.93 fold (chitin) compared to untreated seedlings. Assuming that transcriptional modulation upon MAMP elicitation increases the sensitivity of the plants surveillance system, this result would rather indicate a negative regulating function for FIL1.

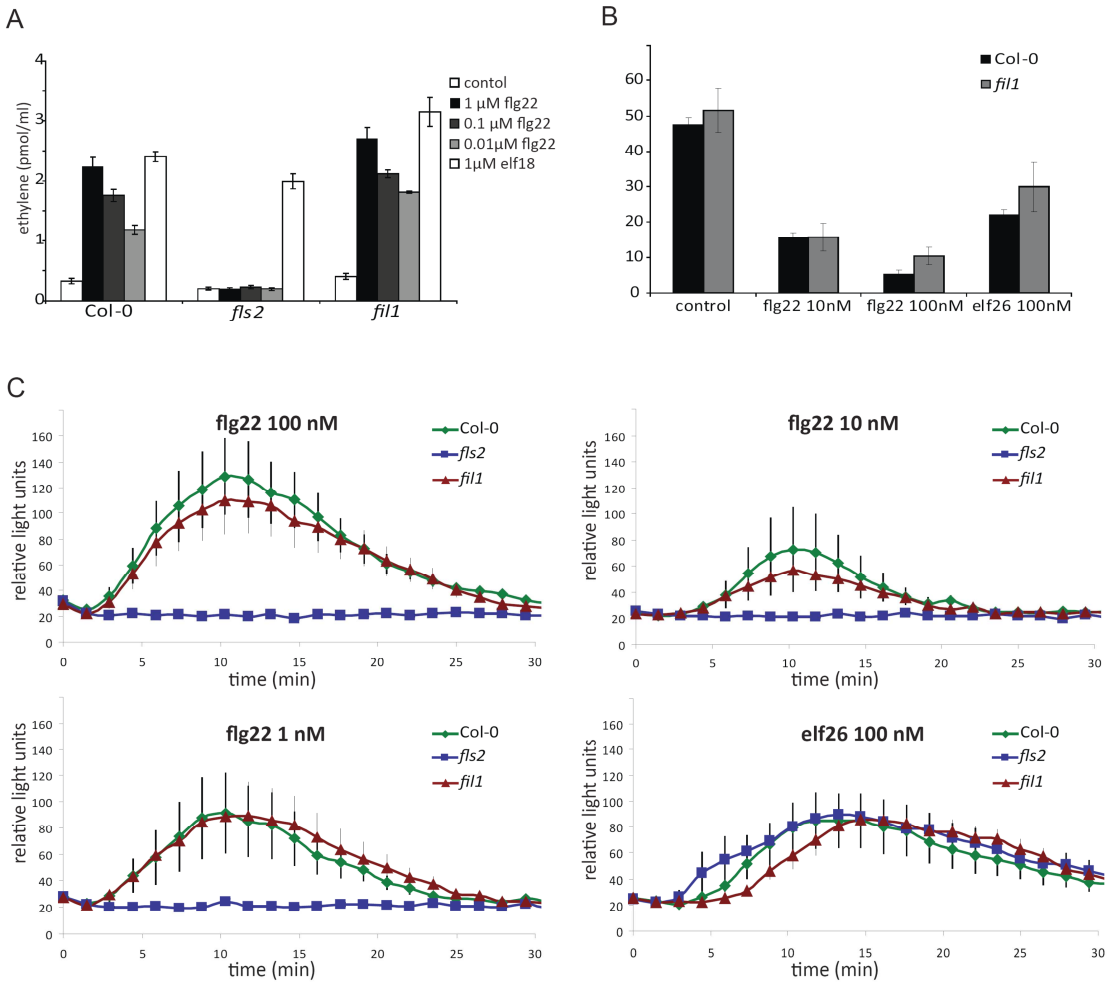


Fig. 6-1 Functional analysis of fil1 T-DNA mutant. Wild-type plants (Col-0), *fls2* mutants and *fil1* mutants were analyzed for ethylene accumulation, seedlings growth inhibition, and ROS generation. A: ethylene accumulation after stimulation with BSA (control), elf26 (1 µM), or different concentrations of flg22 (10 nM – 1 µM). Results shown are a mean of six replicates ± standard error. B: seedlings growth inhibition in presence of BSA (control), 10 nM flg22, 100 nM flg22, and 100 nM elf26. Results shown are a mean of six replicates ± standard error. C: ROS production in response to different flg22 concentrations (1-100 nM) and elf26 (100 nM). Results shown are a mean of eight replicates ± standard error.

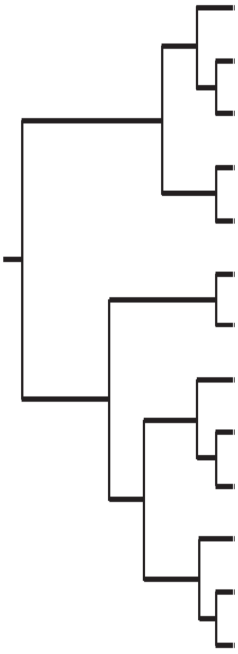
To confirm the interaction of FIL1 with FLS2, we cloned the *FIL1* gene as a YFP-HA fusion protein, under the control of a 1 kb DNA region upstream of the *FIL1* ORF. The sequence was verified by sequencing and used for expression of the *FIL1* transgene by stable transformation of Arabidopsis plants or transient transformation of *N. benthamiana.* However, we could not detect any protein accumulation using anti-HA or anti-GFP antibodies (not shown). For future experiments we propose to use the strong constitutive 35S promoter from CaMV to ensure an expression level which can be used in a biochemical analysis.

Fig. 6-2 Comparison of the amino acid sequence of FIL1 (At2g01210) and its closest homologue (At1g25320) by pair wise alignment. Identical amino acids are highlighted in yellow, similar amino acids in green. Sequences are obtained from GenBank Database.

6.3.3 14-3-3 proteins, potential components of the FLS2 complex

Our mass spectrometric analysis revealed several peptides in the flg22 treated IP sample which led to the identification of 14-3-3 proteins. However, due to the high homology between different isoforms of 14-3-3 proteins, the peptide hits obtained could not unambiguously identify specific candidate proteins. In addition a few 14-3-3 protein derived peptides were found in the untreated sample, but quantification and comparison of the two IP samples showed that such peptides are less abundant in the untreated IP (table 6-1).

As a first step we used an *in silico* analysis to study the possible interaction of FLS2 and the 14-3-3 general regulator family. Therefore we applied the SCANSITE algorithm to predict potential 14-3-3 binding sites on the FLS2 protein, based on its primary amino acid sequence (Yaffe *et al.*, 1997; Obenauer *et al.*, 2003; Ferl, 2004). The best candidate, out of four possible predicted interaction sites, was S-1078, as indicated by a low percentile value (Fig. 6-3). Importantly, this site coincides not only with the putative PEST motif of FLS2 (Robatzek *et al.*, 2006), but also with the only flg22 dependent phosphorylation motif identified in a parallel study (chapter 2). This supports the hypothesis of a 14-3-3 contribution to FLS2 signaling.



isoform	locus	identified in	expression	antibody affinity
MU	At2g42590	plus/minus	0.97	++
EPSILON	At1g22300	plus	1.06	++
PI	At1g78220	not identified	0.91	n. d.
IOTA	At1g26480	not identified	0.21	n. d.
OMICRON	At1g34760	not identified	1.22	n. d.
KAPPA	At5g65430	plus	0.88	-
LAMBDA	At5g10450	plus	0.92	-
PSI	At5g38480	plus	0.91	n. d.
NU	At3g02520	plus/minus	1.14	+++
UPSILON	At5g16050	plus/minus	0.91	+++
OMEGA	At1g78300	plus	0.82	+
PHI	At1g35160	plus/minus	0.89	n. d.
CHI	At4g09000	plus	0.89	n. d.

Tab. 6-1 Outline of Arabidopsis 14-3-3 proteins. The cladogram summarizes the relationship of the different 14-3-3 proteins (De Lille *et al.*, 2001). Peptides referring to different isoforms were either not identified, identified in the IP from flg22 plant material (plus) or found in IPs from untreated and from flg22 treated plant cells (plus/minus). The expression of the corresponding genes in response to flg22 treatment (30 min) is shown in fold change, compared to control. The antibody, which was used for immunoprecipitation to verify the FLS2/14-3-3 interaction (Fig. 6-4), differs in its capacity to bind distinct Arabidopsis isoform (Oeking *et al.*, unpublished), indicated as +++/++/+/- for high/medium/low/no capacity to recognize the corresponding 14-3-3 protein (n.d. = not determined).

To analyze this interaction in further detail, we used anti-14-3-3 antibodies, originally raised against 14-3-3 proteins from spinach, for co-immunoprecipitation (Moorhead *et al.*, 1999). These antibodies recognize most of the Arabidopsis isoforms with varying affinity (tab. 4-1). This tool allowed us to work with a similar experimental setup as used for the mass spectrometric analysis. In immunoprecipitates of FLS2 (Fig. 6-4A; IP: FLS2) a strong signal for FLS2 could be revealed, but neither in absence nor in presence of flg22 stimulation a signal for 14-3-3 proteins was detected in the FLS2 IP using anti-14-3-3 antibodies for Western blot analysis (Fig. 6-4A). In a reciprocal experiment, using anti-14-3-3 antibodies for immunoprecipitation (Fig. 6-4A; IP: 14-3-3), three signals could be revealed which correspond by size to 14-3-3 proteins and refer to different 14-3-3 isoforms. However, no FLS2 protein was detected with anti-FLS2 antibodies in 14-3-3 precipitates irrespectively of elicitor treatment (Fig. 6-4B).

site	score (percentile)	sequence	surface availability
1: S390	4.781 %	LTNLRNLSAHDNLLT	0.971
2: S599	4.526 %	PASLKSLSLLNTFDI	0.385
3: S880	3.427 %	ANIIGSSSLSTVYKG	0.880
4: S1078	1.607 %	MTKQRPTSLNDEDSQ	1.576

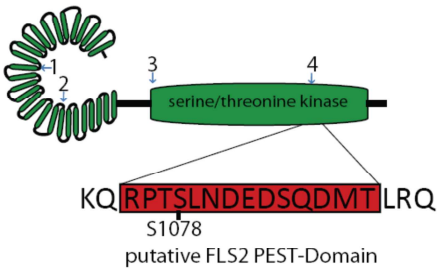


Fig. 6-3 In silico analysis of potential 14-3-3 binding sites on FLS2. FLS2 primary amino acid sequence was analyzed for putative 14-3-3 binding sites using "SCANSITE" algorithm (Obenauer *et al.*, 2003). Shown are the four potential sites predicted together with their surrounding amino acid sequence, a scoring of the quality of prediction, and an estimation of sequence availability. Likelihood increases with lower percentage value, cut off for high stringency is 0.2%. A red box highlights S-1078 which was predicted to be the likeliest 14-3-3 binding site on FLS2. Positions of the potential 14-3-3 binding sites are indicated on a schematic drawing including the potential PEST domain of FLS2 (Robatzek *et al.*, 2006).

As shown in table 4-1 the used antibodies do not recognize all isoforms of Arabidopsis. We therefore tried to verify the FLS2/14-3-3 interaction for each isoforms independently, using transgenic Arabidopsis lines expressing either GFP-tagged 14-3-3 isoforms under the control of their endogenous promoter (obtained from C. Oecking, ZMBP Tübingen) or FLAG-tagged lines, controlled by a constitutive 35S promoter.

Plants expressing the epitope tagged 14-3-3 isoforms were grown in liquid culture, stimulated with flg22 for 5 min and subsequently analyzed again using immunoprecipitation and Western blot analysis. As an example the analysis of plants expressing the GFP tagged 14-3-3 isoform KAPPA was depicted (Fig. 6-4B). FLS2 could be detected in the protein extract (input) and the immunoprecipitation of FLS2 (Fig. 6-4B; IP: FLS2), and also in immunoprecipitation of BAK1 upon flg22 (Fig. 6-4B; IP: BAK1), demonstrating the formation of a FLS2/BAK1 upon elicitor treatment. In contrast no FLS2 protein was detected in immunoprecipitations of 14-3-3-GFP fusion proteins with anti-GFP antibodies (Fig. 6-4B; IP: GFP).

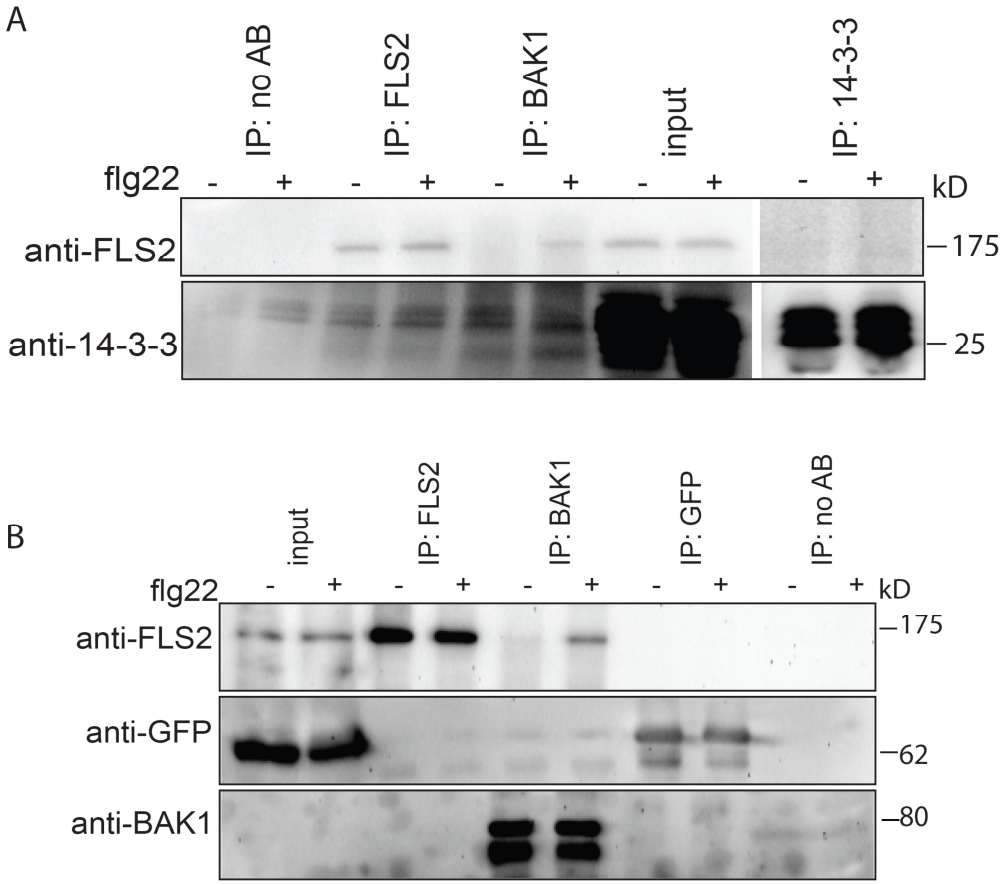


Fig. 6-4 Analysis of FLS/14-3-3 protein interaction by immunopurification. A: wild-type Arabidopsis cells were treated (or not) for 5 min with flg22 (1 µM). After extraction, solubilized membrane proteins (input) were immunoprecipitated with anti-FLS2, anti-BAK1, or anti-14-3-3 antibodies or were incubated with proteinA-sepharose without antibodies as control (noAB). Resulting precipitates were separated by SDS-PAGE and probed with anti-FLS2 or anti-14-3-3 antibodies. B: leaf stripes from plants expressing GFP tagged 14-3-3 isoform KAPPA were treated (or not) with flg22 (1 µM). Anti-FLS2, anti-BAK1 and anti-GFP were used for both, IP and Western blot analysis of the resulting precipitate.

According to the strong signal which was detected in the plant extract (Fig. 6-4B; input) the KAPPA-GFP transgene is strongly expressed in the transgenic lines. Also the immunopurification of the KAPPA-GFP fusion protein, using anti-GFP antibodies, was possible as demonstrated by the strong Western signal in the precipitate of KAPPA-GFP (Fig. 6-4B; IP: GFP). Surprisingly in untreated and flg22 treated precipitates of FLS2 and BAK1 a faint signal is visible which corresponds to the KAPPA-GFP fusion protein. However, a similar strong signal is visible in a control IP, which was performed without any antibody, demonstrating that the low amount of KAPPA fusion protein which co-purified with FLS2

and BAK1 results from unspecific binding of the fusion protein. Hence, the interaction of FLS2 and the 14-3-3 isoform KAPPA could not be verified using this experimental approach.

In a similar way we analyzed all other 14-3-3 candidates (not shown). In none of the FLS2 or BAK1 IPs we could reveal a specific 14-3-3 signal when compared to control IP. Also reciprocally we could not reveal any FLS2 signal in IPs of the tagged 14-3-3 fusion proteins. Therefore further investigation based on a different experimental setup is necessary to reveal a possible function of 14-3-3 proteins in immunity.

6.3.4 FLS2 recruits components of vesicle trafficking

A surprisingly high number of proteins identified in the flg22 stimulated IP appears to be involved in vesicle trafficking (appendix A.1). This group comprises different isoforms of the vesicle-coat recruiting Arf-GTPases, dynamine-like proteins which function in vesicle formation, as well as SNARE proteins and RAB-GTPases (Tab. 4-2) which provide together the specificity of vesicle fusion events (Vernoud *et al.*, 2003).

RAB-GTPases, which were reproducibly identified in the flg22 treated IP, account for the largest number of the identified proteins, with isoforms from the three different subgroups A, D2 and E1. To verify the interaction between RAB proteins and the FLS2 receptor, we used the so called wave-line collection, a series of Arabidopsis lines expressing RAB-YFP-Myc fusion-proteins under the control of an ubiquitin promoter, constructed for microscopic analysis of vesicles (Geldner *et al.*, 2009). Several marker-proteins used in this collection are GTPases from the same subclade or are even identical with RAB-GTPase from our FLS2 IP. The use of these transgenic lines allows the analysis of the RAB-GTPase by IP and subsequent Western analysis. As control we used also lines expressing RAB proteins from clades which were not identified in our mass spectrometric analysis.

The expression-level of the RAB-YFP-Myc transgene varied strongly between the different transgenic lines, resulting in strong differences in the amount of RAB protein purified from different lines by the anti-Myc antibody. In all precipitates which contained a detectable amount of RAB protein, FLS2 was co-immunoprecipitated, verifying the ability of RAB-GTPases to interact with FLS2. However, FLS2 immunoprecipitated not only

together with IPs of RAB protein candidates (D2b, E1d, A1e), but a FLS2 signal was revealed also in precipitates of RAB C1, D1, F2b, G3f which were used as control (Fig. 6-5A). Further a clear difference between IPs from flg22 stimulated and untreated tissue is not reproducibly observed. In reciprocal experiments where FLS2 precipitates were analyzed for the co-purifying RAB-GTPases a similar isoform and stimulus independent binding was observed (Fig. 6-5B). This is in contrast to the result of the mass spectrometric analysis, where RAB-GTPases were only found in IPs from flg22 treated cells. On the basis of these results we cannot exclude that the YFP-Myc tag or the overexpression of the protein in the used transgenic Arabidopsis lines is leading to loss of specificity in these biochemical experiments.

Isoform	Locus	Reproduced	WAVE-line	flg22
A1a	AT1G06400	Yes		0.77
A1c	AT5G45750	Yes		0.89
A1d	AT4G18800	Yes		1.53
A1e	AT4G18430		Yes	3.09
A1f	AT5G60860			0.76
A1g	AT3G15060	Yes	Yes	0.94
A1h	AT2G33870			0.73
A2a	AT1G09630			1.69
A2b	AT1G07410	Yes		0.87
A2c	AT3G46830	Yes		0.8
A4b	AT1G16920	Yes		0.73
A5a	AT5G47520			0.81
D2a	AT1G02130		Yes	0.87
D2b	AT5G47200	Yes	Yes	1.02
D2c	AT4G17530	Yes		0.82
E1a	AT3G53610	Yes		0.88
E1c	AT3G46060	Yes		0.84
E1d	AT5G03520	Yes	Yes	0.71
E1e	AT3G09900			0.98

Tab. 6-2 Outline of identified RAB-GTPases. The table indicates the different RAB-GTPase isoforms which were identified in the mass spectrometric analysis of FLS2 precipitates from flg22 treated material. The analysis was repeated twice, isoforms found in both experiments are marked as verified. For several isoforms Arabidopsis lines expressing the YFP-Myc tagged GTPase were available (WAVE lines). The fold expression change of the corresponding genes upon 30 min flg22 treatment, based on public available micro array data, indicated differential regulation only for one gene (flg22, dark green) (Zimmermann *et al.*, 2004).

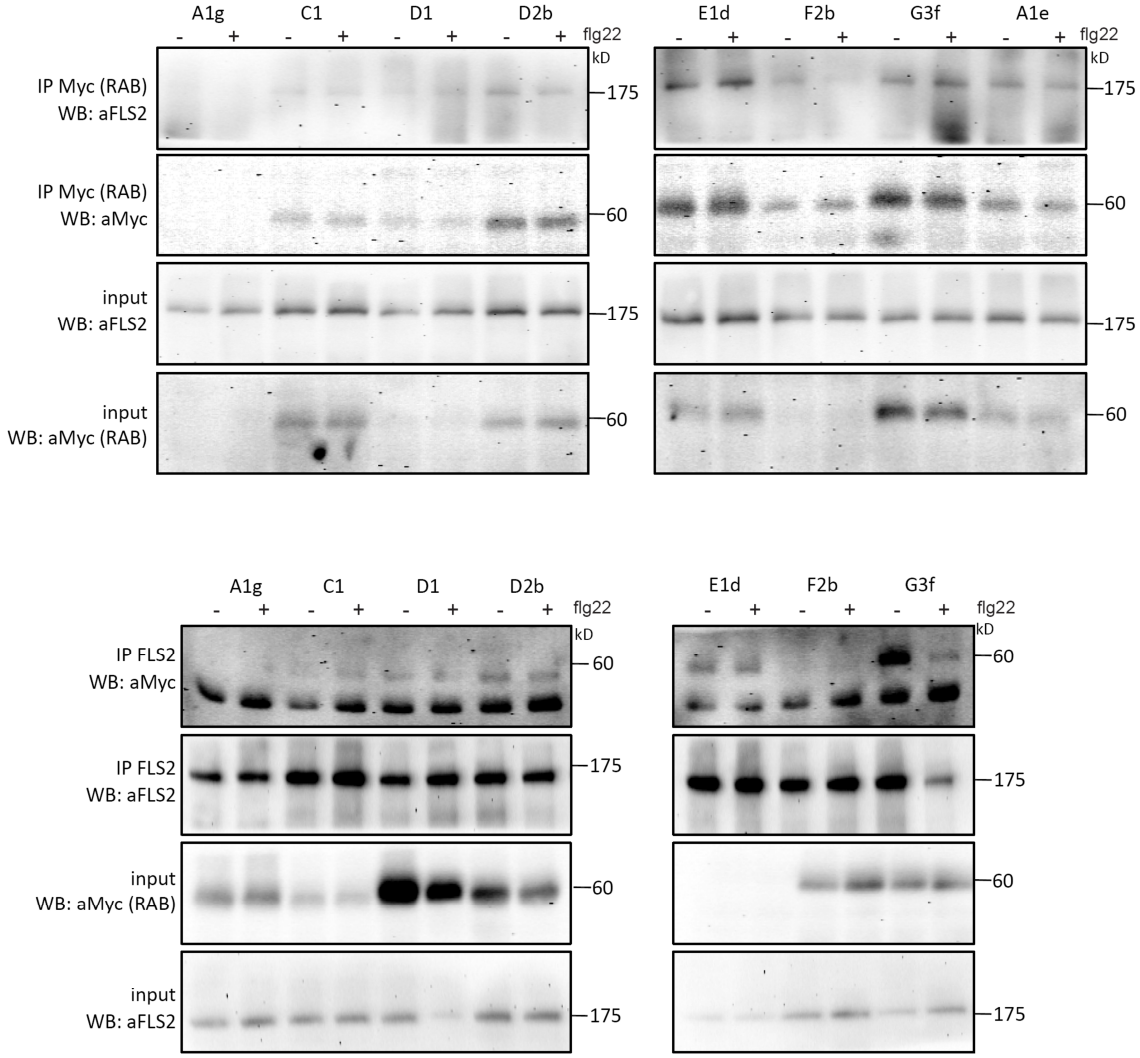


Fig. 6-5 Analysis of FLS2/RAB-GTPase interaction by immunopurification. Leaf material of plants expressing various YFP-Myc tagged RAB-GTPase isoforms were cut in fine stripes and treated (or not) with flg22 (1 µM). After extraction, solubilized membrane proteins (input) were immunoprecipitated with anti-FLS2 (A) or anti-Myc which precipitates tagged RAB proteins (B). After separation by SDS-PAGE, precipitates were analyzed using anti-Myc and anti-FLS2 antibodies.

Interestingly, the analysis of public microarray data supports a function in immunity for at least one isoform. The RAB-GTPase *A1e* is upregulated by flg22 as shown by a 3.1 fold increase in expression 30 min after an flg22 stimulus (Fig. 6-5) (Zimmermann *et al.*, 2004; Thilmony *et al.*, 2006). The lack of a differential expression for the remaining GTPases does not exclude such a function, since most RAB genes are little regulated in their expression level (Rutherford and Moore, 2002).

6.4 Discussion

By the identification of receptors we scratched, taken literally, the surface of plant immunity research, but we know little about events and factors functioning downstream of pathogen recognition. Immunoprecipitation of FLS2 and concurrent co-immunopurification of immediate interaction partners represents a method to directly approach this point and moreover it allows to imitate the process of FLS2 stimulation by comparing IPs from flg22 elicitated and untreated tissue. Using this approach it is possible to investigate, how PRRs recruit additional proteins to assemble an active receptor complex and trigger their downstream signaling.

6.4.1 All SERK proteins are present in the flg22 treated FLS2 complex

One of the first events in FLS2 signaling is clearly the complex formation of the receptor with BAK1, which occurs within 1 second upon flg22 stimulation (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Schulze *et al.*, 2010). Importantly, also SERK1, 2, 4, and 5, the four Arabidopsis paralogues of BAK1, were identified in the immunoprecipitation of FLS2. Hence, BAK1 may be a major component of the FLS2 signaling complex, but presumably other BAK1 homologues contribute to this complex in addition. *bak1-4*/*serk3* mutants which lack the BAK1 RLK are not fully insensitive to flg22, and also other single T-DNA mutants of SERK1, 2, 4, and 5 still respond to the elicitor flg22, indicating a functional redundancy in the Arabidopsis genome for these proteins (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Chinchilla *et al.*, 2009). The flg22 dependent upregulation of SERK4 (Zipfel *et al.*, 2004) is in agreement with this functional redundancy of BAK1 and its paralogues and also other recent findings support this hypothesis: experiments using plants expressing FLAG-tagged SERK2 clearly demonstrate an flg22 dependant FLS2/SERK2 interaction (Dietsch *et al.*, unpublished). An additional line of evidence is provided by a recent genetic analysis: while the crossing of *bak1* and *serk4* null mutants results in plants which die within the first two weeks (He *et al.*, 2007), a weak EMS mutant of BAK1 (*bak1-5*) which is affected in flg22 and elf26 but not in brassinosteroid responses, allows the generation of *bak1-5/serk4* double mutants (Schwessinger *et al.,* unpublished). The analysis of these *bak1-5/serk4 double* mutants showed complete insensitivity for flg22, confirming that also SERK4 is

indeed involved in flg22 and elf26 perception (Chinchilla *et al.*, unpublished). All together these results verify the SERK/FLS2 interaction found in our precipitation.

An additional putative component of the FLS2 signaling complex is the transmembrane protein FIL1, which co-purified reproducibly with the FLS2 receptor. FIL1 belongs to the class III of LRR-RLK proteins, which contain only three LRR repeats in the extracellular domain and an RD signature within the kinase domain. In a preliminary analysis of *fil1* mutant plants no differences were found for ethylene accumulation, ROS generation and seedlings growth inhibition in response to flg22 treatment. A second LRR-RLK (At1g25320), which shows strong homology with FIL1, might be functionally redundant and a double mutant needs to be generated and analyzed for its flg22 responses. However, FIL1 could also be involved in other, yet unknown, signal responses initiated by FLS2. Also verification and a more detailed analysis of the FLS2/FIL1 interaction are necessary. The development of Arabidopsis lines which express tagged versions of FIL1, driven with a constitutive promoter will allow such an analysis.

6.4.2 14-3-3 proteins are involved in plant immunity

After formation of the FLS2 complex, BAK1 and FLS2 are phosphorylated (Schulze *et al.*, 2010). This phosphorylation event may provide the basis for the recruitment of another potential complex constituent, the 14-3-3 proteins. These proteins function as general regulators and as scaffold proteins and bind to the phosphorylated target-proteins. Their binding usually induces a subsequent change in structure and activity of the target protein (De Lille *et al.*, 2001; Ferl, 2004; Kenny and O'Neill, 2008). In recent years, evidence has accumulated which suggests that 14-3-3 proteins may be also associated with plant disease resistance. Using far-Western screening and *in vitro* binding assays, Konagaya *et al.* (2004) detected interaction between several 14-3-3 isoforms and the tobacco N protein, a TIR-NBS-LRR that confers resistance to tobacco mosaic virus. Also RPW8 a transmembrane protein which confers broad-spectrum resistance to the biotrophic fungal pathogens *Golovinomyces spp.* was shown to interact with the 14-3-3 protein LAMBDA (Yang *et al.*, 2009). Furthermore, knockdown of 14-3-3 LAMBDA compromises basal resistance against powdery mildew, confirming its role in disease resistance. A function of 14-3-3 proteins

which is related to FLS2 signaling is indicated by the observation that in Arabidopsis leaves challenged with *Pseudomonas syringae* pv. *tomato* DC3000 14-3-3 proteins showed a greater than 2 fold increase on protein level (Jones *et al.*, 2006). Furthermore, 14-3-3 proteins interact also with SERK1, identified in this study as potential interaction partner of FLS2 itself (Rienties *et al.*, 2005).

Putative binding sites for 14-3-3 proteins within the FLS2 protein were identified using SCANSITE algorithm (Obenauer *et al.*, 2003). Among the 52 serine and threonine residues in the FLS2 C-terminus S-1078, within the putative PEST domain of FLS2 (Robatzek *et al.*, 2006), appeared to be the most probable 14-3-3 binding motif (Fig. 4-4). Importantly, this site correlates with a position identified in a parallel analysis, which is phosphorylated in response to FLS2 activation (chapter 2 of this thesis). The co-occurrence of the identification of this potential phosphorylation site with the prediction of the 14-3-3 binding motif suggests a model in which flg22 recognition and FLS2 complex formation leads to phosphorylation of the Serine 1078 followed by the binding of 14-3-3 proteins.

To identify 14-3-3 isoforms which might be relevant for the FLS2 signaling and also to verify the FLS2/14-3-3 interaction we performed co-immunoprecipitation experiments, using either wild-type Arabidopsis cells in combination with an anti-14-3-3 antibody recognizing most of the 14-3-3 proteins, or Arabidopsis plants expressing epitope tagged 14-3-3 isoforms. Despite repeated attempts, we were not able to detect interaction between these proteins. This could be due to a too transient or weak interaction between FLS2 and the respective 14-3-3 protein. However, a false positive interaction cannot be excluded and further independent experiments are necessary to allow a clear statement regarding the interaction of 14-3-3 proteins with the FLS2 receptor and their function in flg22 signaling.

6.4.3 Vesicle trafficking in innate immunity

Among the proteins which were specifically found together with flg22 stimulated FLS2 the general regulators of the 14-3-3 family are the only proteins which are clearly involved in signaling processes. Conversely, a high number of potential FLS2 interactors are

associated with vesicle trafficking processes. These include the two SNARE proteins VAMP721 and VAMP722, RAB-GTPases from 3 different subclasses, ARF-GTPases and dynamine-like proteins.

In recent years a body of evidence accumulated which proves the importance of vesicle trafficking and secretion for plant immunity and a directed, focal secretion seems thereby of particular importance. For ascomycota and oomycota it was shown, that infection induces a structural rearrangement of the actin cytoskeleton and a movement of organelles including golgi stacks, ER membranes and nucleus to a position in proximity of the infection site (Schmelzer, 2002; Takemoto *et al.*, 2003; Koh *et al.*, 2005). The secretion of specific compounds results consequently in the formation of papillae beneath the infection sites, not only during infections with cell penetrating fungal pathogens but also below colonies of bacteria which form within the apoplast of the plant cell (Bestwick *et al.*, 1995; Soylu *et al.*, 2005). This secretion process not only forms a physical barrier, but also delivers defense related proteins and possible antimicrobial substances or proteins to defeat the proliferating pathogens (Bestwick *et al.*, 1997; Soylu *et al.*, 2005; van Loon *et al.*, 2006; Kalde *et al.*, 2007).

Furthermore, components of vesicle trafficking are targeted by bacterial effector proteins, emphasizing their importance for bacterial directed defense reactions. AtMin7, an ARF-GEF, a class proteins which function as key-components in vesicle trafficking, is targeted by the *Pseudomonas syringae* effector HopM1 (Badel *et al.*, 2003; Nomura *et al.*, 2006). Also the *Pseudomonas* effector AvrPto targets RAB-GTPases which control the specificity of vesicle targeting (Martin *et al.*, 2003; Speth *et al.*, 2009).

In the course of a fungal infection vesicle trafficking functions via a SNARE complex, constituted of the syntaxins Pen1 and SNAP33 at the cell membrane and the v-SNARES VAMP721 and VAMP722 which are localized at the vesicle surface (Collins *et al.*, 2003; Kwon *et al.*, 2008). These proteins contribute to the identity of the vesicle and confer certain specificity, being at the same time the motor for the vesicle fusion process. Mutants or silenced plants lacking components of the PEN1/SNAP33/VAMP721/722 SNARE

bundle, form no or a delayed papilla resulting in a loss of or a reduced resistance against the powdery mildew *Blumeria graminis* (Collins *et al.*, 2003; Kwon *et al.*, 2008).

During a bacterial infection the Pen1 homologue Syp132 seems to play a central role. This syntaxin is phosphorylated in response to flg22 stimulation in Arabidopsis (Nühse *et al.*, 2003) and *N. benthamiana* plants silenced for *SYP132* expression exhibit a delayed extracellular accumulation of PR-1 as well as a reduced resistance to *Pseudomonas* HrpA$^-$ mutants (Kalde *et al.*, 2007).

Many questions regarding vesicle trafficking in plant defense remain to be answered. Are there different systems for the defense against bacterial and fungal pathogens and if so, which v-SNAREs function in the delivery of vesicles directed against bacterial threats? And what is the cargo carried by such vesicles? However, one of the most burning questions is how vesicles locate to the site of fungal penetration or bacterial colonization. The results of our mass spectrometric analysis provides the opportunity to answer several questions at one go. VAMP721 and VAMP722 were found to co-purify together with flg22 stimulated FLS2. Even though this interaction has yet to be validated, it indicates that the SNARE complex employed for bacterial directed defense responses contains the identical v-SNARE proteins as found for fungal directed vesicles. The recent finding, that also flg22 and elf18 trigger the formation of a ternary SNARE complex with Pen1 and VAMP721/722, supports this hypothesis (Hye *et al.*, 2008). Additionally a reduced growth inhibiting effect of flg22 after VAMP721/722 knockdown in Arabidopsis seedlings was observed (Hye *et al.*, 2008). This strongly indicates, that bacterial directed immune responses employ a similar secretion machinery as observed for powdery mildew infections.

An interaction of VAMP721/722 with FLS2 can answer only partially how the vesicles are targeted to the infection sites. Complexes of t-SNAREs and v-SNAREs contribute to the specificity of vesicle fusion, but mostly they are the workhorses which provide the required energy for the membrane fusion process (Rutherford and Moore, 2002). In contrast RAB proteins play a major role in assigning the correct fusion partners (Rutherford and Moore, 2002). These GTPases bind to the membrane at the vesicle site and can then interact with proteins in the target membrane, which brings the fusing membranes into proximity and

initiates the fusion process (Jahn *et al.*, 2003). The observation that RAB-GTPases, after a treatment with flg22, purify together with FLS2, indicates that such a complex, leading vesicles to their correct fusion site, might be assembled between the activated FLS2 and the RAB proteins. This RAB/FLS2 interaction provides therefore a possibility to explain how vesicles are located to the infection site.

Importantly, a focal and FLS2 regulated secretion could explain the riddle of FLS2 endocytosis and expression. The FLS2 receptor is endocytosed and subsequently degraded within 60 min after ligand perception, possibly for down-regulation of signaling capacity or degradation of `used´ FLS2 receptors (Robatzek *et al.*, 2006). In parallel FLS2 expression is strongly upregulated already 30 min after the perception of flg22 (Zipfel *et al.*, 2004). If focal and FLS2 regulated secretion is indeed part of resistance against bacteria, it would represent a mechanism to refill the FLS2 pool at the infection site. This would also explain the association of FLS2 with components of the secretory pathway. Isoforms of the ER-resident chaperone BIP2 was not only shown to be essential for resistance against *Pseudomonas syringae* (Wang *et al.*, 2005a), but also it was reproducibly co-purified with FLS2 after flg22 stimulation, indicating an increased secretion of FLS2 instantaneous after elicitation (appendix A.1).

To support any of these models further experiments are required, which prove the interaction between FLS2, RAB-GTPases and VAMP721/722. For this reason we carried out co-IP experiments, using Arabidopsis lines expressing epitope tagged RAB-GTPases under the control of an ubiquitin promoter (Geldner *et al.*, 2009). Using these lines we observed a constitutive interaction of FLS2 with the identified RAB-GTPases, but also with unrelated RAB-GTPases. This contradicts strongly the findings of the mass spectrometric analysis. Possibly the overexpression of the candidate proteins or the expression as fusion proteins causes this unspecific interaction in this biochemical experiment.

It is likely that independent methods will allow the verification of a FLS2/RAB protein complex formation, since RAB-GTPases emerged reproducibly in our MS/MS analysis and in all cases in an absolutely elicitor dependent manner. Therefore we initiated a microscopic analysis of the localization of various RAB-GTPase candidates and FLS2 in lines expressing

both proteins as fluorescence tagged fusion protein. This method might allow to catch two flies with one stroke: to gain proof of the direct or indirect interaction of these proteins as well as to get more insights in the hypothesized vesicle trafficking process.

Taken together the analysis of FLS2 immunoprecipitations could identify new putative components of the FLS2 signaling complex and it will be important to verify and to further analyze the role of these protein factors in the activation of FLS2 mediated downstream signaling. In addition the proteins identified in this study provide a model to explain the mechanism of focal secretion during plant defense. A further analysis of these putative FLS2 interaction partners may therefore help to explain both: how plants spot enemies, and how they defeat them.

7 Final discussion

In the past years it got apparent that recognition of MAMPs and DAMPs provides the basis for pathogen resistance. The identification of new elicitors and of some of their corresponding receptors has strongly driven this research topic (Boller and Felix, 2009). Nonetheless, it is unclear how such "danger-information" is transmitted into and integrated within the plant cell and what physiological responses finally lead to resistance against given pathogens. In this study new aspects appear which add information to these different steps of plant immunity.

7.1 PEPR1 and PEPR2 represent a model for DAMP-perception

Perception of DAMPs in plants is a topic in research which is still at its beginning and lacks an appropriate model system. Notably receptors for oligogalacturonides or for the proteinaceous DAMPs as HypSys, RALF and systemin are unknown (Boller and Felix, 2009). Until recently the only indentified DAMP receptor in Arabidopsis, PEPR1, which recognizes seven endogenous peptides known as *At*Pep peptides, was never verified by a reverse genetic analysis. Also the process for *At*Pep1 perception is not well studied yet (Huffaker *et al.*, 2006; Yamaguchi *et al.*, 2006).

In this thesis we further characterized the Pep-perception in Arabidopsis. The analysis of a *pepr1* T-DNA insertion mutant showed clearly that *At*Pep1 is not perceived by the PEPR1 receptor solely (chapter 1). This finding provided a basis for a candidate based search for additional Pep-receptors, which led to the identification of PEPR2, the homologue of the published Pep-receptor. PEPR1 and PEPR2 act mostly redundantly in the perception of *At*Pep1, but the signaling of PEPR2 causes significantly stronger inhibition of root growth of seedlings upon *At*Pep1 treatments, compared to the response mediated by PEPR1 (chapter 1). This indicates individual functions for the two receptors, but may be also be explained by different levels of expression of the two receptors in diverse tissues. A parallel study by the group of Prof. C. Ryan verified PEPR2 as the second Pep-receptor and could

demonstrate different affinities of the two receptors for different *At*Pep peptides, which supports a model for a distinct role of PEPR1 and PEPR2 (Yamaguchi *et al.*, 2010).

Importantly, the function of *At*Pep signaling is still obscure. Other DAMP signals like oligogalacturonides are obviously a result of pathogenic action leading to degradation of the host structures (Nothnagel *et al.*, 1983). It was speculated that *At*Pep peptides function during wounding and are released when the plant cell is destroyed (Huffaker *et al.*, 2006), but this hypothesis is based on their upregulation during wound response only. In an alternative model the *At*Pep-signaling functions as amplifier of resistance (Huffaker and Ryan, 2007). The upregulation of *At*Pep expression during MAMP signaling and their subsequent recognition by PEPR1 and PEPR2 would multiply the MAMP response in a positive feedback loop. Also this theory is speculative, and based on transcriptional upregulation of defense related components only. Furthermore, the similarity of the *At*Pep-system with systemin, an endogenous elicitor from tobacco which was believed to serve as long distance signal earlier (Pearce *et al.*, 1991), inspires consideration of *At*Pep as long distant signal during immunity.

To investigate the function of *At*Pep1 signaling, several basic questions should be approached. Do *At*Pep precursors, so called PROPEPs, need to be processed to gain activity or can they function as full length proteins? Assuming *At*Pep peptides can function apart from wounding, how are they released into the apoplast to get access to the ligand binding domain of the Pep-receptors? Furthermore it would be very interesting to analyze the relationship of MAMP and DAMP signaling: do they function additively or even synergistically? The knowledge of the *At*Pep/PEPR perception system provides now a basis to further investigate such points. Using *pepr1/pepr2* double mutant plants, different models can be tested. For example, expressing modified or chimeric receptors, constructed of different domains from DAMP and MAMP PRRs, allows a functional analysis of the Pep-receptors and their similarities to MAMP receptors in their native background *A. thaliana*. And, importantly, the *pepr1/pepr2* double mutants can be analyzed for their susceptibility to different pathogens, herbivores or other biotic stresses.

7.2 Multiple receptor like proteins contribute to MAMP and DAMP perception

In contrast to *At*Pep peptides the function of the MAMPs flg22 and elf26 is apparent and their relevance for resistance to bacterial pathogens has well been demonstrated (Zipfel *et al.*, 2004; Zipfel *et al.*, 2006; Nekrasov *et al.*, 2009). Using these elicitors and their corresponding receptors as model allowed to obtain insights into the process of MAMP perception. It could be shown that the flg22 receptor FLS2 functions through the associated kinase BAK1 to transmit the signal into the inside of the cell (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). Strikingly, our analysis indicates that also other SERK-family members contribute to this process, since also SERK1, 2, 4, and 5 interact with FLS2 in response to flg22 treatment (chapter 4). This finding is verified by recent interaction studies with SERK2 and BAK1 as well as the analysis of *bak1/serk4* double mutants (Dietsch *et al.,* unpublished; Schwessinger *et al.,* unpublished). Interestingly, SERK1, 2, 4, and 5 single mutants do not exhibit reduced flg22 or elf26 sensitivity, demonstrating that membrane proteins of the SERK-family function redundantly in MAMP signaling (Chinchilla *et al.*, 2007).

In addition to the SERK-proteins a LRR-RLK from family III, termed FIL1, was identified as putative FLS2 interactor (chapter 4). Since the interaction of FIL1 seems to be flg22 dependent, this RLK may also contribute to the process of elicitor perception and signaling, but its function remains elusive. Structurally the very short apoplastic domain, constituted of three LRRs only, resembles the 4 LRR long apoplastic domain of the SERK proteins, indicating a similar function as associated receptor.

We also could verify the interaction of EFR and BAK1, which was previously shown by the functional analysis of *bak1* mutant and phosphorylation analysis of proteins in response to elf26 elicitor (Chinchilla *et al.*, 2007; Schulze *et al.*, 2010). This interaction is independent of kinase activity, since kinase inactive EFR still interacts with BAK1 and also mutational inactivation of the BAK1-kinase or the application of a kinase inhibitor does not interfere with the complex formation of FLS2 and BAK1 (Schulze *et al.*, 2010).

BAK1 and the other SERK-family members are crucial for several MAMP receptors of the LRR-class and this raises the question whether BAK1 also functions in the perception DAMP signals. The analysis of single T-DNA insertion mutants for all 5 SERK genes did not show a contribution of BAK1 and its paralogues to *At*Pep1-perception via PEPR1 and PEPR2 (chapter 1). All lines responded similar as wild-type controls to the application of the DAMP peptide *At*Pep1. However, interaction of BAK1 and the Pep-receptors was shown for full length proteins in plants and for the cytoplasmic domains in yeast (Postel *et al.*, 2009; Schulze *et al.*, 2010). Also an electrophysiological analysis demonstrated a reduced Pep1 sensitivity of *bak1* mutants (Krol *et al.*, 2010). In addition, a very recent analysis of *bak1/serk4* double mutants indicated the importance of SERK-proteins for Pep-signaling (Chinchilla *et al.,* unpublished). The SERK proteins are obviously involved in a multitude of processes. These include the perception of different MAMPs and likely also the perception of DAMPs, as just discussed, but also the containment of cell death, somatic embryogenesis, and the perception of the plant hormone brassinosteroid (Li and Chory, 1997; Nam and Li, 2002; Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Kemmerling *et al.*, 2007; Chinchilla *et al.*, 2009). It remains unclear to what extent SERK-proteins are specific for the different downstream pathways. Are these SERK-proteins just highly redundant or are different SERKs responsible for different pathways? Possibly combinations of different SERK-family members create the specificity to the cognate downstream signal. In an alternative model, the specificity is mediated through the receptor itself and the SERKs provide their kinase activity only.

7.3 Activation of pattern recognition receptors

The identification of receptors and associated RLKs leads to the question how these proteins function together to activate the downstream signaling. The PRRs which were analyzed in this study and their complex partner BAK1 contain serine/threonine kinases. The *in vitro* characterization of the FLS2-kinase and EFR kinase demonstrates that PRR kinases are less active with regard to auto- and trans-phosphorylation of artificial substrates than the CLV1 and HAESA kinases, which do not function in plant immunity (chapter 2). This observation, which points to a more stringent regulation of such kinases,

correlates also with the absence of an arginine-aspartate motif within the kinase domain (Wesche *et al.*, 1997; Shane *et al.*, 2006; Stein *et al.*, 2006a). How these, so called non-RD kinases, are regulated is known neither for FLS2, EFR, or any other plant RLK, nor for mammalian non-RD kinases. Also our mutational approach did not reveal the mechanism which controls their activity (chapter 2).

Most of the PRRs which are involved in plant defense belong to the non-RD class of kinases and importantly also the majority of the animal homologues of the plant RLKs, the IRAK kinases, which mediate mammalian pattern recognition, are non-RD type kinases (Shiu and Bleecker, 2001; Dardick and Ronald, 2006). Similar to the low FLS2-kinase activity, the non-RD kinases IRAK-1, IRAK-2 and IRAK-M are not active *in vitro*. IRAK-4 is a RD-type kinase and in contrast to the other IRAKs, IRAK-4 is able to phosphorylate IRAK-1 (Li *et al.*, 2002b). The current understanding is that RD kinases, like IRAK-4, act upstream of non-RD kinases and activate them (Meylan and Tschopp, 2005; Gan and Li, 2006).

Our *in vitro* analysis supports a similar model for the activation of plant PRRs. The FLS2-kinase is not able to phosphorylate BAK1 *in vitro*, but a kinase inactive FLS2 cytoplasmic domain is a substrate for the BAK1-kinase, placing BAK1-kinase activity upstream of FLS2 (chapter 2). And similar to the mammalian system, the recruitment of the non-RD kinase (FLS2) and RD kinase (BAK1) in a common complex would present a crucial step to activate downstream signaling. BAK1 is also active in a complex with the brassinosteroid receptor BRI1, but in the BRI1/BAK1 complex the BAK1-kinase acts downstream of BRI1, since the initial phosphorylation event is a BRI1 catalyzed phosphorylation of BAK1 (Wang *et al.*, 2008). In addition, PRRs do not require kinase activity for their interaction with BAK1, while kinase inactive BRI1 is not capable to form a BRI1/BAK1 complex (Schulze *et al.*, 2010; Wang *et al.*, 2008; chapter 2). Also phosphorylation and interaction of receptor and the regulator BAK1 follow very different kinetics for brassinosteroid and MAMP perception (Wang *et al.*, 2005c; Wang *et al.*, 2008; Schulze *et al.*, 2010). Hence the process of receptor activation for BRI1 differs from the activation of PRRs like FLS2 (Fig. 7-1).

An *in vivo* labeling approach, used in a parallel study, could detect FLS2 and BAK1 phosphorylation already seconds after flg22 application to Arabidopsis cell cultures

(Schulze *et al.*, 2010). This impressively fast phosphorylation points out a role of phosphorylation in the activation of the receptor and its downstream signaling clearly. Also our *in vitro* analysis with full length FLS2 and BAK1 showed such a phosphorylation of the two proteins in response to an *in vivo* flg22 treatment (chapter 2). To investigate the impact of phosphorylation on flg22 signaling, the FLS2 *in vivo* phosphorylation pattern, before and after flg22 stimulation, was analyzed. This led to the identification of two putative phosphorylation sites on the kinase domain: one which was constitutively found independent of elicitor treatment and one which appeared only upon flg22 treatment (chapter 2). However, functional analysis of the identified positions by site direct mutagenesis did not reveal the function of these phosphorylations yet. It cannot be excluded that additional sites on FLS2 are phosphorylated during flg22 signaling, which were not detected by the mass spectrometric analysis. In our analysis we purified FLS2 which was activated by flg22, but very likely also significant amounts of FLS2 which did not perceive any elicitor. Possibly the sensitivity of the mass spectrometric analysis can be increased by purifying only activated FLS2, either via a tagged ligand or indirectly through a purification of its complex partner BAK1.

Also the Pep-receptors are phosphorylated in response to the *At*Pep1 treatment, as shown by *in vivo* labeling techniques (Schulze *et al.*, 2010). Interestingly the DAMP receptors PEPR1 and PEPR2 do not belong to the class of non-RD kinases. A comparison of the MAMP and DAMP kinases could possibly help to understand, if and how these receptors differ in their catalytic properties.

7.4 Signal transduction in pattern recognition

Interestingly, overexpression of catalytically inactive variants of the non-RD kinases IRAK-1, in mammalian cells indicates, that different pathways diverge at the IRAK-kinases and that only a subset of these responses requires the full IRAK1-kinase (Li *et al.*, 2001). Again our analysis reveals significant analogies between mammalian and pattern recognition in Arabidopsis. Inactive EFR can trigger the accumulation of ethylene upon elf26 stimulation, but does not induce the phosphorylation of MAP-kinases and the

generation of reactive oxygen species (chapter 3). This indicates a divergence of these pathways at the receptor. A recent analysis of Ca^{2+}-dependent protein kinases in MAMP signaling, revealed that also Ca^{2+}-influx and ROS generation are not connected through a linear pathway with the MAP-kinase activation (Boudsocq *et al.*, 2010). This indicates the existence of at least three pathways in a branched signaling network.

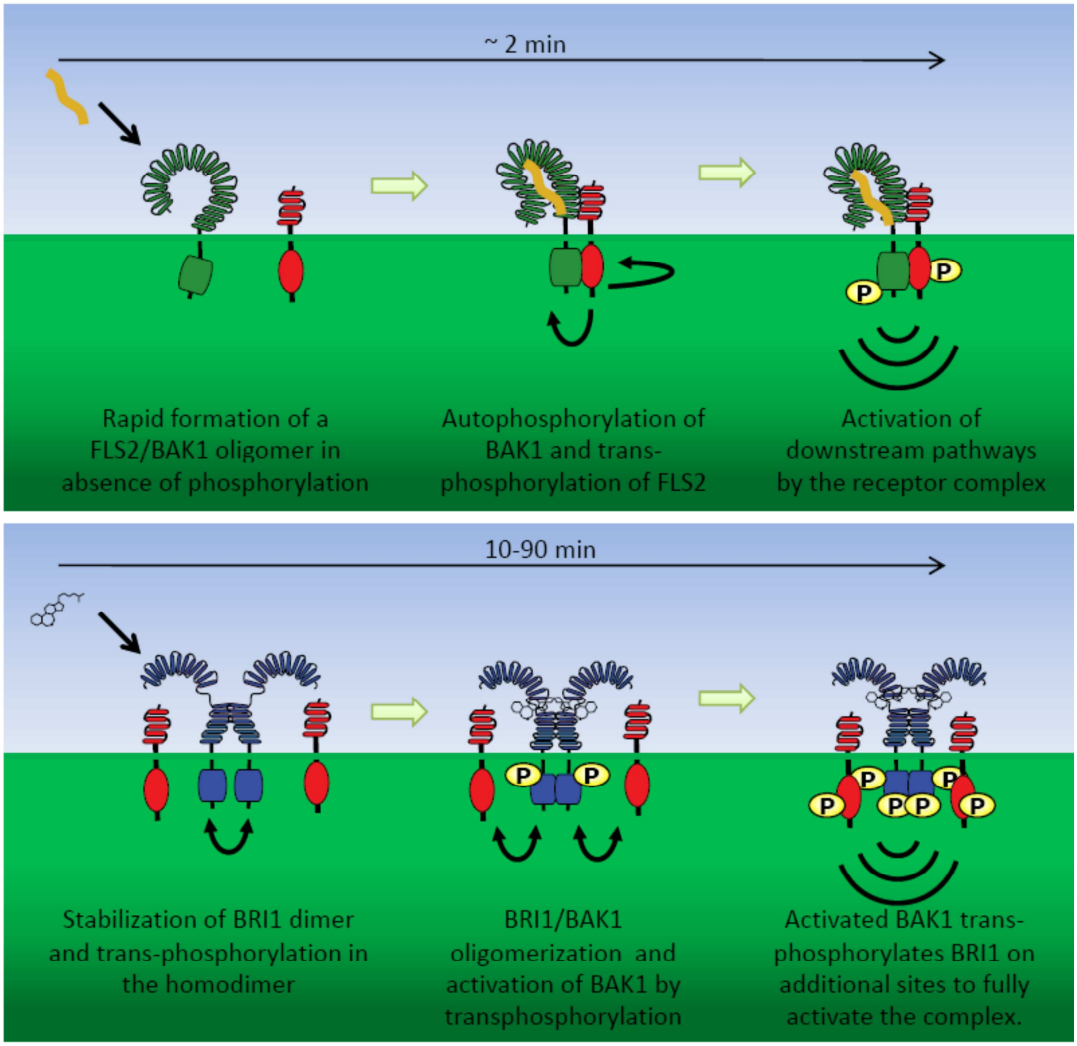


Fig. 7-1 Activation of BRI1 and FLS2 signaling complexes. BRI1 and FLS2 follow a different scheme of activation. The FLS2/BAK1 complex can form in absence of kinase activity and BAK1 is likely to act as upstream kinase which may auto-phosphorylate and trans-phosphorylate FLS2. Conversely the formation of the BRI1 complex is a slow process which requires BRI1 kinase activity for oligomerization and initial phosphorylation of BAK1. The phosphorylated and thereby activated BAK1 is then again trans-phosphorylating BRI1 which leads to full activation of the complex.

Signal transduction is likely to be conserved between DAMPs and MAMPs as exemplified by the use of the common regulator BAK1. Our analysis showed, that also *At*Pep1

treatment induces ROS, ethylene accumulation and inhibition of seedling growth, when applied into the growth medium (chapter 1). Also other DAMPs like OGs or systemin are known to trigger similar signaling events (Doares *et al.*, 1995; Felix and Boller, 1995; Huffaker *et al.*, 2006; Galletti *et al.*, 2008). But beside these overlaps in the signaling, differences can be observed. *At*Pep1 triggered ROS is strongly reduced compared to ROS biosynthesis upon flg22 treatment. More importantly, the sets of genes which are induced upon elicitor application differs for flg22 and oligogalacturonides, a cell wall derived DAMP (Denoux *et al.*, 2008). This reveals that individual transcriptional modulation for DAMPs and PAMPs are similar but not identical. Possibly pattern perception uses a combination of conserved and individual signaling components. Alternatively, differences in timing of the perception, differences in level and localization of expression as well as activity of the different receptors may explain a different processing of signals in the downstream pathway or network. In any case, the diverging pathways, as they were shown in this study, provide a basis for an individual response.

But the existence of diverging signal pathways is important for an additional reason. They provide redundancy, in the information transmission within the cell, which is the logical continuation of the redundancy observed already for receptors and their complex partners. Adapted pathogens interfere with pattern triggered immunity by the secretion of effector proteins into the plant cell. For the bacterial pathogen *Pseudomonas syringae* more than 30 different types of effectors are known and the genome sequence of the fungal pathogen *Phytophthora infestans* revealed more than 600 genes coding for putative effectors (Haas *et al.*, 2009; Studholme *et al.*, 2009). A liner pathway would be a perfect target for such effectors. However, effectors attack different steps in immunity signaling, demonstrating that there is not such a simple pathway and successful infection by microbes requires interference at different points.

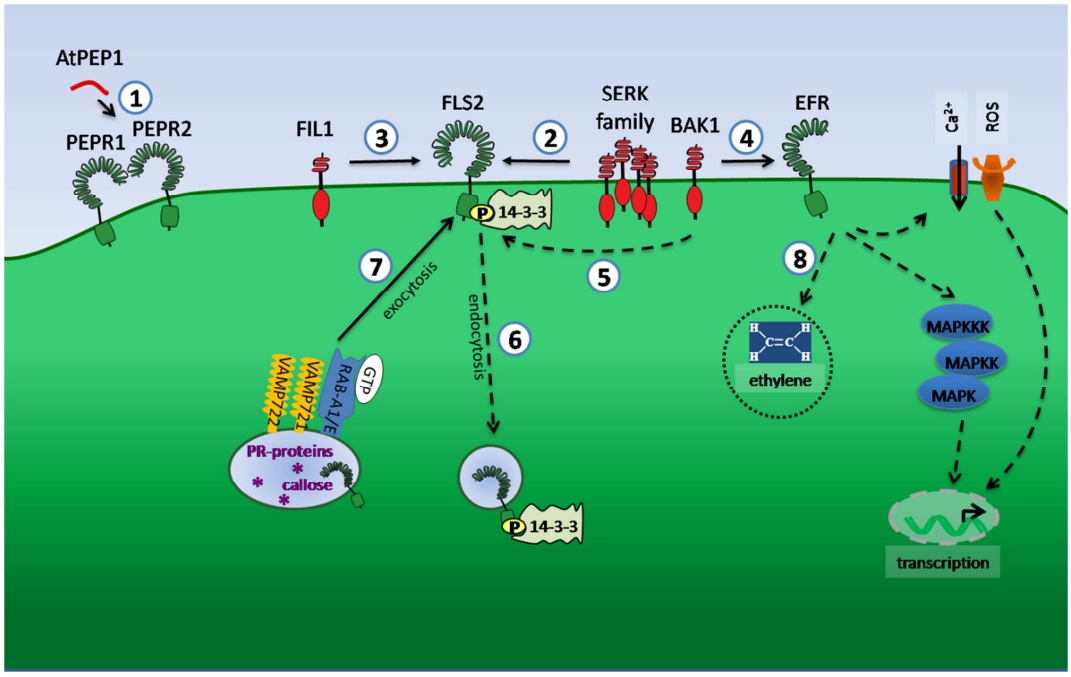


Fig. 7-2 New aspects of plant immunity. The scheme summarizes process and putative interactions, which were analyzed in this study. 1 The DAMP elicitor *At*Pep1 is recognized by two receptors, PEPR1 and PEPR2. 2 FLS2 interacts not only with BAK1, but in addition with all members of the SERK-family in a flg22 dependent manner. 3 In addition to SERK-proteins FIL1, another RLK, interacts with FLS2 upon flg22 treatment. 4 Also EFR interacts with BAK1; this interaction does not require the kinase activity of the receptor. 5 BAK1 is likely to function as upstream kinase which phosphorylates FLS2. 6 This phosphorylation might regulate the endocytosis of FLS2. 7 In parallel the active receptor complex recruits vesicles via an interaction with RAB-GTPases and VAMP721/722. 8 For the activation of MAP-kinases and Ca2+ influx, the EFR receptor requires kinase activity. Independent of these signaling events, the receptor can initiate the accumulation of ethylene without catalytic activity.

7.5 Secretion in plant immunity

The final response, which defeats the pathogen, includes ABA mediated stomata closure or reinforcement of the cell wall trough callose depositions and cross-linking of cell wall components via ROS generation (Gómez-Gómez *et al.*, 1999; Melotto *et al.*, 2006). Also the synthesis of antimicrobial substances and the expression of so called pathogen related proteins is upregulated (van Loon and van Strien, 1999; Kunze *et al.*, 2004; Zipfel *et al.*, 2004; Bednarek *et al.*, 2009). In parallel the secretion machinery is induced which is necessary to deliver such toxic compounds and proteins (Wang *et al.*, 2005a; Thilmony *et al.*, 2006; Li *et al.*, 2009). But simple overexpression of antimicrobial compounds does not lead to a convincing degree of resistance and the key seems to be to target antimicrobial compounds to the pathogen to reach an effective concentration (Punja, 2001; Peschen *et*

al., 2004). Plants achieve this by focal secretion, which allows a directed delivery of toxic load and cell wall components to the site of infection (Aist, 1976; Bestwick *et al.*, 1995; Soylu *et al.*, 2005). A few components of the responsible secretion machinery were identified and their relevance for bacterial and fungal infections was demonstrated in earlier studies (Kalde *et al.*, 2007; Kwon *et al.*, 2008). However, up to now it could not be explained how such vesicles are directed to the site of infection. The different RAB-GTPases and SNARE-proteins, which were identified in this study as putative FLS2 interacting proteins, allow to set up a model how such a process may function (chapter 4). The activated PRR complex, in this case FLS2 and BAK1, could interact directly or indirectly with RAB-GTPases and SNARE-proteins (VAMP721/722) in the membrane of the vesicle. This interaction would target vesicles to the correct membrane site and initiates a fusion process. Thereby the toxic load of vesicles could be released and in parallel the system could be sensitized by delivering an increased amount of receptors.

Interestingly it was reported that several effector proteins interfere with such exocytosis processes. HopM1 mediates the destruction of AtMIN7, an ARF-GEF which is crucial for RAB-GTPase activation, which finally causes reduced callose deposition (Badel *et al.*, 2003; Nomura *et al.*, 2006). Another consequence of HopM1 expressed in Arabidopsis is the disappearance of FLS2 in the plasma membrane (Hann *et al.* unpublished). Also the effector protein XopJ inhibits protein secretion and the effector AvrPto was found to interact with RAB-GTPases of the subfamily E, which were identified in this study as putative FLS2 interactors (chapter 4) (Martin *et al.*, 2003; Bartetzko *et al.*, 2009; Speth *et al.*, 2009).

The nature of these vesicles is still elusive. Do they belong to a default secretory pathway or is this defense completed by specialized vesicular structures? RAB-GTPases are important determinants of vesicle identity. Strikingly, a localization study of RAB-A1 and RAB-D2, showed that both GTPases localize unexpectedly to a not yet clearly defined compartment in proximity to the golgi stacks (Geldner *et al.*, 2009). Combinatorial microscopic studies with golgi markers and RAB-A1 and RAB-D2 show clearly, that these compartments are distinct from the golgi stacks. Interestingly, A1 and D2 do not co-localize, demonstrating the existence of at least two independent post-golgi

compartments, with a still undefined role in the secretory pathway (Geldner *et al.*, 2009). Since the majority of the here identified RAB-GTPases belong to the subfamilies RAB-A1, RAB-D2 and RAB-E (chapter 4), it is tempting to speculate, that these compartments with unknown function represent the vesicles involved in pathogen defense. It is possible that both compartments differ in cargo and are specialized for different pathogens. Such individualized secretion is also supported by the observation that the secretion machinery in fungal and bacterial defense differs, using distinct sets of SNARE-proteins (Kalde *et al.*, 2007; Kwon *et al.*, 2008).

For FLS2, however, no focal secretion but a ligand dependent endocytosis was reported so far (Robatzek *et al.*, 2006). This process may be necessary to remove an access of membrane material or to recycle or degrade activated FLS2 receptors. In addition a possible function for endocytosis in FLS2 signaling is under discussion. The existence of such a signaling process through endocytosis into endosomal compartments was demonstrated already for BRI1 (Geldner *et al.*, 2007). Increasing the endosomal localization of this hormone receptor, using Brefeldin-A, a drug affecting vesicle transport and causing destruction of golgi structures, enhances the activation of its downstream pathway, possibly by concentrating signaling components in a so called signaling compartment (Geldner *et al.*, 2007).

However, a mutation in the putative PEST domain of FLS2 at position P-1076 blocks flg22 induced internalization, but still allows FLS2 to mediate an oxidative burst in response to flg22 (Salomon and Robatzek, 2009). This finding indicates first, that the putative PEST domain is involved in the regulation of FLS2 internalization and second, that the endocytosis is not involved in the downstream signaling of FLS2 (Robatzek *et al.*, 2006). Endocytosis often correlates with a mono-ubiquitination of the target proteins. Therefore it was speculated that also mono-ubiquitination of FLS2, at the putative PEST motif, triggers the internalization of the receptor (Robatzek *et al.*, 2006). Strikingly, both serine residues, which were identified as putative FLS2 phosphorylation sites in this study (chapter 2), are part of this PEST motif and S-1078 which is phosphorylated in response to flg22 is just two amino acids away from P-1076 which impaired FLS2 internalization when mutated to

alanine (Salomon and Robatzek, 2009). Furthermore, according to our *in silico* analysis S-1078 represents the best binding motif for the general regulating protein of the 14-3-3 family (chapter 4). Since also 14-3-3 proteins were found in this study as potential flg22 dependent FLS2 interactors, one may speculate that the phosphorylation of FLS2 at position S-1078 and possibly also on S-1084, together with the binding of 14-3-3 proteins, regulates FLS2 endocytosis. The finding that FLS2 endocytosis is blocked by kinase inhibitors supports such a hypothesis (Robatzek *et al.*, 2006). A microscopic analysis of the YFP tagged FLS2-S1078A and FLS2-S1084A versions, created in this work (chapter 2), will be used to approach this question.

7.6 Concluding remarks

The observations obtained in this study apply to different processes of pattern signaling (Fig. 7-2). With the identification of PEPR2 we could clarify that the endogenous DAMP signal *At*Pep1 is perceived at the plasma membrane by two receptors, which is an important prerequisite for numerous further investigations. After ligand perception receptors need to transfer this information into the inside of the cell. We found that not only BAK1 functions in this process, but other SERK-proteins can interact as well with FLS2 and this interaction does not require kinase activity of the receptor. In the cytosol this interaction results in phosphorylation of FLS2 by its upstream kinase BAK1, as indicated by our *in vitro* analysis. How exactly this complex triggers downstream events remains elusive, but our analysis reveals also aspects of signaling network in plant immunity. Multiple pathways diverge at the receptor and only a part of these may require the catalytic activity of the receptor kinases. In parallel it seems that activated receptor complexes can direct focal secretion via an interaction with RAB-GTPases and SNARE-proteins. All together this work provides a basis to further study process of receptor activation, to explore the signaling network of plant immunity and to investigate the molecular mechanisms which finally lead to resistance against plant pathogens.

8 Material and methods

8.1 Plant material

The Arabidopsis plants used in this study were grown as one plant per pot at 20–21°C with an 8 h photoperiod, or on plates containing MS salts medium (Duchefa), 1% sucrose, and 0.8% agar under continuous light. The T-DNA insertion lines SALK_059281 (*pepr1*), SALK_098161 (*pepr2*), SALK_044334 (*efr*) and SAIL_691C4 (*fls2*) were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK) (Zipfel *et al.*, 2006). *pepr1/pepr2* mutants, homozygous for both T-DNA insertions, were kindly provided by B. Kemmerling (Zentrum für Molekularbiologie der Pflanzen, Tübingen).

Arabidopsis cells (ecotype *Landsberg erecta*) were grown as shaking cultures (110 rpm, 24°C, continuous light) in 50 ml AT-medium (MS-medium containing 0.5 mg/L naphthaleneacetic acid, 0.05 mg/L kinetin, 3% (w/v) sucrose) (May and Leaver, 1993). The cell suspensions were sub-cultured every two weeks by inoculating new medium with 2.5 ml of the old culture. For all experiments cells were used at day 6 after sub-cultivation.

8.2 Peptides

Peptides of elf26 (acSKEKFERTKPHVNVGTIGHVDHGKTT), flg22 (QRLSTGSRINSAKDDAAGLQIA) and *At*Pep1 (ATKVKAKQRGKEKVSSGRPGQHN), obtained from EZBiolabs, were dissolved in water (stock solutions of 1 mM) and diluted in a solution containing 1 mg/mL of BSA and 0.1 M NaCl or MS-medium (Felix *et al.*, 1999; Kunze *et al.*, 2004; Huffaker *et al.*, 2006).

8.3 Primers

All used primers were obtained from Microsynth AG (Balgach, Switzerland) without additional purification.

Primers for RT-PCR

Primer	Sequence
FRK1 fwd/rev	ACTTGCTTACCGACACATTCGTTAC
	GATCTCCTTGCCAGTTCTTCTTCAC
RPL4 fwd/rev	CCACCACCACGAACTTCACCGCGAGTC
	GTGATAGGTCAGGTCAGGGAACAAC

Primers for site directed mutagenesis

Primer	Sequence
FLS2 K898A fwd/rev	GGGACAGTGATTGCAGTAGCAGTATTGAATCTAAAGG CCTTTAGATTCAATACTGCTACTGCAATCACTGTCCC
EFR K741A fwd/rev	CTCGTCGCGGTTGCAGTTTTGAACCTCCTAAAGC GCTTTAGGAGGTTCAAAACTGCAACCGCGACGAG
BAK1 K317R fwd/rev	GGTACTTTAGTGGCCGTTCGAAGGCTAAAAGAGG CCTCTTTTAGCCTTCGAACGGCCACTAAAGTACC
BAK1 Del-SPGR fwd/rev	CGAATACCCCTAGGGTCCAAGATAACACCC GGGTGTTATCTTGGACCCTAGGGGTATTCG
FLS2 C996R for/rev	GGTTTTCCCATCGTTCATCGTGATCTGAAGCCAGC GCTGGCTTCAGATCACGATGAACGATGGGAAAACC
FLS2 S1078D fwd/rev	CGAAACAGAGACCAACTGACTTGAATGATGAAGATTCAC GTGAATCTTCATCATTCAAGTCAGTTGGTCTCTGTTTCG
FLS2 S1078A fwd/rev	CGAAACAGAGACCAACTGCGTTGAATGATGAAG CTTCATCATTCAACGCAGTTGGTCTCTGTTTCG
FLS2 S1084A fwd/rev	GAATGATGAAGATGCACAAGACATGACTTTGCG CGCAAAGTCATGTCTTGTGCATCTTCATCATTC
FLS2 S1084D fwd/rev	CGTTGAATGATGAAGATGACCAAGACATGACTTTGCGCC GGCGCAAAGTCATGTCTTGGTCATCTTCATCATTCAACG

Primers for cloning of genes

Primer	Sequence
FLS2 fwd/rev	AAAAAGCAGGCTATGAAGTTACTCTCAAAGACC AGAAAGCTGGGTGAACTTCTCGATCCTCGTTACGATC
FLS2-kin fwd/rev	AAAAAGCAGGCTGCATTGAAAATTCATCAGAGTCCTCA AGAAAGCTGGGTGAACTTCTCGATCCTCGTTACGATC
EFR fwd/rev	AAAAAGCAGGCTGTGGTCCCCATTTCTTGGTGTGC AGAAAGCTGGGTGCTACATAGTATGCATGTCCGTATTTA
EFR-kin for/rev	AAAAAGCAGGCTGCAACAATGCCAGTGATGGTAACCCA AGAAAGCTGGGTGCTACATAGTATGCATGTCCGTATTTA
BAK1-kin fwd/rev	AAAAAGCAGGCTGCCGAAGGAAAAAGCCGCAGGACC AGAAAGCTGGGTGTTATCTTGGACCCGAGGGGTATTCG
FIL1 fwd/rev	AAAAAGCAGGCTCACCATAACGACAATAAGGGCCGGAC AGAAAGCTGGGTCATCGCCGGCCACGGGTAATCTG
RAB-D2c fwd/rev	AAAAAGCAGGCTTCATGAATCCTGAATATGACTATTTG AGAAAGCTGGGTTTAAGAGGAGCAGCAGCCTG
RAB-E1a fwd/rev	AAAAAGCAGGCTAAATGGCTGCTCCTCCTGCTAG AGAAAGCTGGGTTTATGTGCCGCAACATGCTGATTTC

8.4 Antibodies

Primary antibodies	
Rabbit anti-FLS2	Polyclonal antibody (Chinchilla *et al.*, 2006)
Rabbit anti-BAK1	Polyclonal antibody (Schulze *et al.*, 2010)
Sheep anti-14-3-3	Polyclonal antibody (Moorhead *et al.*, 1999)
Rat anti-HA	Monoclonal high affinity antibody (clone 3F10); Roche Applied Science
Mouse anti-p44/42 MAPK	p44/42 MAPK (Erk1/2) antibody 9102; Cell Signaling Technology
Rabbit anti-GFP	Monoclonal antibody (clones 7.1 and 13.1); Roche Applied Science
Rabbit anti-Myc	Monoclonal antibody (Clone 9E10); Roche Applied Science
Secondary antibodies	
Goat anti-rabbit IgG alkaline phosphatase conjugate	Polyclonal antibody; Sigma-Aldrich A3687
Goat anti-rat IgG alkaline phosphatase conjugate	Polyclonal antibody; Sigma-Aldrich A8438
Goat anti-mouse IgG alkaline phosphatase conjugate	Polyclonal antibody; Sigma-Aldrich A3562
Donkey anti-sheep IgG alkaline phosphatase conjugate	Polyclonal antibody; Sigma-Aldrich A5187

8.5 Generation of constructs for plant transformation and heterologous protein expression

For heterologous expression, the cytoplasmic domains of FLS2 (aa 837-1173), EFR (aa 679-1101) and BAK1 (aa 249-616) were cloned from cDNA by PCR and integrated into pDonor207 (Invitrogen) using Gateway technology. The cytoplasmic domain of CLV (NBRC stock N1G75820ZE_K) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, USA) in pDonor201 (Invitrogen). After verification by sequencing the

inserts were transferred into pGex-6pD21 (provided by E. Wanker, MDC Berlin), again using Gateway recombination technology (Invitrogen). The final constructs code for a N-terminal GST-tag in frame with the cytoplasmic portion of the RLK. The expression construct for GST-HAESA-kin in pGex-2T (Horn and Walker, 1994) was kindly provided by Prof. J. Walker (University of Missouri, Columbia).

For stable transformation of plants the full length coding sequence of the FLS2 or EFR genes was amplified by PCR from genomic DNA using Phusion Polymerase (Finnzymes) and cloned into pDonor207 (Invitrogen). For generation of transgenic plants we used pEarly301 vector (Earley *et al.*, 2006) as an acceptor plasmid to express FLS2 or EFR as YFP-HA fusion proteins under the control of the constitutive 35S-promoter.

8.6 Site directed mutagenesis

For single amino acid exchanges site directed mutagenesis was performed using pDonor constructs as templates. Oligonucleotide primers containing the desired mutation, each complementary to opposite strands of the vector, were extended by PCR using Phusion Polymerase (Finnzymes) to generate the mutated plasmid. The resulting PCR product was *Dpn*I treated to select for mutation-containing synthesized DNA. *Dpn*I endonuclease is specific for methylated and hemimethylated DNA and digests only the *E. coli* derived parental template DNA. The nicked vector DNA containing the desired mutations was then transformed into DH5α competent *E. coli* cells for multiplication. The success of the mutagenesis procedure was checked by sequencing of the full insert.

8.7 Protein purification from *Escherichia coli* bacteria

Proteins for *in vitro* analysis were expressed in *E. coli* BL21, in 1 L cultures of LB-medium provided with appropriate antibiotics at 37°C. Expression of the transgenic protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranosid at a density of OD_{600} = 0.8. After 3 h at 28°C cultures were harvested by centrifugation and lysed in extraction buffer (20.000U/ml Lysozyme, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) by sonication (100 watt, 30 pulses of 1 sec). After clearing by centrifugation (30.000 g, 10 min) the protein extract was supplied with GST-sepharose 4B (Amersham Bioscience)

for affinity purification of the tagged protein and incubated at light shaking at 4°C for 1 h. The GST-sepharose beads were harvested by centrifugation (500 g, 5 min) and washed 3 times with 5 volumes of phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The purified proteins were eluted using a glutathione contain buffer (50 mM Tris-HCl pH 8.0, 20 mM reduced glutathione) and stored at -20°C in 50% glycerol. The quantity and quality of the purified proteins were determined by Bradford analysis and Coomassie protein staining after separation by SDS-PAGE.

8.8 *In vitro* assays for characterization of kinase assay and substrate specificity

To analyze auto- and trans-phosphorylation activity of kinases we used GST-tagged cytoplasmic domains of different RLKs. 0.1 – 1 µg of various affinity purified recombinant proteins or artificial substrates were premixed in a 25 µl volume of kinase buffer (100 mM HEPES pH7.5, 10 mM $MgCl_2$, 0.1% βM). To start the kinase reaction the assay was supplied with a mixture of cold carrier ATP (5 µM) and radiolabeled γ-P^{32} ATP (1 µCi; 20 nM; Hartmann Analytic GmbH, Germany) and incubated at 25°C for 30 min. The reaction was stopped by adding SDS-sample buffer with 20 mM EDTA and analyzed by SDS-PAGE and autoradiography.

8.9 Stable transformation of Arabidopsis plants

Stable transformants of *Arabidopsis thaliana* were obtained using a floral dip procedure (Clough and Bent, 1998). *Agrobacteria*, carrying the DNA-construct of interest, were grown over night in YEB-medium, harvested by centrifugation (2000 g, 15 min) and resuspended in a solution of 5% sucrose (Carl Roth GmbH, Germany) and 0.02% Silvett L-77 (Lehle Seeds, USA) at a density of OD_{600} = 0.8. Arabidopsis plants in their flowering stage were submerged into the bacteria solution for 30 sec. After the dipping procedure plants were further grown for two days under a cover to provide high humidity, then without cover till seed ripening. T1 transformants were selected by spray application of a 300 µM glufosinate-ammonium solution (BASTA, Omya AG Switzerland). Segregating T2 lines were again selected for glufosinate resistance before functional analysis.

8.10 Analysis of transgene expression

Leaf material (50 mg) from three week old soil grown Arabidopsis plants was frozen in liquid nitrogen, ground in a bead mill, and resuspended in 0.1 ml of cold extraction buffer (50 mM Tris-HCl pH 8, 50 mM NaCl and protease inhibitor cocktail (Sigma)). After denaturation in standard SDS-loading buffer equal amounts of protein were separated by SDS-PAGE and analyzed by Western blot.

8.11 Immunoprecipitation experiments

Leaf material (250 mg) from three week old soil grown Arabidopsis plants was cut into fine stripes (> 1 mm) and floated on water for 8 h. This material was stimulated with 1 µM elicitor for 5 min and frozen in liquid nitrogen, extracted by ground in a bead mill, and resuspended in 0.5 ml of cold extraction buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol, 1% (w/v) Nonidet P-40 and protease inhibitor cocktail (Sigma)). After incubation at 4 °C with gentle shaking for 1 h, this preparation was centrifuged three times at 20.000 g for 10 min to remove cell debris. The supernatant was incubated at 4 °C for 1 h with proteinG-sepharose beads (Amersham Biosciences) and monoclonal anti-HA (Sigma) for IPs of EFR-YFP-HA or with proteinA-sepharose beads (Amersham Biosciences) and polyclonal anti-BAK1 antibodies, polyclonal FLS2 antibodies or polyclonal GFP antibodies (for 14-3-3-GFP and RAB-GTPase-YFP IPs). Beads were collected and washed three times with cold extraction buffer and once with 50 mM Tris-HCl pH 8. Immunoprecipitated proteins were separated by SDS–PAGE and analyzed by Western blot.

For mass spectrometric analysis of FLS2 phosphorylation and identification of FLS2 interacting proteins 100 g of Arabidopsis cells were stimulated with 1 µM flg22 for 5 min. After removal of the culture medium by filtration, cells were resuspended in 100 ml cold extraction buffer with phosphatase inhibitors (50 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol, 1% (w/v) Nonidet P-40, protease inhibitor cocktail (Sigma), 20 mM Microcystin-LR, 100 nM Calyculin A, 20 mM sodium fluoride) and ground with sand in a pre-cooled mortar at 4°C. The homogenized cells were supplied with 100 ml of detergent containing buffer (extraction buffer with 1% (w/v) sodium deoxycholate and 2% (w/v) Nonidet P-40) to

solubilize membrane proteins and incubated with light shaking for 3 h. After solubilization cell debris was removed by one filtration and centrifugation (50.000 g, 30 min). For immunoprecipitation the protein extract was incubated with 30 µg of polyclonal FLS2 antibody, coupled to 300 µl of proteinA-sepharose, for 3 h. After immunoprecipitation sepharose beads were washed 10 times with 2 ml of detergent containing buffer (sodium deoxycholate 0.5% (w/v), 1% (w/v) (Nonidet P-40)) and twice with Tris-HCl pH 8. After the elicitor treatment all steps were performed in a cooled room at 4°C to avoid warming of the sample.

8.12 Oxidative burst analysis

Reactive oxygen species released by leaf tissue was assayed by H_2O_2 dependent luminescence of luminol. Leaf pieces of 4 week old Arabidopsis plants were cut in 2 mm^2 pieces and floated over night in water. One piece per well was transferred into a 96 well plate (LIA White, Greiner Bio-One) containing 1 mg horseradish peroxidase (Sigma) and 100 nM luminol (Sigma). Luminescence was measured in a plate reader (MicroLumat LB96P, Berthold Technologies) for 30-60 min after addition of elicitor.

8.13 Ethylene accumulation analysis

For measurement of ethylene biosynthesis leaf material of 4 week old plants were cut into 1 mm thick stripes and floated over night in water. Afterwards three leaf stripes (20 mg) were transferred in 6 ml glass vials containing 0.5 ml of an aqueous solution of the elicitor to be tested. The tubes were closed with rubber septa and ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu) after 3 h incubation.

8.14 MAP-kinase activation

Seedlings, grown for 5 days on MS-agar plates, and 10 days in liquid MS-medium were overlaid for 12 min or 24 min with indicated elicitors (1 µM) diluted in liquid MS-medium or, as control, for 12 min with MS-medium only. For each treatment 8 seedlings were frozen in liquid nitrogen and ground in a beadmill. 50 mg plant powder was taken up in 0.1 ml of cold extraction buffer (50 mM Tris-HCl pH 8, 50 mM NaCl and protease inhibitor

cocktail (Sigma)). Extracted proteins were fractionated by 10% SDS–PAGE and analyzed by Western blot analysis using a polyclonal phosphospecific MAPK antibody (anti-pERK p44; New England Biolabs).

8.15 Elicitor induced inhibition of seedling growth

Seedlings grown for 5 days on MS-agar plates were transferred to liquid MS-medium supplied with the elicitors indicated (two seedlings per 500 µl of medium in 24-well-plates). The effect of treatment with different peptides on seedling growth was analyzed by weighing fresh weight after 10 days. The elicitor effect on root growth was determined by measuring the length of the longest root hair.

8.16 Resistance to *Agrobacterium* infection

Qualitative analysis of symptom development was monitored by pressure infiltration of *Agrobacteria* into leaves of 4 week old soil grown Arabidopsis plants (Zipfel *et al.*, 2006). *Agrobacteria* (GV3101) were grown over night in YEB-medium, harvested by centrifugation and resuspended in infiltration buffer ($MgCl_2$ 10mM, MES 10mM, pH 5.7) at an OD_{600} of 0.3 and injected into Arabidopsis leaves using a syringe. The infiltrated plants were grown under a cover to provide high humidity for two to three days and four additional days without cover. At day seven infiltrated leaves were detached and symptoms were documented by photography. 8 leaves from 4 plants were used per experiment.

For quantification of resistance by transformation efficiency, 7 day old seedlings, grown on MS-medium (solid) were covered with a solution of *Agrobacteria*, carrying the GUS transgene in pBIN19g, resuspended in liquid MS-medium (OD_{600}=0.03). For biological replicates seedlings were grown on separate plates. After five days 30 seedlings were pooled, washed with water (3x 2ml), frozen and ground in a beadmill. For quantification of the glucuronidase activity a MUG-assay was performed (Tian *et al.*, 2003). Soluble proteins were extracted from 100 mg of tissue powder by resuspending the material in 300 µl buffer (100 mM Tris-HCl pH 8.0, 0.1 mg/ml BSA, 10 mM ß-mercaptoethanol) and subsequent clearification of the suspension by centifugation (15.000g, 10 min). For the enzyme reaction 10 µl of the resulting protein extract were incubated with 190 µl of 2 mM

4-methylumbelliferyl β-D-galactopyranoside (MUG; Sigma) dissolved in water at 37°C for 3 h. The enzyme reaction was stopped by transferring 20 µl of the reaction mixture into 230 µl of a sodium carbonate solution (200 mM). For blank controls 20 µl of reaction mixture were stopped at time point 0 h. Accumulation of the fluorescent reaction product was quantified in a plate reader (Fluorostar Optima, BMG Labtech) with 355 nm exitation wave length and a filter for emission wavelength of 460 nm. The presented values correspond to the relative fluorescence at timepoint 3 h minus fluorescence at time point 0 h.

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Appendix A: Mass spectrometric data

A.1 Identification of FLS2 interaction partners by co-immunopurification

Potential FLS2 interacting proteins were identified by the detection of tryptic peptides in FLS2 immunopurification using LC/MS/MS analysis. The resulting candidate proteins were found in either precipitates from untreated cells (table A-1), in precipitates from cells treated for 5 min with flg22 (table A-2), or in both purifications (table A-3).

Table A-1 Proteins identified in FLS2 immunoprecipitates from untreated cells

Signaling	
Locus	**Description**
At3G48750	CDKA;1
At1G66750	CDKA1;2
At4G23230	Protein kinase family protein
At1G52290	Protein kinase family protein
At2G22560	Kinase interacting protein-related
At4G30250	AAA-type ATPase family protein
At4G05380	AAA-type ATPase family protein
At4G25835	AAA-type ATPase family protein
At1G43910	AAA-type ATPase family protein
At2G18193	AAA-type ATPase family protein
At2G18190	AAA-type ATPase family protein
At3G50930	AAA-type ATPase family protein
At3G28570	AAA-type ATPase family protein
At3G50940	AAA-type ATPase family protein
At5G17730	AAA-type ATPase family protein
At5G17740	AAA-type ATPase family protein
At5G17760	AAA-type ATPase family protein
At5G57480	AAA-type ATPase family protein
At5G40000	AAA-type ATPase family protein
At3G28610	ATPase
Chaperones	
At2G05250	DNAJ heat shock protein
At2G05230	DNAJ heat shock protein
At5G56030	ERD8
At5G56010	Heat shock protein 81-3
At5G56000	Heat shock protein 81-4
At5G52640	HSP90
Proteasome components	
At5G23540	26S proteasome regulatory subunit
At3G51260	20S proteasome alpha subunit D1

At5G66140	20S proteasome alpha subunit D2
At5G42790	20S proteasome alpha subunit F1
At1G47250	20S proteasome alpha subunit F2
Endocytosis	
At3G11130	Clathrin heavy chain
At3G08530	Clathrin heavy chain
Cytoskeleton components	
At1G75780	Tubulin beta chain
At5G62690	Tubulin beta chain
At5G62700	Tubulin beta chain
At5G44340	Tubulin beta chain
At1G20010	Tubulin beta chain
At5G12250	Tubulin beta chain
AT2G29550	Tubulin beta chain
AT5G23860	Tubulin beta chain
AT4G20890	Tubulin beta chain
Metabolism	
AT1G56190	Phosphoglycerate kinase
AT3G26650	Glyceraldehyde-3-phosphate dehydrogenase
AT1G12900	Glyceraldehyde-3-phosphate dehydrogenase
AT1G42970	Glyceraldehyde-3-phosphate dehydrogenase
AT3G12780	Phosphoglycerate kinase
AT5G05000	Chloroplast translocon component
AT2G33040	ATP synthase gamma chain
AT3G54110	Component mitochondrial electron transport chain
ATCG00540	Component mitochondrial electron transport chain
AT5G27520	Mitochondrial substrate carrier family protein
AT5G15640	Mitochondrial substrate carrier family protein
AT4G01100	Mitochondrial substrate carrier family protein
AT5G13490	ADP/ATP carrier
AT4G28390	ADP/ATP carrier
AT5G14040	Mitochondrial phosphate transporter
AT1G65260	Plastidal protein for thylakoid membrane formation
AT5G16390	Plastidal carboxyl-carrier subunit
AT2G30200	S-malonyltransferase
AT2G30200	S-malonyltransferase
AT3G47520	Malate dehydrogenase
AT2G31740	Methyl transferase like protein
AT5G53460	NADH-dependent glutamate synthase
AT3G53580	Diaminopimelate epimerase family protein
AT4G35000	Ascorbat peroxidase
AT2G05990	Enoyl-acyl-carrier-protein
AT1G71380	Glycosyl hydrolase family 9
AT4G28510	Mitochondrial respiratory complex component
AT2G20530	Mitochondrial respiratory complex component
AT5G19440	Cinnamyl-alcohol dehydrogenase
AT5G63400	Mitochondrial adenylate kinase
AT2G19550	Esterase/lipase/thioesterase family protein

AT3G47590	Esterase/lipase/thioesterase family protein
AT3G47560	Esterase/lipase/thioesterase family protein
Nucleolar protein components	
AT5G15090	Porin component
AT3G01280	Porin component
AT5G67500	Porin component
AT4G25630	Fibrillarin, nucleolar snoRNA interacting protein
AT5G52470	Fibrillarin, nucleolar snoRNA interacting protein
AT5G52490	Fibrillarin, nucleolar snoRNA interacting protein
At1G07660	Histone H4
At1G07820	Histone H4
At1G07820	Histone H4
At1G07660	Histone H4
At2G28740	Histone H4
At3G45930	Histone H4
At3G53730	Histone H4
At3G46320	Histone H4
At5G59690	Histone H4
At5G59970	Histone H4
At1G48610	AT hook motif-containing protein
At1G48610	AT hook motif-containing protein
At5G37720	RNA and export factor-binding protein
At5G37720	RNA and export factor-binding protein
At1G48920	rRNA processing
Translation machinery	
At2G17360	40S ribosomal protein
At5G58420	40S ribosomal protein
At5G07090	40S ribosomal protein
At4G34670	40S ribosomal protein
At3G04840	40S ribosomal protein
At2G31610	40S ribosomal protein
At3G53870	40S ribosomal protein
At5G35530	40S ribosomal protein
At3G09200	60S ribosomal protein
At3G09200	60S ribosomal protein
At1G18540	60S ribosomal protein
At2G40010	60S ribosomal protein
At3G11250	60S ribosomal protein
At1G74050	60S ribosomal protein
At1G74060	60S ribosomal protein
At4G36130	60S ribosomal protein
At2G47610	60S ribosomal protein
At5G39740	60S ribosomal protein
At3G62870	60S ribosomal protein
At2G18020	Structural constituent of ribosome
At5G07090	Structural constituent of ribosome
At3G25520	Structural constituent of ribosome
At4G31700	Structural constituent of ribosome

At5G10360	Structural constituent of ribosome
At1G25260	Acidic ribosomal protein P0-related
At1G30230	Elongation factor 1-beta
At2G18110	Elongation factor 1-beta
At5G19510	Elongation factor 1B alpha-subunit 2
Unknown function	
At5G12470	Unknown protein
At5G23060	Unknown protein
At1G69840	Band 7 family protein

Table A-2 Proteins identified in FLS2 immunoprecipitates from flg22 treated cells

Vesicle transport	
Locus	**Description**
At1G06400	RAB-A1a
At5G45750	RAB-A1c
At4G18800	RAB-A1d
At4G18430	RAB-A1e
At5G60860	RAB-A1f
At3G15060	RAB-A1g
At2G33870	RAB-A1h
At1G09630	RAB-A2a
At1G07410	RAB-A2b
At3G46830	RAB-A2c
At1G16920	RAB-A4b
At5G47520	RAB-A5a
At1G02130	RAB-D2a
At5G47200	RAB-D2b
At4G17530	RAB-D2c
At3G53610	RAB-E1a
At3G46060	RAB-E1c
At5G03520	RAB-E1d
At3G09900	RAB-E1e
At1G09180	ARF GTPase
At1G56330	ARF GTPase
At4G02080	ARF GTPase
AT1G04750	VAMP721
At2g33120	VAMP722
At1G59610	Dynamin like GTPase
At1G10290	Dynamin like GTPase
At5G59840	Ras-related GTP-binding family protein, RAB type
14-3-3 proteins	
At1G26480	IOTA
At1G34760	OMICRON
At5G65430	KAPPA
At5G10450	LAMBDA
At1G22300	EPSILON

At1G78300	OMEGA
At4G09000	CHI
At5G38480	PSI
LRR-RLKs	
At1G71830	SERK1
At1G34210	SERK2
At4G33430	SERK3/BAK1
At2G13790	SERK4
At2G13800	SERK5
At2g01210	FIL1
Chaperons/protein folding	
At5G28540	Luminal binding protein BiP-1
At5G42020	Luminal binding protein BiP-2
At5G50920	Heat shock protein
At3G48870	Heat shock protein
At4G37910	Mitochondrial heat shock protein
Metabolism	
At1G53310	PEP Carboxylase
At3G14940	PEP Carboxylase
At2G36580	Pyruvate kinase
At3G52990	Pyruvate kinase
At5G13110	Glucose-6-phosphate 1-dehydrogenase
At3G03780	Putative methionine synthase
At1G06410	Putaive trehalose-6-phosphatase/synthase
At1G23190	Phosphoglucomutase
At5G08670	ATPase subunit
At5G08680	ATPase subunit
At5G08690	ATPase subunit
AtCG00120	ATPase subunit
At4G10340	Light harvesting complex of photosystem II
At1G06950	Chloroplast translocon component
At5G01530	Chlorophyll A-B binding protein
AtCG00480	Chloroplasic subunit of ATP synthase
At2G26260	3-beta-hydroxy-delta5-steroid dehydrogenase
Transcription and translation machinery	
At5G15200	40S ribosomal protein
At1G33120	60S ribosomal protein
At1G33140	60S ribosomal protein
At2G01250	60S ribosomal protein
At2G01250	60S ribosomal protein
At2G44120	60S ribosomal protein
At2G44120	60S ribosomal protein
At2G01250	60S ribosomal protein
At1G26910	60S ribosomal protein
At1G66580	60S ribosomal protein
At1G14320	60S ribosomal protein
At1G56070	Elongation factor

At2G33730	DEAD box RNA helicase
At3G06010	Homeotic gene regulator (chromatin regulator)
Unknown function	
At2G16460	Unknown protein
At1G07970	Unknown protein
At5G64710	Unknown protein
At1G70770	Unknown protein
At4G12070	Unknown protein

Table A-3 Proteins identified in FLS2 immunoprecipitates from treated and untreated cells

14-3-3 proteins	
Locus	**Description**
AT1G35160	PHI
AT2G42590	MU
AT3G02520	NU
AT5G16050	UPSILON
Signaling	
AT4G05370	AAA-type ATPase family protein
Chaperons/protein folding/degradation	
AT1G16030	HSP70
AT1G56410	HSP70
AT3G12580	HSP70
AT3G09440	HSP70
AT5G02490	HSP70
AT5G02500	HSP70
AT2G02560	Cullin associated neddylation dissociated 1
Metabolism	
AT1G13440	Glyceraldehyde-3-phosphate dehydrogenase
AT1G13440	Glyceraldehyde-3-phosphate dehydrogenase
AT3G04120	Glyceraldehyde-3-phosphate dehydrogenase
AT3G08580	ATP:ADP antiporter/ binding
Transcription and translation machinery	
AT1G07920	Elongation factor
AT1G07930	Elongation factor
AT1G07940	Elongation factor
AT1G07930	Elongation factor
AT5G60390	Elongation factor
AT5G14580	Polyribonucleotide nucleotidyltransferase
AT5G61780	Tudor domain-containing protein / nuclease family protein
AT5G07350	Tudor domain-containing protein / nuclease family protein
Unknown function	
AT5G09840	Unknown protein
AT5G64710	Unknown protein

A.2 Coverage of FLS2 phosphorylation site analysis

```
   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 EFPQSITNLR NLTVLTVGFN NISGELPADL GLLTNLRNLS AHDNLLTGPI
 401 PSSISNCTGL KLLDLSHNQM TGEIPRGFGR MNLTFISIGR NHFTGEIPDD
 451 IFNCSNLETL SVADNNLTGT LKPLIGKLQK LRILQVSYNS LTGPIPREIG
 501 NLKDLNILYL HSNGFTGRIP REMSNLTLLQ GLRMYSNDLE GPIPEEMFDM
 551 KLLSVLDLSN NKFSGQIPAL FSKLESLTYL 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 SSESSLPDLD
 851 SALKLKRFEP KELEQATDSF NSANIIGSSS LSTVYKGQLE DGTVIAVKVL
 901 NLKEFSAESD KWFYTEAKTL SQLKHRNLVK ILGFAWESGK TKALVLPFME
 951 NGNLEDTIHG SAAPIGSLLE KIDLCVHIAS GIDYLHSGYG FPIVHCDLKP
1001 ANILLDSDRV AHVSDFGTAR ILGFREDGST TASTSAFEGT IGYLAPEFAY
1051 MRKVTTKADV FSFGIIMMEL MTKQRPTSLN DEDSQDMTLR QLVEKSIGNG
1101 RKGMVRVLDM ELGDSIVSLK QEEAIEDFLK LCLFCTSSRP EDRPDMNEIL
1151 THLMKLRGKA NSFREDRNED REV
```

The scheme illustrates all amino acid stretches (in red) identified by the mass spectrometric analysis for FLS2 phosphorylation. For the cytoplasmic domain (highlighted in grey) the analysis reached coverage of 70%. Beside this high coverage, there are several gaps in the data, including the very C terminus and a stretch of about 57 amino acids in the central kinase domain including several S/T residues. Therefore these amino acid stretches could not be analyzed for their phosphorylation status and additional phosphorylation sites are possible.

Curriculum vitae

Particulars

Name:	Tobias Mentzel
Date of birth:	23. March 1980
Place of birth:	Neustadt (Weinstr.), Germany
Nationality:	German
Civil status:	married

School and university education

11/2005-06/2010

Dissertation (Botanical Institute of the University Basel, Switzerland)
Supervisor: Prof. Dr. Thomas Boller

10/2000 –09/2005

Studies of biology (Ruprecht – Karls Universität, Heidelberg, Germany)
Focus: biochemistry, plant physiology, molecular biology and cell biology.
Master thesis at the Heidelberg Institute of Plant Science on regulation of the sulfur metabolism in plants.

1990-/1999

High School Diploma (Hans-Purrmann-Gymnasium, Speyer)
Main subjects: biology, mathematics

Professional experiences

02/2004 – 05/2004

Chromatin Inc., Chicago IL, USA; scientific lab assistant
Focus: development of artificial plant-chromosomes for the genetic modification of agronomical crop species.

10/2003 – 12/2003

Heidelberg Institute of Plant Science
Scientific lab assistant in the research group of Prof. Dr. Hell
Focus: Analysis of protein composition in seeds.

08/2002 – 09/2003

Center for Molecular Biology Heidelberg
Scientific lab assistant in the research group of Prof. Dr. Dobberstein
Focus: Analysis of protein transport in yeast cells.

08/1999 – 06/2000

Institute for Agronomic Analysis and Research in Speyer
Analysis and preparation of soils for agronomical and toxicological research

Publications

Krol E.*, Mentzel T.*, Chinchilla D., Boller T., Felix G., Kemmerling B., Postel S., Arents M., Jeworutzki E., A. S. Al Rasheid K. A S., Becker D., and Hedrich R. (*contributed equally); Perception of the danger signal *At*Pep in Arabidopsis involves two pattern recognition receptors, *At*PEPR1 and *At*PEPR2; Journal of Biological Chemistry 285 (18) 13471-13479

Schulze B., Mentzel T., Jehle A., Mueller K., Beeler S., Boller T., Felix G., Chinchilla D. (2010) Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1; Journal of Biological Chemistry 285 (13) 9444-9451

Lu X., Tintor N., Mentzel T., Kombrink E., Boller T., Robatzek S., Schulze-Lefert P. and Saijo Y. (2009) Uncoupling of sustained MAMP receptor signaling from early outputs in an Arabidopsis ER glucosidase II allele; Proceedings of the National Academy of Sciences of the United States of America 106 (52) 22522-22527

Göhre V., Spallek T. , Haeweker H., Mersmann S., Mentzel T., Boller T., de Torres M., Mansfield J.W . and Robatzek S. (2008) plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB; Current Biology 18 (23) 1824-1832

Conference contributions

14th International Congress on Molecular Plant-Microbe Interactions, Quebec, Canada
Poster: Mentzel T., Jenni D., Chinchilla D., Schulze B., Bittel P., Clouse S., Boller T., (2009) Analysis of kinase activity and phosphorylation of pattern recognition receptors

5th Tri-National Arabidopsis Meeting, Zürich, Switzerland
Poster: Mentzel T., Schulze B., Chinchilla D., Jenni D., Bittel P. and Boller T. (2008) Signaling in plant innate immunity: activation and phosphorylation of the FLS2 receptor kinase

24th Annual Missouri Meeting of Plant Biology: Plant Protein Phosphorylation-Dephosphorylation, Missouri-Columbia, MO, USA
Poster: Mentzel T., Chinchilla D., Schulze B. and Boller T. (2007) Downstream signaling of the flagellin receptor kinase FLS2

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