Characterization of a novel bacterial PAMP – Elongation Factor Tu – and its role in *Arabidopsis thaliana* defense and immunity.

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Hamm (Westf.), Deutschland

Basel Juni 2005

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

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Basel, den 05.07.2005

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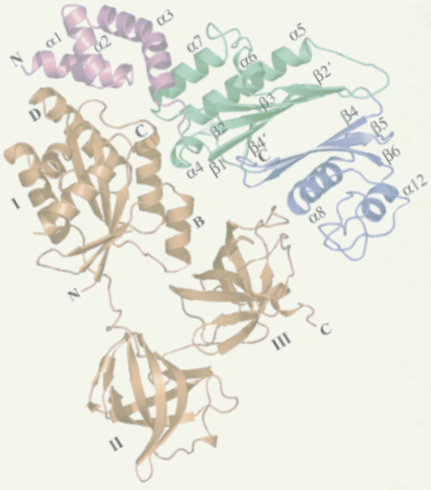

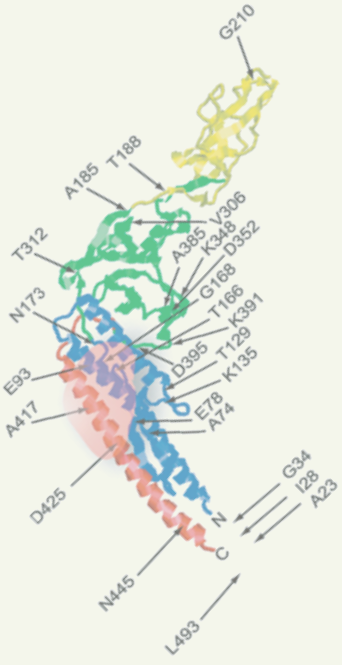

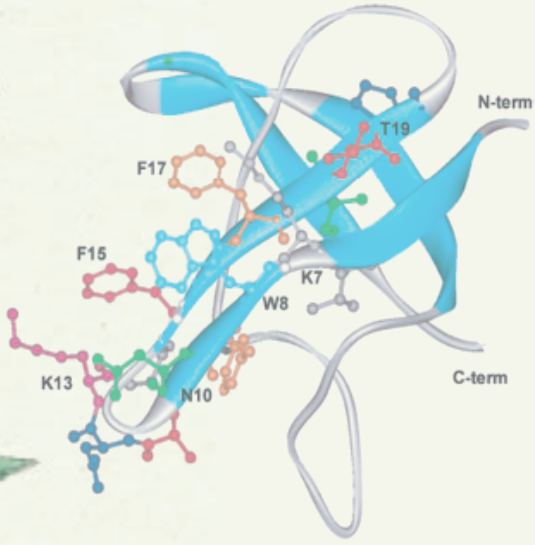

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Abbreviations

Avr	aviurlence gene.
CARD	caspase-activating and recruitment domain
CC	coiled coil
CCD	controlled cell death
ChBD	chitin-binding domain
CSP	cold shock protein
EC_{50}	concentration required to induce a half-maximal response
EF-Tu	elongation factor Tu
EIX	elicitor ethylene-inducing xylanase
elfNN	peptide representing the acetylated NN amino acid residues of N-terminus of EF-Tu
FLG	flagellin
FLS	flagellin sensing
GNBP	Gram-negative bacteria binding proteins
GTPase	guanosine triphosphatase
HG	β-1,3β-1,6 heptaglucoside
HR	Hypersensitive response
Hrp	hypersensitive response and pathogenicity
hsp	Heat-shock protein
IL-1	Interleucin 1
IRAK1	(IL-1R associated) kinase
LBP	LPS binding protein
LPS	lipoplysaccharide
LRR	Leucine rich repeat
MALDI-TOF	ionization-time of flight mass spectrometry
MAP	Mitogen activated protein kinase
MS	mass spectrometry
MyD88	myeloid-differentiation factor 88
NBS	nucleotide binding domain
NOD	nucleotide-binding oligomerisation domain
Nod-factor	Nodulation factor
NPP1	necrosis-inducing Phytophthora protein 1
OGA	oligogalcturonides
PAMP	pathogen-associated molecular pattern
PCD	Programmed cell death (animals)
PGIP	Polygalacturnoase-inhibiting proteins
PGN	peptidoglycan
PGRP	peptidoglycan-recognition proteins
PGs	polygalacturonases
PPM	eukaryotic phosphatases (Mg(2+)-or Mn(2+)-dependent protein phosphatase
PRR	pattern recognition receptor
RLCKs	receptor-like cytoplasmatic kinases
RLP	receptor-like protein
RLU	relative light units
ROS	Reactive oxygen species
RSKs	receptor serine/threonine kinases
RTKs	receptor tyrosine kinases
sCD14	soluble LPS-binding protein ()
Stp	(Mn(2+)-dependent serine-threonine phosphatase
SYMRK	symbiosis receptor-like kinas
TLR	Toll-like receptor
TTSS	type III secretion system
Xac	Xanthomonas axonopodis pv. citri
Xcc	Xanthomonas camptestris pv. campestris
YE	Yeast extract-derived elicitor

Summary

The discrimination of self and nonself is a primary challenge for all living organisms to detect microbial invasion and to protect and defend against the invader. If a pathogen manages to overcome constitutive barriers, highly specific recognition systems are able to identify common pathogenic signals and activate the innate immune system as a first line of defense. Specialized cells and a circulatory system that is able to spread somatically generated adaptive immune responses to the infection side exist only in animals. Plants lack an adaptive immune system comparable like this, but they independently co-evolved the capability to detect microbial invasions by perception of specific molecules, so called PAMPs (pathogen associated molecular patterns), and subsequent activation of innate immune responses.

Flagellin, the major subunit of the bacterial motility organ flagellum (Yonekura, K. et al. 2001), can be regarded as the best characterized bacterial PAMP in plants. Flg22, a synthetic peptide comprising the highly conserved epitope of the flagellin N-terminus, is recognized by the plant cell and is sufficient to activate innate immune responses. In this work based on the model plant *Arabidopsis thaliana,* experiments with bacterial extracts devoid of elicitor active flagellin, show still the capability to induce a broad set of plant defense reactions as flagellin, suggesting that at least one additional perception system for another elicitor exist. This novel elicitor and the corresponding active site were identified as the first 18-26 amino acids from the N-terminus of bacterial Elongation factor Tu (elf18-elf26). This essential protein, involved in the delivery of aminoacyl-tRNA to the ribosome during the translation process, is highly conserved over all organisms (Appendix 1) and is the most abundant protein in the bacterial cell (Helms, M. K. and Jameson, D. M. 1995). Furthermore it is considered to be the slowest evolving protein (Gaucher, E. A. et al. 2003) containing all characteristics for a classical PAMP (see definition included in General Introduction). Further characterization of the EF-Tu/*Arabidopsis thaliana* interaction showed that all known plant defense mechanisms are activated upon EF-Tu elicitation. This includes extra cellular medium alkalinization of suspension cultured cells of Arabidopsis, production of reactive oxygen species (ROS) (Laloi, C. et al. 2004) and increase in the biosynthesis of plant hormone ethylene. Like shown previously for flagellin (Zipfel, C. et al. 2004), pre-treatment of Arabidopsis with elf-

peptides led to enhanced resistance against plant pathogenic bacteria *Pseudomonas syringae* pv. *tomato* (DC3000). Gene expression changes were analyzed using Affymetrix ATH1 array and about 1000 genes with induced expression after 30-60 minutes treatment with elf-peptides were identified. These genes were congruent with that affected by flg22 treatment (Navarro, L. et al. 2004, Zipfel, C. et al. 2004). The same genes were also found to be induced in the flagellin insensitive receptor mutant *fls2* upon elf-treatment. Binding studies with radio labeled elf26-^{125}I-TY show that there is a high affinity binding site for EF-Tu existing in *Arabidopsis thaliana*, which is saturable, highly specific and independent of FLS2. Crosslinking experiments identified a polypeptide band of 150 kDa as potential binding site for EF-Tu. Strikingly the perception of one PAMP (e.g. flg22) leads to a higher amount of binding sites for the other elicitor (e.g. elf18). Furthermore the perception of EF-Tu activates, like flg22, a MAP kinase-based signaling cascade in nearly identical kinetic. Together this study indicate two independent receptors using a converging signaling cascade that leads to the activation of the plant innate immune system with the same broad array of plant defense reactions.

0 General Introduction

In their particular environments plants and all other living organisms are surrounded by potential pathogens from various species (e.g. fungi, oomycetes, viruses, bacteria) and have to defend themselves against attacks from these invaders. Nevertheless disease in wild type populations is the exception and most of the individuals are mostly healthy. Because plants are immobile and cannot escape actively from mobile pathogens, plant cells possess a preformed and an inducible defense system. In the immune system of vertebrates specialized cells can be transferred to the infection site and limit or kill the invading species (Blander, J. M. and Medzhitov, R. 2004).

A key aspect in every case is the early detection of potential invaders. So called pattern recognition receptors (PRR) (Medzhitov, R. and Janeway, C. A. Jr. 2002) are responsible for the perception of characteristic microbial compounds in mammals and insects. The pathogen-associated molecular patterns (PAMPs) are molecules that are common and at least partly conserved for a whole class of microorganisms. Contrary to the definition of PAMPs these motifs are not only restricted to pathogens and are present in non-pathogenic microorganisms as well. Structures that are regarded to act as PAMPs include molecules like lipoplysaccharide (LPS), peptidoglycan (PGN) and flagellin (FLG). Furthermore, these components are essential for the pathogen, do not exist in the potential host and they are released or exposed by the microbe. Upon the PAMP detection the PRR activates signaling cascades leading to subsequent activation of innate immune responses by transcription of defense regulated genes, which for example code for the production of antimicrobial compounds. The known components of perception pathways show striking similarities between PAMP-perception in plants, mammals and insects (summarized in Fig. 1).

0.1 Innate Immunity in Vertebrates

0.1.1 Mammals / TLR

The detection of invader signals is the basis for the activation of the innate immune system. The major players in the perception of extracytosolic and cytosolic PAMPs in mammals are the leucine-rich repeat (LRR)-containing Toll-like receptors (TLRs) (Kollisch, G. et al. 2005) and the nucleotide-binding oligomerisation domain (NOD)-LRR proteins. TLRs are integral membrane proteins with an amino-terminal extracellular LRR and a caboxyterminal cytosolic Toll and IL-1 receptor homology (TIR) domain (Beutler, B. et al. 2005b). Nod proteins represent an intracellular pathogen perception system in mammals and contain also LRRs and a nucleotide binding domain (NBS). They are structurally related to the R proteins in plants that have been demonstrated to be involved in disease resistance (Gomez-Gomez, L. 2004).

10 TLR homologues have been found in the human database (Philpott, D. J. and Girardin, S. E. 2004a) and 12 in mice (Beutler, Bruce 2004). PAMPs are either recognized by single or combined TLR perception. 23 NOD-LRR genes have been identified in mammals of which at least 2 have been implicated (NOD1, NOD2) to play a role in peptidoglycan (PGN) perception (Girardin, S. E. and Philpott, D. J. 2004a) (Table 1).

Table 1: Partial summary of involved elements in sensing and affecting the innate immune system Adapted from (Beutler, B. 2004)

	Afferent (sensing)	**Efferent (effectors)**
Humoral	LBP, CD14, collectins Properdin, C3b, pentraxins	Cytokines, antimicrobial peptides, lysozyme, BPI, complement (esp. late components), lactoferrin, acute phase reactants
Cellular	TLRs, Dectin-1, CD14, FMLP receptor, NOD1, NOD2	Antimicrobial peptides, porteases, lipases, glycosidases, cell adhesion molecules, ROS, nitric oxide, peroxynitrite, others

Exemplary TLR4, which is important for sensing LPS, and TLR5 that perceives flagellin, are described in more details (Fig. 1). The PAMPs LPS and flagellin are molecules that show elicitor active characteristics in plants and the similarities and differences in the perception systems are discussed separately. (Bacterial Elicitors in plant innate immune system)

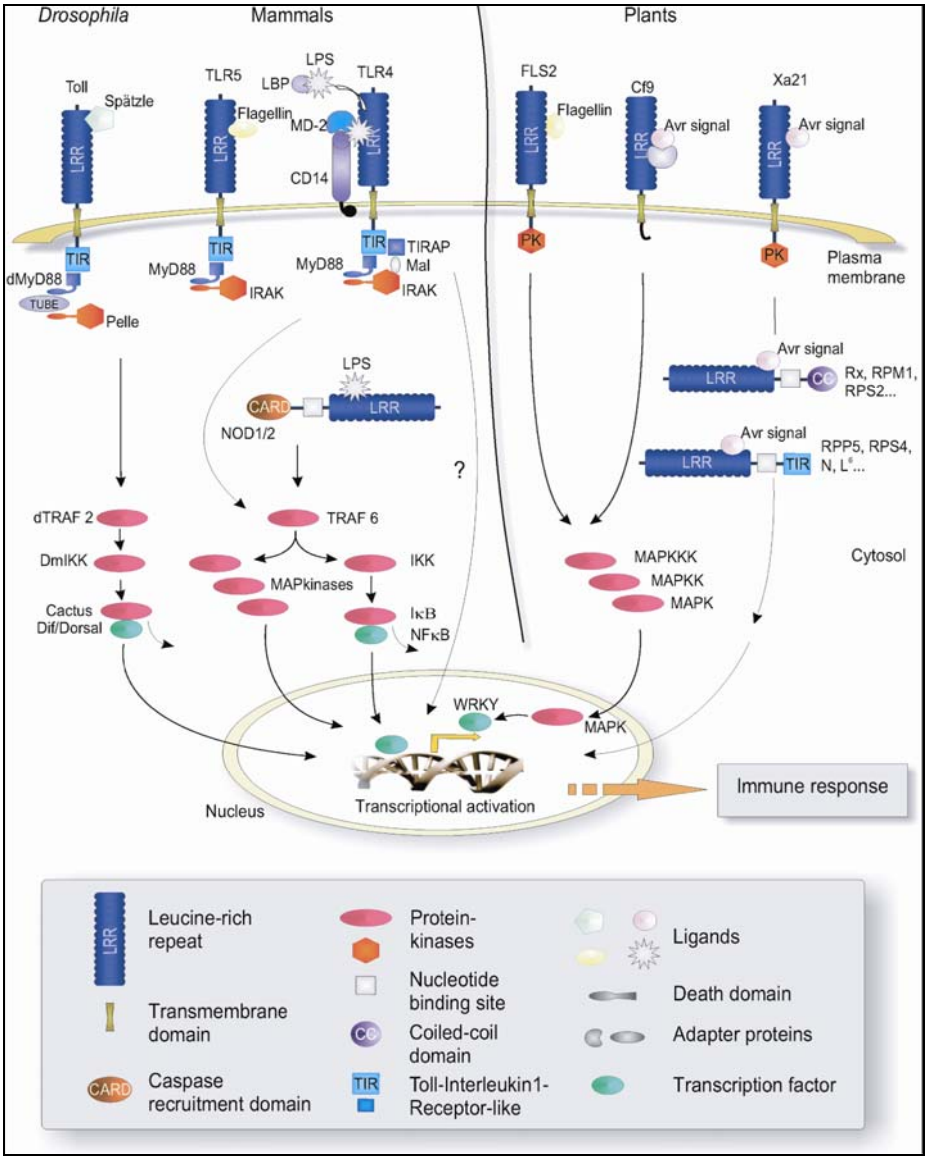

Fig. 1: Overview of signaling pathways based on the perception of PAMPs leading to the activation of innate immunity responses in insects, mammals and plants; adapted from (Nürnberger, T. et al. 2004).

0.1.2 LPS/TLR4

LPS, the amhiphilic lipopolysaccharides bound to the outer membrane of gram-negative bacteria can be divided in two major parts - the lipid and the polysaccharide part. The lipid A component is anchored in the bacterial outer membrane, whereas the core oligosaccharide is linked to the lipid A. The LPS perception with TLR4 (Beutler, B. et al. 2005a, Hajjar, A. M. et al. 2002) requires as a first step that LPS is cleaved from the bacterial membrane. This is done by the soluble LPS-binding protein (LBP) sCD14. The LPS-LBP-sCD14 complex is transferred to CD-14 (55-kDa glycosyl-phosphatidylinositol (GPI)-anchored protein, containing LRR), that was previously believed to be the relevant LPS receptor (Wright, S. D. et al. 1990). MD2 was found to be associated with TLR4 and is essential for LPS recognition. The studies of (Visintin, A. et al. 2001a, Visintin, A. et al. 2001b) demonstrated that the LPS complex is bound to MD2 which, in turn binds to TLR4 and induces aggregation and signal transduction. The TLR4-MD-2 complex receptor can function in two separate modes: one in which full signaling occurs and one limited to MyD88-based signaling, depending on the present LPS chemotypes (Jiang, Z. et al. 2005).

TLR4 induce MyD88, a TIR domain containing adaptor that activates IRAK1 by phosphorylation. The posphorylated IRAK1 (IL-1R associated) kinase associates with TRAF6, a TNF-receptor associated factor. This complex activates signaling elements like MAP-kinase based cascades and mediates activation of transcription factors (NF-κB) through inactivation of the repressor protein IκB. This finally led to the expression of immune response genes and the production of pro-inflammatory cytokines. Interestingly, a recent pharmaceutical study show evidence that the production of ROS after LPS treatment is independent of TLR4 (Qin, L. et al. 2005), indicating unknown existing signaling components for LPS perception. Furthermore several other components like integrins, heat-shock proteins, CXCR4 or CD55 (reviewed in (Triantafilou, M. and Triantafilou, K. 2005)) have been found in association with LBP and to be involved in LPS perception. They might act as secondary LPS transfer molecules without experimentally evidence for direct TLR4-interaction. Intracellular detection of LPS is mediated by the NBS-LRR receptors

NOD1 and NOD2 (recognizes also different moieties of bacterial peptidoglycan (PGN) (Carneiro, L. A. et al. 2004, Viala, J. et al. 2004a) (reviewed in (Philpott, D. J. and Girardin, S. E. 2004b)) both carrying a CARD-domain. This is a structural difference to intracellular NBS-LRR proteins in plants that contain either CC (coiled coil) or TIR domains.

Several reports show that TLR2 and TLR4 are able to recognize several self-proteins, like members of the heat shock protein family (hsp60, hsp70) (Asea, A. et al. 2002, Vabulas, R. M. et al. 2002a, Vabulas, R. M. et al. 2002b). However purification of hsp-proteins abrogates their capability to induce TLR4 (Gao, B. and Tsan, M. F. 2003). Imperfect discrimination of ligands can lead to recognition of self-molecules by TLRs that has been described for TLR9 that senses immuno-stimulatory unmethylated CpG DNA from bacterial or viral genomes (Wagner, H. 2004).

0.1.3 Flagellin/TLR5

Flagella filaments are composed of flagellin monomers, sticking together and forming long protofilaments (Smith, K. D. et al. 2003). The Flagella as motility organ, is essential and contributes for the virulence of pathogenic bacteria and the invasion of host surfaces through general chemotaxis (Ramos, H. C. et al. 2004, Vande Broek, A. and Vanderleyden, J. 1995). Recently the flagellum has also found to be important for sensing environmental signals like external wetness (Wang, Q. et al. 2005) and therefore regulate not only its own biogenesis (shorter or longer flagellum), but also affects other physiological functions.

This bacterial-specific protein consists of high conserved N- and C-terminal domains, which are widespread in bacterial species and dedicated to filament polymerization. The central region is highly variable in the known flagellin sequences and exposed on the surface of the flagellum. The conserved domains of flagellin are embedded at the inner part of the flagellum structure, but are exposed in flagellin monomers (Fig. 2) and therefore potential primary targets for receptor perception. The role of the

hypervariable region of flagellin with regard to recognition processes is still unknown. Flagellin has been found in bacterial media (Komoriya, K. et al. 1999), probably resulting from degradation to adapt to environmental changes (Wang, Q. et al. 2005), or potential breaks during flagella construction (Gewirtz, A. T. et al. 2001, Shimizu, R. et al. 2003).

Mammalian hosts detect the conserved domain on flagellin through TLR5 (Fig.2). It has been demonstrated that flagellin is a major stimuli of proinflammatory cytokine production in dendritic cells (Means, T. K. et al. 2003) and lung epithelial cells (Tallant, T. et al. 2004). Sequence changes in TLR5 led to increased disease susceptibility (Hawn, T. R. et al. 2003), indicating that TLR5 alone is essential for mediating flagellin recognition in human cells. Interestingly, signaling with a heteromeric complex TLR5/TLR4 seemed to be required for the expression of nitric oxide synthase and nitric oxide production (Mizel, S. B. et al. 2003).

Recent studies about flagellin signaling via TLR5 propose, that gangliosides might act as a potential co-receptor (Ogushi, K. et al. 2004), but current status is that gangliosides can inhibit flagellin signaling in the absence of an effect towards binding of flagellin to TLR5 (West, A. P. et al. 2005). These findings still indicate that direct interaction between flagellin and TLR5 occurs and the inhibitory effect of gangliosides is mediated by a distinct mechanism.

The discussed MyD88-IRAK1 (LPS perception)pathway, is also part of the flagellin perception via TLR5 (Chalifour, A. et al. 2004, Hayashi, F. et al. 2001, Hayashi, F. et al. 2003, Means, T. K. et al. 2003, Tsujita, T. et al. 2004). No additional co-factor seemed to be needed for binding of the flagellin domain D1 (Fig.2) with TLR5 , but some recent reports (Tallant, T. et al. 2004) speculate that additional factors may be required for an efficient signal propagation in response to flagellin recognition.

Interestingly TLR5 detects a different epitope of flagellin (Smith, K. D. et al. 2003) (Fig.2) than FLS2 (Innate Immunity in plants), indicating that the perception systems in mammals and plants have evolved independently. The same bacterial protein is targeted by different organisms and detected by the use of similar constructed receptors.

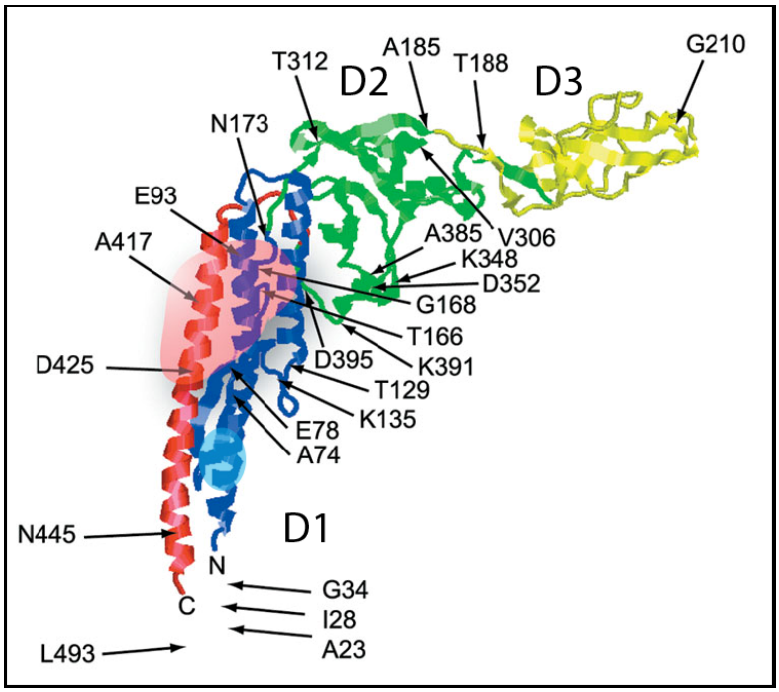

Fig 2: Flagellin domain structure. Marked in red the position of abrogate TLR5 recognition, in blue: the conserved N-terminal part of flagellin (flg22) that is recognized by FLS2 in plants. Modified from (Smith, K. D. et al. 2003)

0.1.4 EF-Tu acts as a PAMP?

During protein synthesis the delivery of a charged aminoacetyl-tRNA to the ribosome is an essential step in the translation process. This process is catalyzed by the elongation factor Tu (EF-Tu), a guanosine triphosphatase (GTPase) that increases translational fidelity (Gromadski, K. B. and Rodnina, M. V. 2004) (Fig3). This highly conserved and abundant protein (Navratil, T. and Spremulli, L. L. 2003) forms a stable ternary complex with GTP and the tRNA that binds at the A finger site loop of the 70S ribosome (Fig4). This step can be stalled by the use of antibiotics, which prevents dissociation of EF-Tu from the Ribosome (Hogg, T. et al. 2002). EF-Tu sense further the matching of the tRNA anticodon and mRNA codon, trigger GTP hydrolysis and is released from the ribosomal complex. In this step tmRNA and SmpB, a protein required for tmRNA activity, are regulating the release, but SmpB may also serve to permit tmRNA binding to the A site (Moore, S. D. et al. 2003).

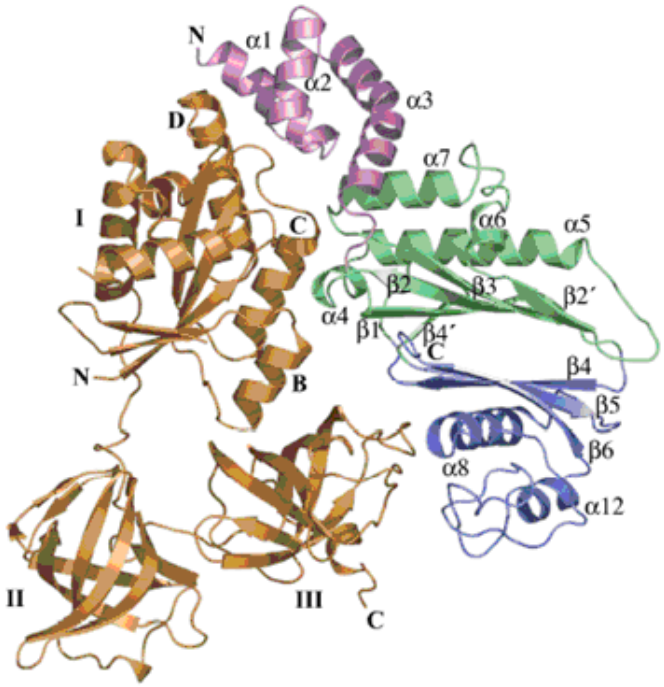

Fig. 3: Crystal structure of Elongation factor Tu (left molecule, uniform colored), adapted from (Jeppesen, M. G. et al. 2005) in complex with EF-Ts (right molecule)

Despite from this function EF-Tu has been reported to be part of a potential bacterial cytoskeleton (Regula, J. T. et al. 2001). Furthermore, EF-Tu that is believed to be a cytoplasmatic protein has been found at the surface of *Lactobacillus johnsonii*. This

probiotic bacteria is able to bind to human epithelial cell lines and can induce the secretion of different cytokines. Studies in human and animals could show that this bacteria has immune adjuvant effects and modulates non-specific immune responses (Ibnou-Zekri, N. et al. 2003)

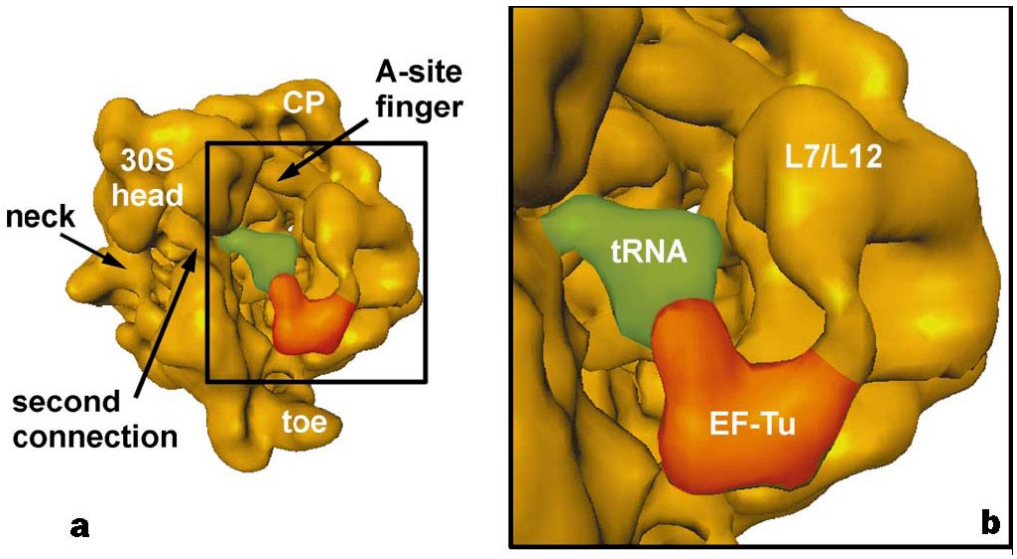

Fig. 4: EF-Tu bound to the ribosome: a) Domain 1 (the G domain) of the EF-Tu is bound both to the L7/L12 stalk and to the 50S body underneath the stalk, whereas domain 2 is oriented towards the S12 region on the 30S subunit b) close-up. Adapted from (Stark, H. et al. 1997)

The surface location of intact EF-Tu was confirmed in recent work of (Granato, D. et al. 2004b) by various experimental techniques (immunoblotting, EM). In addition, studies with human intestinal cells (Caco-2 and HT29) demonstrated that EF-Tu recombinant protein has the capability to induce proinflammatory response in the presence of soluble CD14 (sCD14, LPS perception) and might participate in gut homeostasis. However, because of the involvement of sCD14 it is tempting to speculate, that a TLR-Receptor is responsible for sensing EF-Tu in mammalian immunity.

Furthermore, in the pathogenicity of *Listeria monocytogenes*, a pathogen that cause listeriosis, EF-Tu was identified as a target of Stp (Mn(2+)-dependent serine-threonine phosphatase, similar to PPM eukaryotic phosphatases (Mg(2+)-or Mn(2+)-dependent protein phosphatase) (Archambaud, C. et al. 2005). Listeria has evolved a variety of regulatory elements, in particular many signal transduction systems based on reversible phosphorylation, in order to survive and grow in the host. Post-

translational phosphorylation of EF-Tu prevents the binding to amino-acetylated transfer RNA as well as to kirromycin. A stp deletion mutant was less sensitive to kirromycin, suggesting an important role for Stp in regulating EF-Tu and controlling bacterial survival in the infected host. Another approach (Lock, R. A. et al. 2002) identified by analyzing the proteome from *Helicobater pylori* infected patients with enzyme antibodies (recognizing either serum IgG or IgA), beside chaperonin HspB, urease beta-subunit UreB also EF-Tu and flagellin FlaA, as highly immuno-reactive proteins, indicating a possible role in human innate immunity for this proteins.

0.2 Innate Immunity in Drosophila

Toll, the namesake of TLR family in Drosophila, is involved in immunity and has in addition a developmental function. Eight other Toll paralogs involved in developmental processes are present in flies, but for none an immune function was reported so far (Beutler, Bruce 2004). Interestingly the 11 mammalian TLRs seemed to have no other function as the perception of PAMPs and they are activated by diverse microbial ligands, promoting the suggestion that the nine Tolls of Drosophila could similarly respond to distinct microbial agonists during infection. Peptidoglycan (PGN), a cell wall component of Gram-positive Bacteria can be sensed by Drosophila innate immune system (Royet, J. et al. 2005).

0.2.1 Toll/Imd

The Toll-protein is a transmembrane protein with an extracellular LRR and an intracellular TIR domain, strikingly similar to the TLRs structure found in mammals. The Toll receptor is not directly activated by fungal or bacterial PAMPs (Fig.1). The presence of peptidoglycan-recognition proteins and Gram-negative bacteria binding proteins (PGRP-SA, PGRP-SD, and GNBP-1) are required for the activation process. This led to binding of an activated form of the cytokine Spaetzle (activation components has not been identified so far), that cause dimerization and signaling

activation. The myeloid-differentiation factor 88 (MyD88), like discussed before in the TLR-system, interacts with Toll through the TIR domain, furthermore the proteins Tube and Pelle (IRAK like kinase) are required and led to the degradation of Cactus and transcriptional activation.

Independent from the Toll-pathway the transmembrane receptor PGRP-LC is required for triggering innate immune response towards infection of gram-negative bacteria (Fig. 5)(Kaneko, T. et al. 2004). It is still not clear how PGRP-LC can activate the intra-cytoplasmatic Imd-signaling pathway. TAK1, a MAP3 kinase, can activate IKK-kinase (activated IKK participates with the caspase-8 homologue in the process leading to the cleavage of the NF-*k*B family member Relish) or JNK kinase, leading to subsequent expression of antimicrobial genes (IKK) and genes encoding for cytoskeleton proteins (JNK).

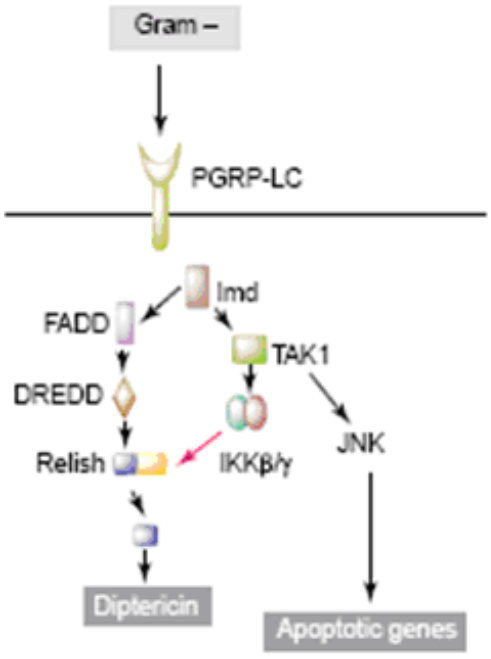

Fig. 5: Antimicrobial perception in Drosophila for Gram-negative bacteria. Signaling occurs by transmembrane receptor PGRP-LC that activates the Imd Pathway by an unknown mechanism. From (Royet, J. et al. 2005)

0.3 Innate Immunity in Plants

Comparable to the innate immune system in animals, sessile plants have evolved the capacity to defend themselves against mobile pathogens. Most plants are resistant to a broad spectrum of microorganisms. The defense mechanisms in the so called “non-host resistance” contain structural barriers and inducible responses (Jones, D. A. and Takemoto, D. 2004).

The plants cell wall is a first constitutive physical barrier (Mellersh, D. G. and Heath, M. C. 2001) for potential pathogens that prevent colonization the host plant. However, if a pathogen was able to enter plant tissue (e.g. wounds, or natural openings like stomata), plant cells have developed a basal perception system for “general elicitors”, molecules that are common to entire classes of pathogens (homologues of animal PAMPs, pathogen-associated molecular patterns (Nürnberger, T. and Brunner, F. 2002)). The so called “Basal-defense” is independent of the genotype of the invader and is activated either by host and non-host pathogen (even human/animal pathogens can trigger plant defense responses (Kunze, G. et al. 2004, Prithiviraj, B. et al. 2005b, Rahme, L. G. et al. 1997). After a general elicitor is perceived by PRRs, the plant cell rapidly onsets defense responses like the production of reactive oxygen species (ROS), an increase in the biosynthesis of plant hormone ethylene, lignin and callose deposition and the induction of genes encoding pathogenesis-related proteins (PR-Genes), regulated by defense associated transcription factors (Eulgem, T. 2005). In plants during this type of interaction a hypersensitive response (HR), homologue to the localized controlled cell death (CCD) in animals, occurs rarely (e.g. Harpin (HrpZ) in high concentrations (Li, C. M. et al. 2005). HR is a process of rapidly induced programmed cell death (PCD) in the infected and neighboring cells (Greenberg, J. T. and Yao, N. 2004b).

The pathogen-race/host plant cultivar specific resistance (R-mediated resistance) allows recognition of distinct races of biotrophic pathogens and use HR to restrict an infection. Probably due to evolutionary pressure pathogens modified the molecules that are detected by the plant and obtain therefore invisibility to this identification

mechanism. Furthermore they evolved virulence factors, often injected with a type III secretion system (TTSS), which are required for the pathogen to evade or suppress plant defense systems (Alfano, J. R. and Collmer, A. 2004b, Wehling, M. D. et al. 2004). Co-evolution of the potential hosts led to the development of resistance genes (*R*). This gene product enables the susceptible host plant to recognize the specific microorganism strain and resist to infection. The gene-for-gene model, established by Flor (Flor, H. H. 1971), predicted that plant resistance will only occur, when a plant posses a dominant resistance gene (*R*) and the pathogen express the complementary dominant aviurlence gene (*Avr*). The Avr proteins act as "specific elicitors" of defense when they are recognized by the R-Protein of the plant (discussed in more details exemplary for AvrPtoB). The basal defense limits the disease to a specific extent (in compatible interactions), whereas the non-host resistance is a result of the preformed defenses. Plant defense mechanisms activated after perception of general elicitors or after R-mediated perception are partly similar (Dangl, J. L. and Jones, J. D. 2001), with the exception of HR-occurrence and systemic signal induction in a time-dependent matter (de Torres, M. et al. 2003). For example Syringolin A, a secreted protein from *Pseodomonas syringae* pv. *syringae* can induce HR-dependent cell death of cells colonized with powdery mildew in wheat and reprogram a compatible interaction in an incompatible one (Amrein, H. et al. 2004). However, the syringolin function in this interaction is still unclear, but treated Arabidopsis seedlings monitored with Affymetrix ATH1 array show similar regulation of genes, like presented in this work (Chapter 2), including a high representation of induced RLKs (personal communication K. Michel, University of Zurich).

0.3.1 R-Proteins/avr (AvrPtoB)

In addition to basal resistance the plant disease resistance can also be based on different cultivars. *Pseudomonas syringae* pv. *tomato* DC3000 is a plant pathogen that uses a type III secretion system to inject effector proteins into the plant cell. These effector proteins can suppress or inhibit plant defenses and therefore promotes bacterial survival and multiplication in host plants. In the last years the interaction between Arabidopsis and DC3000 was used as a model system to

evaluate the molecular basis of plant immunity and disease susceptibility (Nimchuk, Z. et al. 2003). A key aspect of the R protein mediated immunity (Fig. 6) is the hypersensitive response (HR). DC3000 elicits immunity associated programmed cell death in tomato, that posses the Pto resistance protein (Fig.6). Pto is a serine/threonine protein kinase, which interacts with DC3000 effectors AvrPto or AvrPtoB. Prf, another protein and member of the LRR class of disease-resistance genes, is required for Pto mediated HR-based PCD. If Pto is lacking, AvrPtoB suppresses PCD and immunity, whereas AvrPto suppresses cell wall-based defenses like the accumulation of callose (Hauck, P. et al. 2003). In the genome of DC3000 more than 30 type III effectors have been identified (Buell, C. R. et al. 2003). Recent analysis of DC3000 mutants with deletion of *avrPto* and *avrPtoB,* results in a virulent pathogen without the elicitation of host immunity, suggesting that these effectors are the only trigger of Pto-mediated immunity in tomato (Lin, N. C. and Martin, G. B. 2005).

R proteins are present in various plant species and are mostly cytoplasmic nucleotide binding site-Leucine Rich Repeat (NBS-LRR) proteins (Jones, D. A. and Takemoto, D. 2004) that have also reported to be involved in mediated resistance against viruses (Peart, J. R. et al. 2005). The N-terminal domains of this proteins contains either a putitative coiled coil (CC) or a Toll-IL1-Receptor-like (TIR) (Nimchuk, Z. et al. 2003) structure. The most common feature of all R protein classes is the presence of LRR-domains. The LRR domains found in other proteins, are important for protein-protein interaction (Jones, J. D. G. 1997) and therefore the LRR are believed to interact directly with Avr proteins. The only example of such an interaction has been determined between the Pi-ta CC-NBS-LRR protein in rice and the Avr-Pita protein from *Magnaporthe grisea* fungus (Jia, Y. et al. 2000). The facts that such a direct interaction seems to be relatively rare and that this proteins were found to form signal perception complexes (Axtell, M. J. and Staskawicz, B. J. 2003), led to the establishing of the ”guard” hypothesis. In this theory it is proposed that the interaction between an R protein and its corresponding Avr protein is mediated by another host protein that is the target for the effector. The Avr protein functions as an inducer of plant resistance, when the R protein as a “guard of the functional cell” is involved in a signal perception complex.

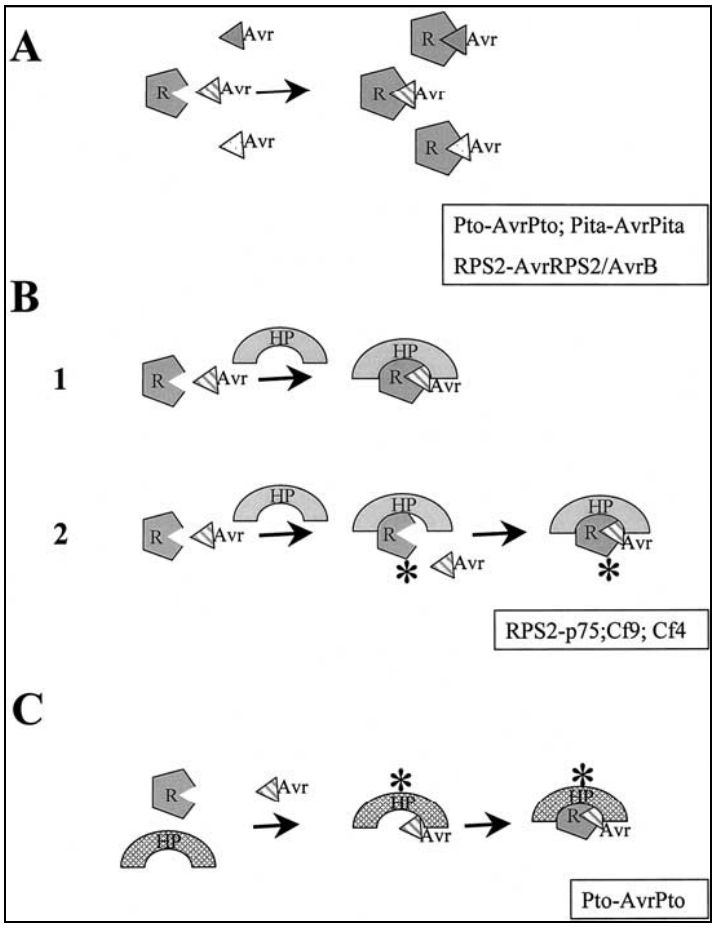

Figure 6: Models for plant R protein and pathogen Avr protein Interaction (Ellis, C. et al. 2002)

A. Direct binding between resistance (R) and avirulence (Avr) proteins; One R protein could recognize more than one Avr peptide (shown AtRPS2 and P. syringae AvrRPT2 and AvrB).
B. Host protein mediated R-Avr binding. The R protein binds the Avr protein only in the presence of a host protein (HP). (RPS2-p75 binding and Cf9 recognition of Avr9) B1. Binding to the Avr protein does not require modification of the R protein. Only if all components are present the complex is formed. B2. The host protein forms a complex with the R protein, which is modified prior to Avr binding.
C. Avr-mediated R-HP binding. In the absence of the Avr peptide, no interaction of the R protein with the host protein occurs. The host protein(s) form a complex with the Avr protein, resulting in a modification or conformational change, which allows binding of the R protein.

(R, resistance protein; Avr, avirulence protein; HP, host protein; ∗protein modification)

0.3.2 Recognition during symbiosis (Nod-factor /SymRK)

Legumes can form symbiosis with rhizobial bacteria and mycorrhizal fungi and through this interaction they can acquire nitrogen and phosphorus, two important macronutrients (Parniske, M. and Downie, J. A. 2003a). In contrast to the broad host range of mycorrhizal fungi, the host range of rhizobial symbiosis and the formation of root nodules are only known from legumes. In order not to defend against “friendly” symbiotic microorganisms, the plant need to sense and to identify the symbionts. The nature of fungal signal molecules that are perceived by the plant root and allow hyphal entry and further development of the fungus is still unknown. In case of the bacteria the lipochito-oligosaccharides (Nod-factors, consisting of substituted β1-4 N-acetylglucosamine (chitin) backbones) could be identified as a morphogenic signal from rhizobial bacteria, inducing the necessary root hair deformation and cell division. Nod-factors, like Avr-proteins, are secreted by the bacteria. The receptor like kinase SYMRK (symbiosis receptor-like kinase) is required for rhizobial and mycorrhizal symbiosis of *Lotus japonicus* (Stracke, S. et al. 2004, Stracke, Silke et al. 2002) (Fig. 7).

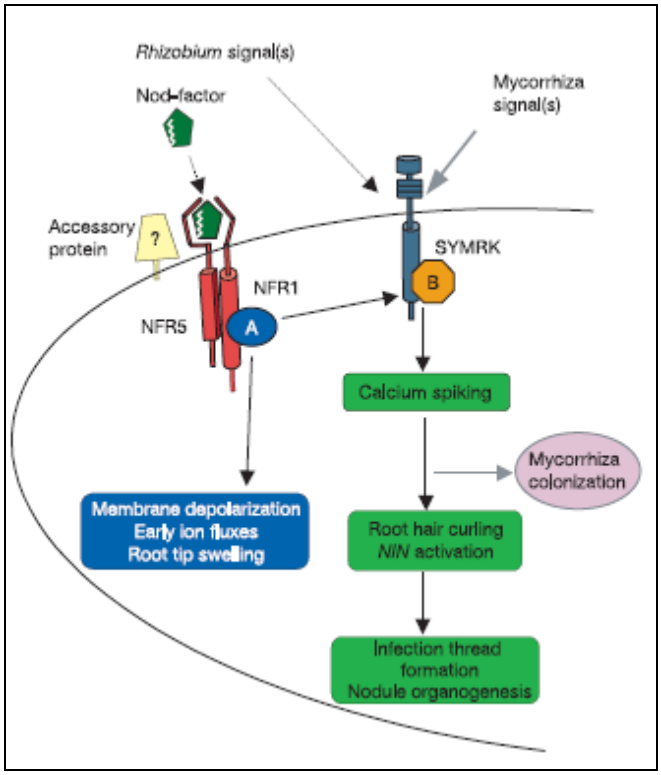

Fig. 7: Model for the role of NFR1 and NFR5 in perception of symbiontic signaling. Adapted from (Radutoiu, S. et al. 2003)

Like found in other perception systems SYMRK consist of an extracellular LRR domain and an intracellular protein kinase domain, a structure that is highly homologous to other known RLKs. *SYMRK* mutants are unable to form root nodules, but root deformation is still occurring, in addition the fungal infection is arrested in epidermal cells, exhibited by aberrant swellings and deformations. The two Lotus mutant's nrf1 and nfr5 show wild-type mycorrhization phenotype but lack the root hair response to rhizobia. NFR1 and NFR5 proteins, witch contains an extracellular LysM (lysine motif) domain (Madsen, E. B. et al. 2003), have been predicted to bind Nod-factor and elicits signaling via their intracellular kinase domain (Fig 6). The NFR receptors interact and activate SYMRK in an unknown mechanism to investigate the symbiosis (Fig 7) and conserved phosphorylation sites might be responsible for determine the activity (Yoshida, Satoko and Parniske, Martin 2005).

0.4 General Elicitors in Plant innate immunity / PAMPs

As discussed before, plants have evolved sensitive perception systems for specific molecules ("general elicitors" or PAMPs) that are characteristic for whole classes of microorganisms. These elicitors, sensed by pattern recognition receptors (PRRs), mediate the activation of plant defense responses in a non-cultivar-specific manner. An increasing number of PAMP-like components that induces plant defense reactions have been identified, but the potential receptors of most of them are still unknown. In all plants only three receptors (all in different species and all of a different type of PRR proteins) for three elicitors have been published until now: The bacterial flagellin receptor AtFLS2 (LRR-RLK), the β-glucan binding protein GnGBP in rice and the Xylanase elicitor receptor LeEIX (LRR-RLP).

0.4.1 Bacterial Elicitors

0.4.1.1 Flagellin / FLS2

Flagellin has been identified to induce proinflammatory responses in animals (Flagellin/TLR5) by direct interaction with TLR5 and also acts as a general elicitor of plant defense response (Felix, G. et al. 1999). Bacterial flagellin contains a highly conserved N- and C-term, whereas the middle part is variable through known species. The elicitor active epitope could be matched to the most conserved region in the N-terminal domain and a synthetic peptide (flg22) was used to analyze the flagellin effect on plant cells. When flg22 is truncated (flg15Δ4) the capability to activate defense responses is lost, but the peptide act as weak competitive antagonist of flg22. This indicates that one part of the peptide is necessary for binding to the specific receptor and another part is responsible for the activation of the immune response. (proposed “address-message concept” by (Meindl, T. et al. 2000). A putative transmembrane receptor kinase, containing an extracellular LRR (LRR-RLK) and a intracellular kinase domain (Gómez-Gómez, L. and Boller, T. 2000) was identified to be required for flagellin sensing. Binding studies has shown that FLS2 (Flagellin sensing 2) is important for binding a radio labeled flg22 peptide (^{125}I-Tyr-flg22) (Bauer, Z. et al. 2001). Recent biochemical approaches indeed demonstrate that FLS2 is the “bona-fide” flg22 receptor in *Arabidopsis thaliana* (Chinchilla, unpublished results) and a homologue exists in tomato (S.Robatzek, personal communication, University of Basle).

Flagellin perception led to several induced defense reactions in most plants (Exception: rice, where different epitopes in a bacterial strain-specific manner are recognized, (Takeuchi, K. et al. 2003). Flg22 triggers the alkalinization of extracellular media of suspension cultured cells of Arabidopsis at subnanomolar concentrations, increases ethylene biosynthesis and production of ROS. Furthermore flg22 activates a MAP kinase based signaling cascade (MEKK1, MKK4/5, MPK3/6) (Asai, T. et al. 2002). Like described in Chapter 2 in more detail for elf26 (EF-Tu), flg22 perception induce the expression of 1000 PR-genes, including a high number of receptor like kinases (RLKs) (Navarro, L. et al. 2004, Zipfel, C. et al. 2004).

0.4.1.2 LPS

Lipopolysaccharides (LPS) are the major component of the membrane of Gram-negative bacteria. Parts of the LPS are highly variable (O-Antigen, Polysaccharide chain), whereas the oligosaccharide core and the Lipid A is conserved in most bacteria. The Lipid A is a potent activator of proinflammatory responses in mammals (Miyake, K. 2004, Scheidle, H. et al. 2005, Zeidler, D. et al. 2004) and is therefore often used in medical studies as positive control-stimuli.

In plants several studies show that LPS is able to induce oxidative burst (Meyer, A. et al. 2001) and when used in very high concentrations (mM range) led to growth inhibition of Arabidopsis seedlings (our unpublished data). *Arabidopsis thaliana* reacts to LPS preparations of several plant pathogenic bacteria, with a rapid burst of NO, a hallmark of innate immunity in animals (Zeidler, D. et al. 2004).

Interestingly symbiotic rhizobial bacteria LPS show the ability to suppress the oxidative burst in host plant cell cultures (Albus, U et al. 2001). Furthermore in host plants of *Sinorhizobium meliloti* the Lipid A part is sufficient to suppress the oxidative burst (Scheidle, H. et al. 2005). A receptor-mediated endocytosis is known for immunity in mammals. In a recent publication (Gross, A. et al. 2005) could show, that labeled LPS binds rapidly to the cell wall and is further internalized into the cell. This process could be successfully restricted by adding an excess of unlabeled LPS or by amantadine, an inhibitor of endocytosis in mammalian cells.

0.4.1.3 Peptidoglycan (PGN) / Cold-Shock protein (CSP)

Peptidoglycan (PGN) is the major building molecule of Gram-positive bacteria cell wall, but exists also to a less extent in Gram-negative bacteria. PGN consist of β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid, conserved in known bacteria species. In Drosophila and mammals, PGN can be sensed by PGRP proteins and

NOD proteins, respectively (Innate Immunity in Drosophila). PGN derived from *Staphylococcus aureus* preparations, trigger defense responses in tobacco cells, but was not analyzed in more details (Felix, G. and Boller, T. 2003).

RNP-1, derived from a crude PGN preparation, is a cold-shock-inducible RNA-binding protein from Gram-positive bacteria. It was identified in the same report and it was demonstrated to induce immune responses in tobacco cells.

Similar to flagellin and EF-Tu, only a conserved part is sufficient as elicitor (csp15). The modified csp15-Ala10 peptide was inactive as elicitor, but also exhibit antagonistic activity, when unmodified csp15 was used as an inducer of medium alkalinization or oxidative burst.

0.4.1.4 EF-Tu

In the study presented in Chapter 1, by using a bacteria strain lacking a flagellin gene (*FLC*-), we demonstrate that the most abundant and conserved bacterial protein, elongation factor Tu (EF-Tu), functions as a PAMP in *Arabidopsis thaliana* (Kunze, G. et al. 2004). By using a combined approach of chromatography, enzymatic digestions and MALDI-TOF/MS we could restrict elicitor-activity to an epitope of 18 amino acids. Elf18, a peptide representing this domain, is able to induce oxidative burst, increased ethylene biosynthesis, massive seedling growth inhibition, and expression changes in a high number of genes at subnanomolar concentrations as potent and nearly congruent as flagellin (flg22) (Chapter 2). Moreover, elf18 signaling acts through a MAP kinase-based signaling cascade, and pre-treatment of *Arabidopsis* with elf18 induces enhanced resistance to subsequent infection with pathogenic *Pseudomonas syringae* pv. *tomato.* Elf12, a shorter peptide can act as competitive antagonist for elf18 elicitation, similar as found in previous studies for flagellin and cold-shock proteins.

The characterization of the EF-Tu binding site in Arabidopsis with radio-labeled ligand and cross-linking analysis indicates the presence of a high affinity binding site of ~150 kDa. This binding is time dependent, highly specific, saturable and functions independently from the flagellin FLS2-based perception system. Strikingly the perception of one PAMP leads to a higher amount of binding sites for the other elicitor (Chapter 2). The perception of flagellin (flg22) is controlled by the receptor kinase FLS2, whereas genetic evidence shows that the receptor kinase EFR acts as the EF-Tu (elf18) receptor (Zipfel, unpublished results, Appendix 4). Together our data indicate two independent receptors using a converging signaling cascade, which leads to the activation of the Arabidopsis thaliana innate immunity system

0.4.2 Oomycetes / Fungal PAMPs

Oomycetes and fungi are known wide spread plant pathogens. The mechanisms of invading and the corresponding plant defense mechanisms are also of industrial interest, because they infect a variety of cereals and grasses, leading to loss of crop.

0.4.2.1 β-1,3β-1,6 heptaglucoside (HG)

The plant species family Fabaceae is able to detect surface structures of oomycete Phytophthora. The main polysaccharide molecules of the oomycetal cell wall are the branched 1,3-1,6-β-glucans. Elicitor activity have been shown for the branched 1,3-1,6-β-heptaglucoside (HG) isolated from *Phytophtora megasperma* (Shibuya, N. and Minami, E. 1998), that triggers the production of phytoalexin in soybean, but not in rice and tobacco (Yamaguchi, T. et al. 2000). Similar to other known systems (e.g. LPS in mammals) a soluble Glucan-binding protein (GBP) has been found (Umemoto, N. et al. 1997), that show 1,3-β-glucanase activity. GBP may release oligoglucoside fragments that may act as ligands for the unknown high affinity binding site (Fliegmann, J. et al. 2004). These fragments are not able to pass the plasma membrane and therefore must be perceived outside of responsive cells.

Fliegmann et al. demonstrate by using different methods in immonhistochemistry, that the 75-kDa GBP is restricted to the cytoplasmic face of the cell wall. Until now the GBP-proteins are the only known receptors for oomycete elicitors. GBP proteins have been identified in other plant families. Interestingly, like stated before, plants outside of the Fabaceae showed no responsiveness to these compounds and experimental attempts to detect potential binding sites for purified β-glucan fractions or synthetic HG failed, when tested in plants outside the Fabaceae.

0.4.2.2 Pep13

Pep-13, a minimal active peptide fragment, within an abundant cell wall glycoprotein (GP42) from phytopathogenic oomycete *Phytophthora sojae* has been shown to be sufficient for receptor-mediated defense gene expression and synthesis of phytoalexin in parsley (Hahlbrock, K. et al. 1995).

Pep-13, constitutes a surface-exposed fragment with a cell wall transglutaminase (TGase, R-glutaminyl-peptide:amine-γ-glutamyltransferase), and is conserved among known Phytophthora TGases (Brunner, F. et al. 2002). Mutational analysis within the Pep-13 motif demonstrates that the amino acid residues shown to be important for elicititation of plant defense were also essential for TGase activity. These findings are consistent with the classical PAMP definition, suggesting a Pep13 function as a genus-specific recognition determinant for the activation of plant defense in host (potato) and nonhost (parsley), and in addition have an essential function for the pathogen (Nürnberger, T. et al. 2004).

0.4.2.3 NPP1 / PaNie / Elicitin

In addition of Pep-13 many oomycete species have now been shown to possess another cell wall protein (24 kDa), that induce a similar set of responses in parsley as Pep-13. The unlike Pep-13, necrosis-inducing Phytophthora protein 1 (NPP1) purified

from *P. parasitica* also induced hypersensitive cell death-like lesions in parsley. Infiltration of NPP1 into leaves of *Arabidopsis thaliana* plants resulted in transcript accumulation of pathogenesis-related (PR) genes, callose apposition, production of ROS and ethylene and HR-like cell death. NPP1-triggerd induction of the *PR1* gene and is salicylic acid-dependent. Structural homologs of NPP1 have been found in oomycetes, fungi, and bacteria, but not in plants. (Fellbrich, G. et al. 2002)

The **PaNie234** protein elicitor has been isolated from *Pythium aphanidermatum* and has been shown to induce programmed cell death in carrot (*Daucus carota*) cell cultures (Koch, Wolfgang et al. 1998). The deduced amino acid sequence contains a putative eukaryotic secretion signal including a proteinase cleavage site. If heterologously expressed the elicitor protein without the secretion signal (21 amino acids, (PaNie213)) triggered programmed cell death and de novo formation of 4-hydroxybenzoic acid in cultured cells of carrot. Furthermore, the infiltration of purified PaNie213 into leafes of Arabidopsis resulted in necrosis and deposition of callose on the cell walls of spongy parenchyma cells and the surrounding necrotic mesophyll cells. Interestingly these findings could be confirmed also in tobacco and tomato, but not in maize and oat, suggesting that monocotyledonous plants are unable to perceive this signal (Veit, S. et al. 2001).

Elicitins forms a family of structurally related proteins that induce HR in plants. Elicitin-induced production of ROS has been demonstrated in tobacco and experimental results indicate that although the recognition of the elicitor signal is cell cycle-independent, the induction of cell cycle arrest and following cell death is dependent on the actual phase of the cell cycle (Kadota, Y. et al. 2004). Plant MAP kinases, involved in many plant defense inductions (Pedley, K. F. and Martin, G. B. 2004) (Fig. 8), are also activated upon *Phytophthora spp.* elicitin treatment. Furthermore they are also activated by wounding of the plant cell, suggesting that this signaling elements are involved in multiple signal transduction pathways (Zhang, S. and Klessig, D. F. 2000), corroborating results from bacterial research (Mayrose, M. et al. 2004). Elicitins of class I (e.g. INF1, a 10 kDa-canonical elicitin from Phytophtohora) are secreted, whereas classIII elecitins are cell-surface-anchored polypeptides. Recent publications give evidence that small sequence changes in elicitin revealed significant differences in intensity, specificity and consistency of the

HR induction. (Huitema, E. et al. 2005) suggest that variations in the resistance of *Nicotiana spp*. to *P. infestans* are shadowed by the variations in the response to INF elicitins, indicating an ongoing evolution on the side of the pathogen. Similar observations have been also described in the bacterial field for a type III effector of *Xanthomonas oryzae* pv*. oryzae*, where the virulence factor lost avirulence activity, but retain effector function to avoid host recognition (Yang, B. et al. 2005).

0.4.3 Fungal elicitors

Fungi are also able to secrete a broad spectrum of general elicitors which can be sensed by the plants (e.g. glycopeptides and Xylanase (Basse, C. W. et al. 1993, Boller, T. 1995).

0.4.3.1 Xylanase (EIX)

The plant hormone ethylene affects plant growth, development, and plays in addition a role in plant defense. The fungal elicitor ethylene-inducing xylanase (EIX) can elicits HR and other plant defense responses in tobacco and tomato independently of its xylan degradation activity.

The identified pentapeptide TKLGE was detected at an exposed β-strand of the EIX protein and is responsible for inducing the HR (Rotblat, B. et al. 2002). In the study of (Ron, M. and Avni, A. 2004) the EIX receptor could be identified and characterized. The deduced amino acid sequences, encoded by LeEix1 and LeEix2, contain a Leucine-zipper, an extracellular Leucine-rich repeat domain with glycosylation signals, a transmembrane domain, and a C-terminal domain with a mammalian endocytosis signal - similar like found before in other potential receptors. Therefore these receptor-like proteins (RLPs) can be structurally classified as typical PAMP receptors. Both can bind EIX independently, but only LeEix2 is an activator of HR.

Recently a minimal promoter element in the tomato Acs2 gene (1-aminocyclopropane-1-caboxylic acid synthase, responsible for the ethylene biosynthesis; Expression of Acs2 is induced by EIX) was found to be essential for the induction by EIX (Matarasso, N. et al. 2005), but also other elements seems to play a role in this interaction (Cys-protease (LeCp), ubiquitin-related modifier protein).

0.4.3.2 Chitin

Chitin is a major building block of the cell wall of higher fungi and the exoskeleton of insects. An important defense in response to fungal infection is the attack of the fungal cell wall by plant chitinases that produces fragments of the β-1,4-linked linear N-acetylglucosamine. This N-acetylchitooligosaccharides have been identified to act as elicitor (e.g. induction of ethylene, (Mauch, F. et al. 1992)) in several plant species, but the exact mechanism is still unknown. After perception, chitin induces the expression of defense-related genes (Zhang, B. et al. 2002) in *Arabidopsis thaliana*. Identified loss of function T-DNA insertion mutants for chito-oligomer responsive genes, show higher susceptibility to the fungal pathogen powdery mildew (Ramonell, K. et al. 2005).

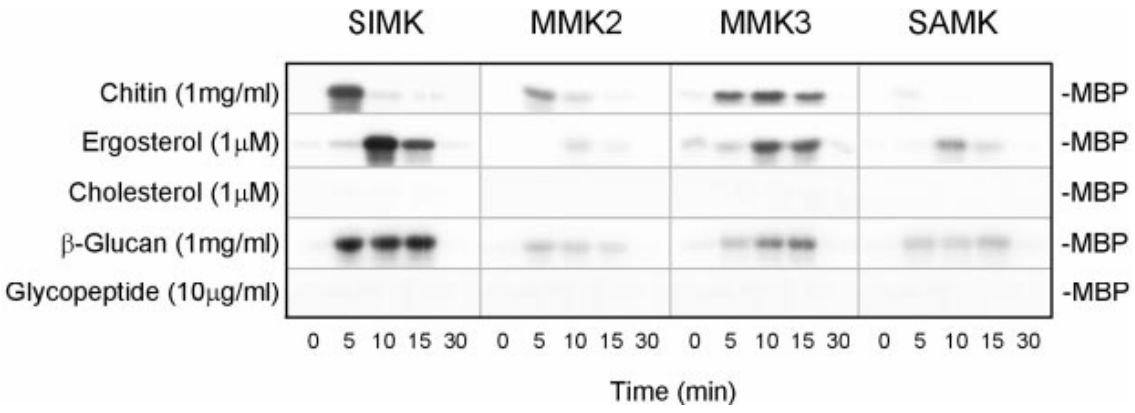

Fig. 8: Differential activation of MAP kinase based pathways by various components in Yeast elicitor (YE) preparations. Time course analysis of the activation: the activity was monitored by immunokinase analysis and myelin binding protein (MBP) was used as substrate. Adapted from (Cardinale, F. et al. 2000)

Like discussed in other elicitor-perception systems an involvement of a MAP kinase based signaling cascade has also demonstrated for chitin (Fig.8) (Cardinale, F. et al. 2000, Zhang, B. et al. 2002). Cardinale et al. demonstrate in addition that a minimum length of three glucosamine residues of the sugar backbone is required to activate the MAP kinases.

The Anti-fungal activity of plant chitinases (Rabea, E. I. et al. 2003) is restricted to chitinases containing a noncatalytic, plantspecific chitin-binding domain (ChBD) (Hevein domain). Interestingly, studies with the race-specific elicitor AVR4 of the tomato pathogen *Cladosporium fulvum* seems to protect fungi cell wall from being degraded by plant chitinases (van den Burg, H. A. et al. 2004).

When suspension cultured cells of *Arabidopsis thaliana* were treatment with chitin and changes in the proteome were analyzed with ionization-time of flight (MALDI-TOF) mass spectrometry (MS), an increase of level from phosphorylated putative endochitinase, a polygalacturonase inhibiting protein and a putative receptor-like protein kinase (without transmembrane domain) was monitored. This findings implicate the existence of an extracellular phosphorylation network which might be involved in intercellular communication (Ndimba, B. K. et al. 2003).

0.4.3.3 Ergosterol

Similar to chitin, ergosterol (a fungal membrane component) induced specific modification of proton fluxes (Granado, J. et al. 1995) and membrane hyperpolarization in motor cells of *Mimosa pudica*. After ergosterol treatment a desensitization (cells did not react to a second ergosterol application) take place, a phenomenon that appears similar to receptor adaptation in animal systems. The same remark can be made concerning the action of chitosan and thus argues for the existence of a receptor for this compound as described in previous work of (Shibuya, N. et al. 1996), that is distinct from the unknown ergosterol receptor (Amborabe, B. E. et al. 2003). MAP kinases are also induced by ergosterol treatment (Fig. 8) and are part of the ergosterol signaling pathway (Sablowski, R. and Harberd, N. P. 2005).

0.4.4 Oligo-α-galacturonides (OGA) endogenous elicitor

Pectin belongs to a family of complex polysaccharides that are present in all plant cell walls. In order to infect the plant, pathogenic fungi (e.g. *Botrytis cineria*) secrets polygalacturonases (PGs) to digest the plants cell wall. Because of the fact that the enzymes themselves are not elicitor-active, they were believed to be important pathogenicity factors (D'Ovidio, R. et al. 2004). Polygalacturnoase-inhibiting proteins (PGIPs) are plant cell wall proteins that protect the plant from fungal invasion. They interact with PGs secreted from the fungi, inhibit their enzymatic activity, and support the accumulation of long chain oligogalcturonides (OGAs), which activate plant defense mechanisms like the ROS production. This elicitor (OGA) is therefore plant-derived and can be regarded as the only endogenous elicitor to induce plant defense reactions known in plant-microbe interaction. For example OGA pre-treatment of grapevine leaves reduce the infection by Botrytis to more than 50% (Aziz, A. et al. 2004) and are therefore speculated to be of use in crop-protection. Interestingly, PGIPs are members of the Leucine-rich repeat (LRR) family (like flagellin receptor FLS2) and their negatively charged LRR structures might be involved in direct binding of polygalacturnoase (Di, Matteo A. et al. 2003).

1 *Plant Cell,* ***16 (12):*** **3496-3507**

The N-terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants

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The abbreviations used are: EF-Tu, elongation factor Tu; EC_{50}, concentration required to induce a half-maximal response; PAMP, pathogen-associated molecular pattern; CSP, cold shock protein; elf18, peptide representing the acetylated 18 amino acid residues of the N-terminus of EF-Tu ; RLU, relative light units

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1.1 Abstract

Innate immunity is based on the recognition of pathogen-associated molecular patterns (PAMPs). Here, we show that elongation factor Tu (EF-Tu), the most abundant bacterial protein, acts as a PAMP in *Arabidopsis* and other *Brassicaceae*. EF-Tu is highly conserved in all bacteria and known to be N-acetylated in *E. coli*. *Arabidopsis* plants specifically recognize the N-terminus of the protein and an N-acetylated peptide comprising the first 18 amino acids, termed elf18, is fully active as inducer of defense responses. The shorter peptide elf12, comprising the acetyl group and the first 12 N-terminal amino acids, is inactive as elicitor but acts as a specific antagonist for EF-Tu-related elicitors. In leaves of *Arabidopsis* plants, elf18 induces an oxidative burst and biosynthesis of ethylene and it triggers resistance to subsequent infection with pathogenic bacteria.

1.2 Introduction

The discrimination between "self" and "infectious non-self" is a principal challenge for multicellular organisms to defend themselves against microbial pathogens. Both plants and animals have evolved sensitive perception systems for molecular determinants highly characteristic of potentially infectious microbes. These "pathogen-associated molecular patterns" (PAMPs) play key roles as activators of the innate immune response in animals. PAMPs recognized by the innate immune systems of animals and plants are highly conserved determinants typical of whole classes of pathogens. The classic example for a PAMP acting as general elicitor of defense responses in plants is the β-heptaglucoside, part of the β-glucan forming the cell walls of oomycetes (Sharp et al., 1984). Likewise, elicitin-proteins secreted by almost all pathogenic oomycetes (Ponchet et al., 1999) and the pep13 domain, forming a conserved epitope of the transglutaminase enzyme involved in crosslinking of the oomycetes cell wall, can signal presence of oomycetes to plants (Brunner et al., 2002). As summarized in recent reviews (Jones and Takemoto, 2004; Nürnberger et al., 2004), plants have been reported to respond to structures characteristic for true fungi such as the wall components chitin, chitosan and glucan, the membrane component ergosterol and the N-linked glycosylation characteristic of fungal

glycoproteins. With regard to recognition of bacteria, plants have evolved perception systems for flagellin, cold-shock protein and lipopolysacharides. Flagellin also acts as a PAMP in the innate immune system of animals where it triggers pro-inflammatory responses via the toll-like receptor TLR5 (Hayashi et al., 2001). However, while plant cells recognize a single stretch of 22 amino acids represented by the flg22 peptide (Felix et al., 1999), animals interact with a different domain of flagellin formed by an N-terminal and a C-terminal part of the peptide chain (Smith et al., 2003), indicating that these perception systems have evolved independently.

In recent work we have observed that pretreatment of *Arabidopsis* plants with crude bacterial extracts or the elicitor-active flagellin peptide flg22 induces resistance to subsequent infection with the bacterial pathogen, *Pseudomonas syringae* pv. *tomato* (Zipfel et al., 2004). In plants mutated in the flagellin receptor gene *FLS2*, flg22 treatment has no effect, but treatment with crude bacterial extracts still inhibits subsequent disease development. This suggests that bacterial extracts contain additional factors, different from flagellin, which act as inducer of resistance. Here, we describe the identification of one such new general elicitor from bacteria, namely the most abundant protein in the bacterial cell, the elongation factor EF-Tu. We localized the epitope recognized as a PAMP to the N-terminus of the protein and show that synthetic peptides representing the N-acetylated N-terminus with ≥18 amino acids act as potent elicitors of defense responses and disease resistance in *Arabidopsis*.

1.3 Results

1.3.1 Crude bacterial extracts contain PAMP(s) different from flagellin

Altered ion fluxes across the plasma membrane are among the earliest symptoms observed in plant cells treated with bacterial preparations (Atkinson et al., 1985). Extracellular alkalinization, a common consequence of these altered ion fluxes, can serve as a convenient, rapid, sensitive and quantitative bioassay to study PAMP perception. Suspension-cultured cells of *Arabidopsis* exhibited typical alkalinization when challenged with crude preparations obtained from bacterial species known to

lack elicitor-active flagellin like *Ralstonia solanacearum* (Pfund et al., 2004), *Sinorhizobium meliloti* (Felix et al., 1999) and *Escherichia coli* GI826, a strain carrying a deletion in the *FliC* gene encoding flagellin. As shown for the examples in Figure 1A, extracellular pH of cultured *Arabidopsis* cells started to increase after a lag phase of 5 to 8 min, reaching a maximum (ΔpH_{max}) after 30 to 40 min. Although ΔpH_{max} varied with age, cell-density and the initial pH of different batches of the cell culture (0.8 to 1.6 pH units), the response to a given dose of a preparation was highly reproducible within a given batch of cells. Higher doses of the *E. coli* preparations did not lead to stronger alkalinization, indicating saturation of the response. In contrast, lower doses exhibited clear dose-dependence and indicated that the boiled preparation was ~10-fold more potent in inducing alkalinization than the preparations of living bacteria and the cell-free supernatant (data not shown). The alkalinization-inducing activity in the bacterial preparations was not affected by heating in SDS (1% v/v, 95°C for 10 min) but was strongly reduced by treatment with proteases like endo-protease Glu-C (Fig. 1A) and pronase (Fig. 1B). These results indicate presence of a novel, proteinaceous factor in *E. coli* and other bacteria that elicits alkalinization in *Arabidopsis* cells.

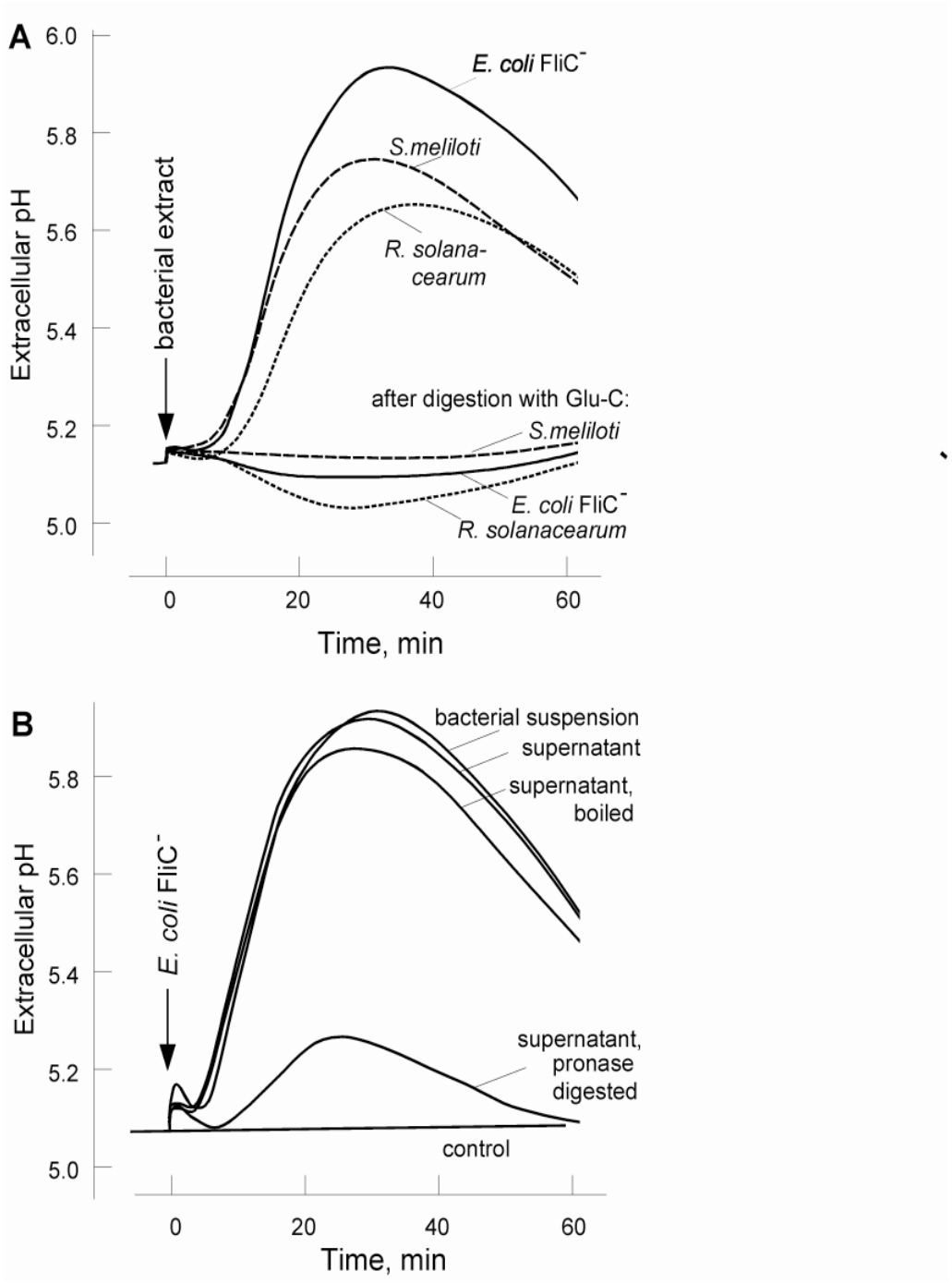

Fig. 1: Induction of extracellular alkalinization by bacteria and bacterial extracts

(A) Extracellular pH in *Arabidopsis* cells after treatment with crude, cell-free extracts from *E. coli* strain GI826 (FliC$^-$), *Ralstonia solanacearum* and *Sinorhizobium meliloti.* At t=0 min, cells were either treated with 10 μl/ml of bacterial extracts or bacterial extracts that were pre-incubated with endoproteinase Glu-C (50 μg/ml for 6 h at 25 °C). (B) Response to treatment with a suspension of living *E. coli* FliC$^-$ cells or the cell-free supernatant of this suspension, either without further treatment or after heating (95°C, 10 min) or digestion with pronase (100 μg/ml, 15 min, 25 °C).

1.3.2 Purification of the elicitor-active protein from E. coli GI826 (FliC-) and its identification as EF-Tu

As a first step of purification crude bacterial extract was fractionated on a MonoQ ion exchange column. Activity eluted as a single peak and fractions with elicitor activity were pooled, proteins precipitated by 80 % acetone, and separated by SDS-Page (Fig. 2A). After staining and drying, the gel was cut in 2 mm segments and eluates obtained from these slices were tested for induction of alkalinization (Fig. 2B). Activity was observed to co-migrate with the major polypeptide band of ~43 kDa apparent molecular weight. The tryptic digest from the eluate with highest elicitor activity resulted in a mass fingerprint that covered 43 % of elongation factor Tu (EF-Tu) (Fig. 2C, underlined parts). While demonstrating presence of EF-Tu, this result did not exclude the possibility that elicitor activity was attributable to a different, minor protein co-migrating with EF-Tu on SDS-PAGE. To prove elicitor activity of EF-Tu directly, we tested highly purified EF-Tu from *E. coli* (M. Rodnina, University of Witten-Herdecke, Germany) and His-tagged EF-Tu (L. Spremulli, University of North Carolina, US). Both samples of EF-Tu proved to be very potent elicitors in *Arabidopsis* and induced half-maximal alkalinization (EC_{50}) at concentrations of ~4 nM (Fig. 2D, shown for non-tagged EF-Tu).

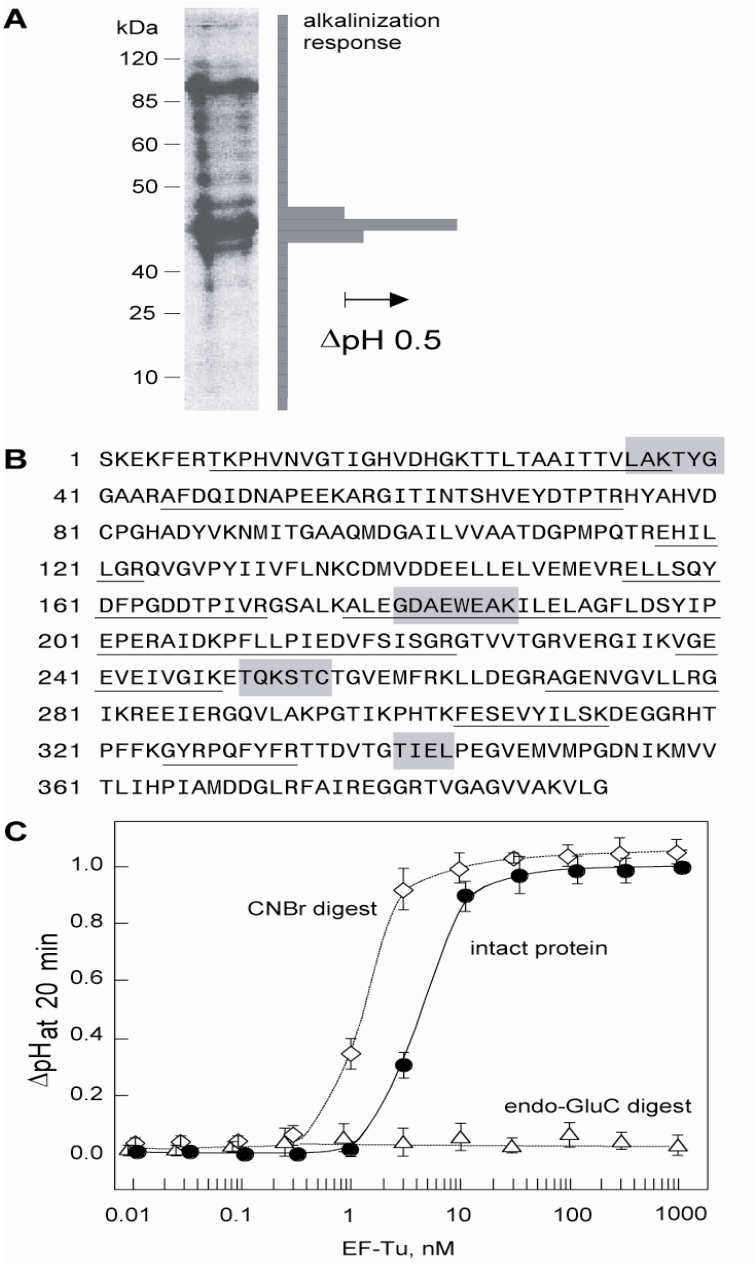

Fig. 2: Identification of the elicitor-active protein as EF-Tu.

(A) Alkalinization-inducing activity in extract from *E. coli* strain GI826 was pre-purified on MonoQ-Ion exchange chromatography and separated by SDS-PAGE. (B) The dried, Coomassie-blue stained gel was cut in slices and the eluates of these slices were assayed for alkalinization-inducing activity by measuring extracellular pH in *Arabidopsis* cells after 20 min of treatment. (C) Amino acid sequence of mature EF-Tu protein from *E. coli* (Laursen et al., 1981). Eluate with highest elicitor activity was digested with trypsin and peptide masses were compared to the masses calculated for the proteome of *E. coli*. Underlined sequences indicate peptides with masses matching the ones calculated for EF-Tu. With the exception of the amino acids indicated with a shaded background, EF-Tu is highly conserved with identical amino acids in >90 % of the sequences from different bacteria (n>100 sequences in the database). (D) Activity of EF-Tu and of EF-Tu digested with endoproteinase Glu-C or CNBr. Different doses of purified, intact EF-Tu (closed circles), EF-Tu after digestion with endoprotease Glu-C (open triangles) and EF-Tu after cleavage with CNBr (open diamonds) were assayed for induction of alkalinization in *Arabidopsis* cells. Extracellular pH was measured after 20 min of treatment. Data points and bars represent mean and standard deviation of 3 replicates.

1.3.3 The elicitor-active epitope resides in the N-terminus of EF-Tu

In previous work with the bacterial elicitors flagellin (Felix et al., 1999) and CSP (Felix and Boller, 2003), we succeeded to localize elicitor-activity to particular domains of the respective proteins. As a guide for this localization we used the hypothesis that plants recognize functionally essential, highly conserved epitopes of these proteins as PAMPs. Apart from some small regions, however, the entire EF-Tu sequence is highly conserved and exhibits identities >90% for sequences from many different bacteria (Fig. 2C). To delineate the epitope responsible for elicitor-activity, we thus resorted to proteolytic cleavage of the protein. Enzymatic cleavage of EF-Tu with trypsin or the endoproteases Arg-C, Asp-N, Lys-C and Glu-C completely inactivated its elicitor activity (data shown for Glu-C in Fig. 2D). In contrast, chemical cleavage with CNBr at methionine residues did not lead to inactivation but led to a slight increase in its specific activity (EC_{50} of <2 nM, Fig. 2D). Thus, we concluded that the elicitor-active epitope of EF-Tu includes the amino acids K, R, E and D but no M. The CNBr-fragments were separated on a C8 reversed-phase column, and the fractions containing activity were re-run on the column using a more shallow gradient (Fig. 3A). Alkalinization-inducing activity was associated exclusively with the second of the two major peaks eluting from the column. This peak contained peptides with masses of 10044 + n*28 (Fig. 3B). This heterogeneity of mass, probably due to formyl-adductions occurring in the CNBr-cleavage reaction in 70% formic acid, did not allow direct, unequivocal mapping to a domain in EF-Tu. However, masses of fragments obtained after further digestion with trypsin all matched the ones calculated for tryptic peptides of the N-terminal CNBr-fragment of EF-Tu (amino acids 1-91, Fig 3C), indicating that it is the N-terminal part of EF-Tu (Fig. 3D) which is recognized by the plant.

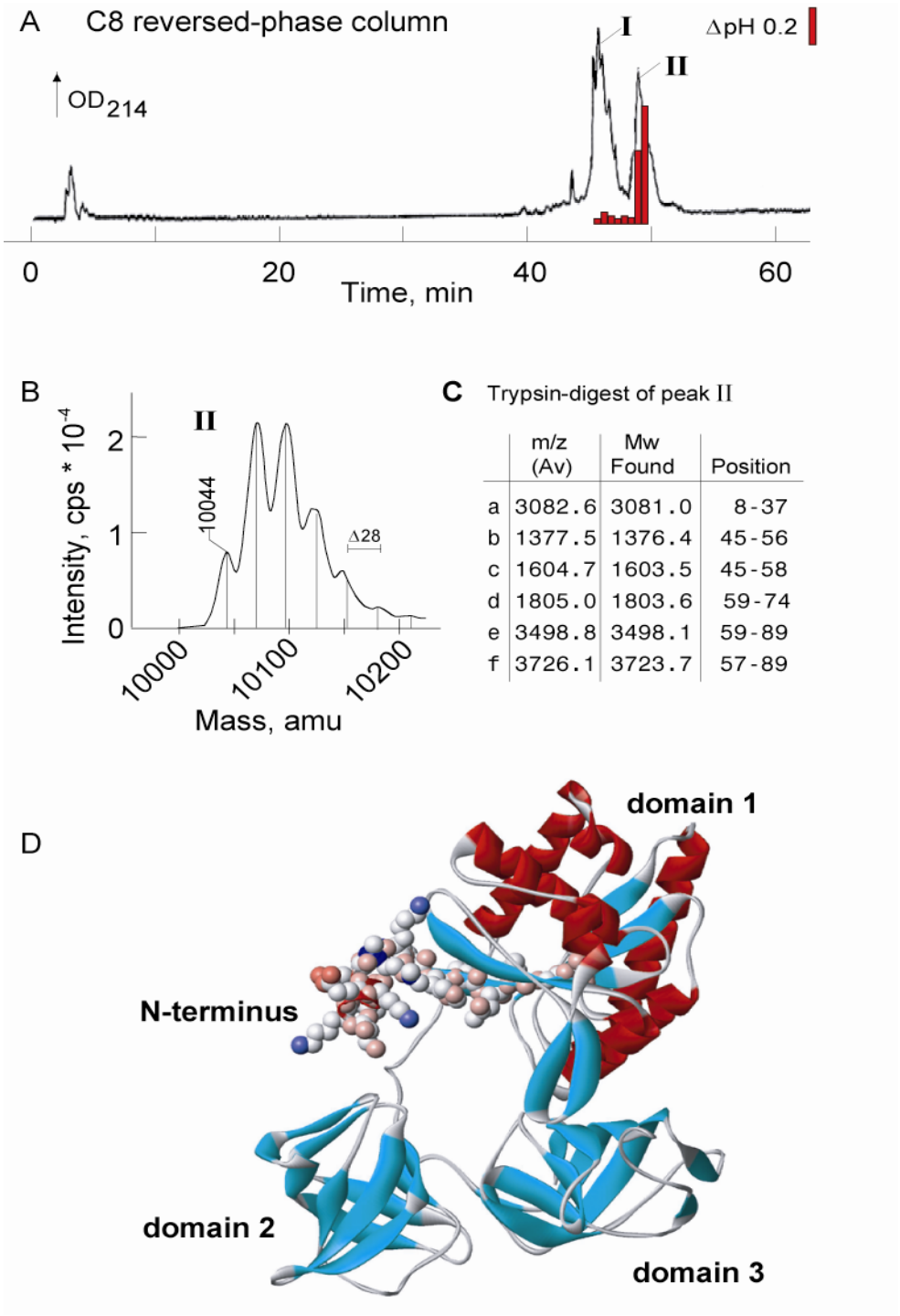

Fig. 3: Identification of the CNBr- fragment carrying elicitor activity
(A) The CNBr-digest of EF-Tu was separated on C8 reversed-phase column. Fractions containing activity were re-run on C8 using a more shallow gradient and eluate was assayed for UV-absorption (OD214 nm) and elicitor activity (bars). (B) Masses found in peak II with nanospray analysis. (C) Peptide masses observed after trypsin-digestion of peptides in peak II that map to the CNBr-fragment of EF-Tu 1-91. (D) Structure of whole, unmodified, Ef-Tu (Song et al., 1999) completed with a tentative, computer-assisted prediction (Geno3D (Combet et al., 2002)) for the 8 N-terminal amino acid residues. Ribbon model with the N-terminal part shown as ball and stick (drawn with WebLab ViewerLite, Molecular Simulations Inc., Cambridge, UK).

1.3.4 Activity of different EF-Tu peptides

Two domains in the N-terminal fragment EF-Tu 1-91 contain E, D, K and R within a stretch of <30 amino acid residues, and were therefore considered as candidates for the elicitor-active epitope. Whereas a synthetic peptide corresponding to EF-Tu 45-71 exhibited no activity even at 10 μM (data not shown), the peptide representing EF-Tu 1-26 was as active as the intact EF-Tu protein and induced medium alkalinization with an EC_{50} of ~4.5 nM (Fig. 4). A peptide variant with N-α-(9-fluorenylmethyloxycarbonyl) (Fmoc), the protective group used in the peptide synthesis, still attached to the N-terminus showed an even higher elicitor activity (EC_{50} of ~0.7 nM, Fig. 4). In early work on EF-Tu from *E. coli*, the protein was found to start with a serine residue modified by N-acetylation (Laursen et al., 1981). N-terminal acetylation of the peptide EF-Tu 1-26 indeed resulted in a peptide with a ~20-fold higher specific activity, inducing alkalinization with an EC_{50} of ~0.2 nM (Fig. 4). In contrast, N-terminal prolongation by a formyl-group, a methionine residue or a formyl-methionine residue group had little effect (Fig. 4, values shown for Met-1-26 only). The peptide EF-Tu ac-1-26 was termed **elf26**, referring to the acetylated N-term of *el*ongation *f*actor with the first 26 amino acid residues. To determine the minimal length required for activity, we tested peptides progressively shortened at the C-terminal end. Full activity was observed also for elf22, elf20 and elf18, peptides comprising at least the acetyl group and the first 18 residues of EF-Tu (Fig. 5). The peptides elf18 to elf26 were equally active and were used interchangeably in further experiments. In different batches of the cell culture used to compare the relative activity of the various peptides the EC_{50} values of fully active peptides varied between 0.1 nM and 0.4 nM, indicating high reproducibility and robustness of the alkalinization assay. Since elf18 contains no Asp residue full activity of this peptide was somewhat surprising with respect to the sensitivity of the elicitor-activity to endoprotease Asp-N described above. Most likely, inactivation was due to the minor activity of this enzyme at Glu-N indicated by the supplier. The peptide elf16 showed significantly lower activity and only residual activity was found with elf14. The peptide elf12 did not induce an alkalinization response even when applied at concentrations of 10 to 30 μM (Fig. 5).

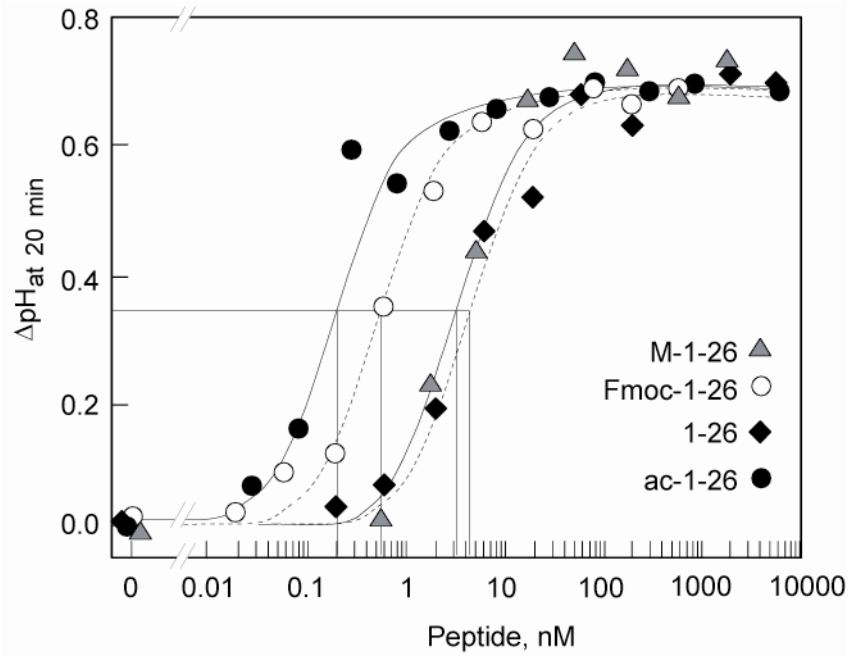

Fig. 4: Elicitor activity of peptides representing the N-terminus of EF-Tu

Different doses of synthetic peptides representing the amino acids 1 to 26 of EF-Tu, either with the N-terminal NH2-group left free (1-26) or coupled to an extra methionine residue (M-1-26), an acetyl group (ac-1-26) or Fmoc (N-α-(9-fluorenylmethyloxycarbonyl)) used as protective group in the peptide synthesis (Fmoc-1-26), were assayed for induction of alkalinization in *Arabidopsis* cells. Extracellular pH was measured after 20 min of treatment, pHi at the beginning of the experiment was 4.8.

The peptide elf18 served as a core peptide to test the effect of individual amino acid residues on the activity of the EF-Tu peptides. Peptides with an Ala residue replacing the residues at position 1, 3, 6, 9, 10, 11, 12 or 13 retained full activity and EC_{50} values between 0.15 nM and 0.6 nM (Fig. 5). In contrast, replacements at positions 2, 4, 5 and 7 led to 10 to 400-fold lower activity. Changing the two residues 2 and 5 to Ala residues resulted in a combined effect and 50'000-fold lower activity. Permutations of the last four amino acids in elf18 had little effect on activity but swapping VNV (position 12-14) with GTI (position 15-17) strongly reduced activity (Fig. 5).

The N-terminal EF-Tu sequences of many species of enteric bacteria as well as those of *Erwinia amylovora* and *E. chrysanthemi* are identical to the one described for *E. coli*. We tested further peptides representing the exact sequences of EF-Tu's encoded by some other plant-pathogenic or plant associated bacteria. The peptide representing the N-terminal 18 aa residues in *A. tumefaciens* and *S. meliloti,* differing in positions 1, 3, 8 and 14, exhibited full activity. In contrast, peptides representing

EF-Tu from *Pseudomonas syringae* pv tomato DC3000 and *Xylella fastidiosa* showed reduced activity and EC_{50} value of ~15 nM and ~30 nM, respectively (Fig. 5).

Sequence conservation for elongation factors extends beyond bacteria, and homologous sequences can be found in eukaryotes, notably for the elongation factors of plastids and mitochondria. Therefore, we also tested peptides corresponding to the plastid, mitochondrial and cytoplasmic homologues from *Arabidopsis*. In their non-acetylated forms, the peptides representing the cytoplasmic sequence exhibited no activity while the plastid and mitochondrial peptides induced alkalinization with EC_{50} values of 800 to 1000 nM, respectively. Acetylation of the cytoplasmic peptide led to a somewhat higher activity and an EC_{50} value of ~300 nM (Fig. 5).

In summary, these results demonstrate that *Arabidopsis* cells have a sensitive perception system specifically recognizing the N-terminus of EF-Tu, an epitope predicted to protrude from the surface of the protein (Fig. 3D). A minimal peptide with N-terminal acetylation and a sequence comprising 'acetyl-xKxKFxRxxxxxxxxx' appears to be required for full activity as elicitor in *Arabidopsis*.

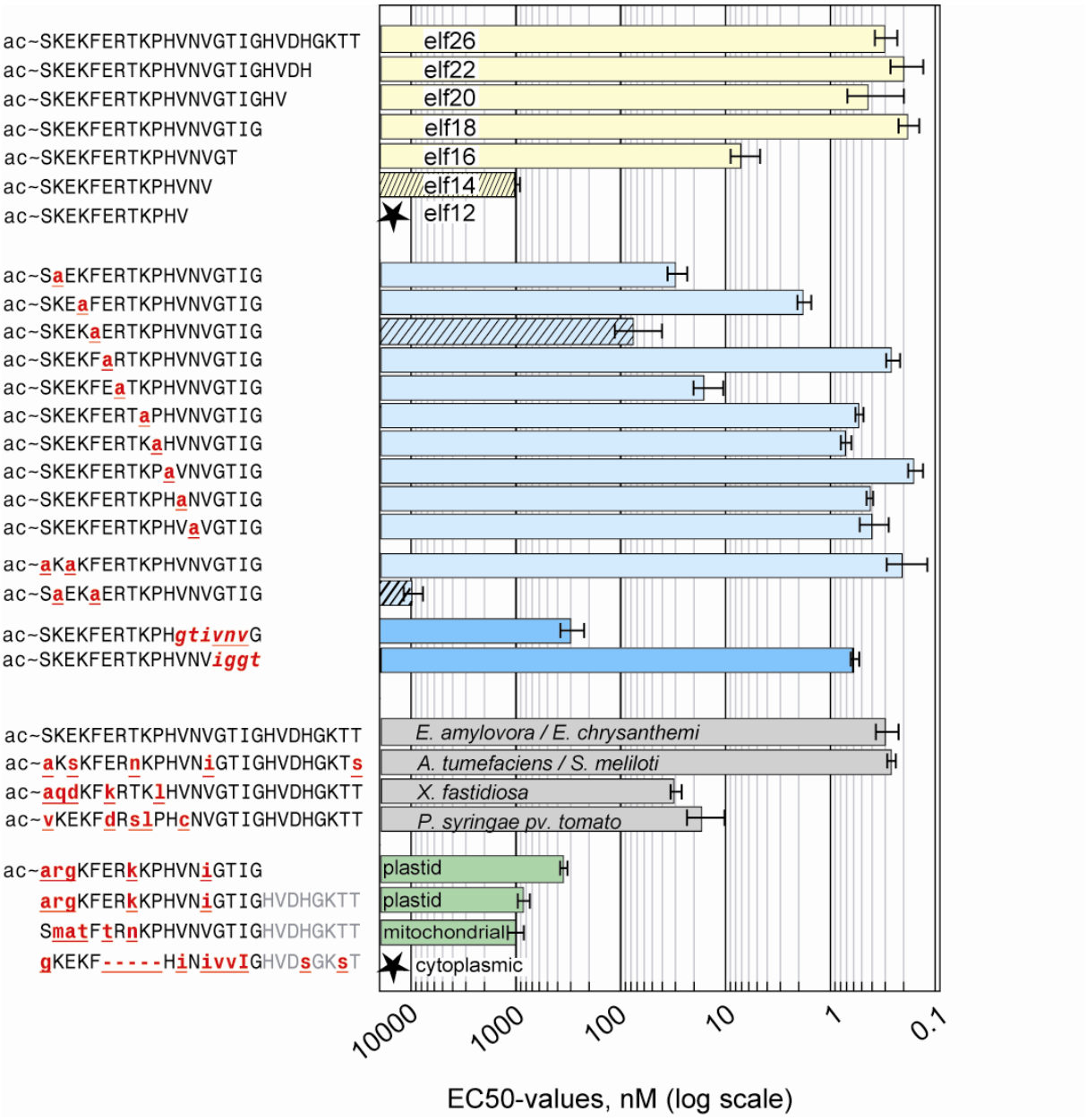

Fig. 5: Alkalinization-inducing activity of EF-Tu N-terminal peptides

Summary of EC_{50} values determined from dose response curves with the different peptides. Peptide sequences and N-terminal acetylation (ac~) are indicated in the left part. Bars and error bars in the right part represent EC_{50} values and their standard errors on a logarithmic scale. Hatched bars indicate activity of peptides that act as partial agonists, inducing 50 % of the pH amplitude observed for full agonists at the concentrations indicated but fail to induce a full pH change even at the highest concentrations of 30 µM tested. No activity could be detected with peptides denoted with asterisks (EC_{50} >>10^4nM).

1.3.5 The peptide elf12 antagonizes elicitor activity of EF-Tu

Inactive, structural analogs of elicitors may act as specific, competitive antagonists for the elicitor they were derived from. Examples for this include the oligosaccharide part of the glycopeptide elicitor (Basse et al., 1992) and C-terminally truncated forms of the flg22 elicitor (Bauer et al., 2001; Meindl et al., 2000). Indeed, elf12, which shows no elicitor activity even when applied at micromolar concentrations (Fig. 5), exhibited antagonistic activity for EF-Tu-related elicitors but not for the structurally unrelated elicitor flg22 (Fig. 6A). Inhibitor-activity of elf12 was rather weak and, as expected for a competitive antagonist, could be overcome by increasing concentrations of the agonist (data not shown). Nevertheless, elf12 applied at micromolar concentrations could serve as diagnostic tool to test for the presence of EF-Tu-related activity in crude bacterial extracts (Fig. 6B). For example, elf12 inhibited the activity present in the cell-free supernatant of *E. coli* GI826 and also strongly reduced response to extracts from *Agrobacterium tumefaciens* and *Ralstonia solanacearum*, indicating that the EF-Tu was the predominant elicitor activity in these preparations.

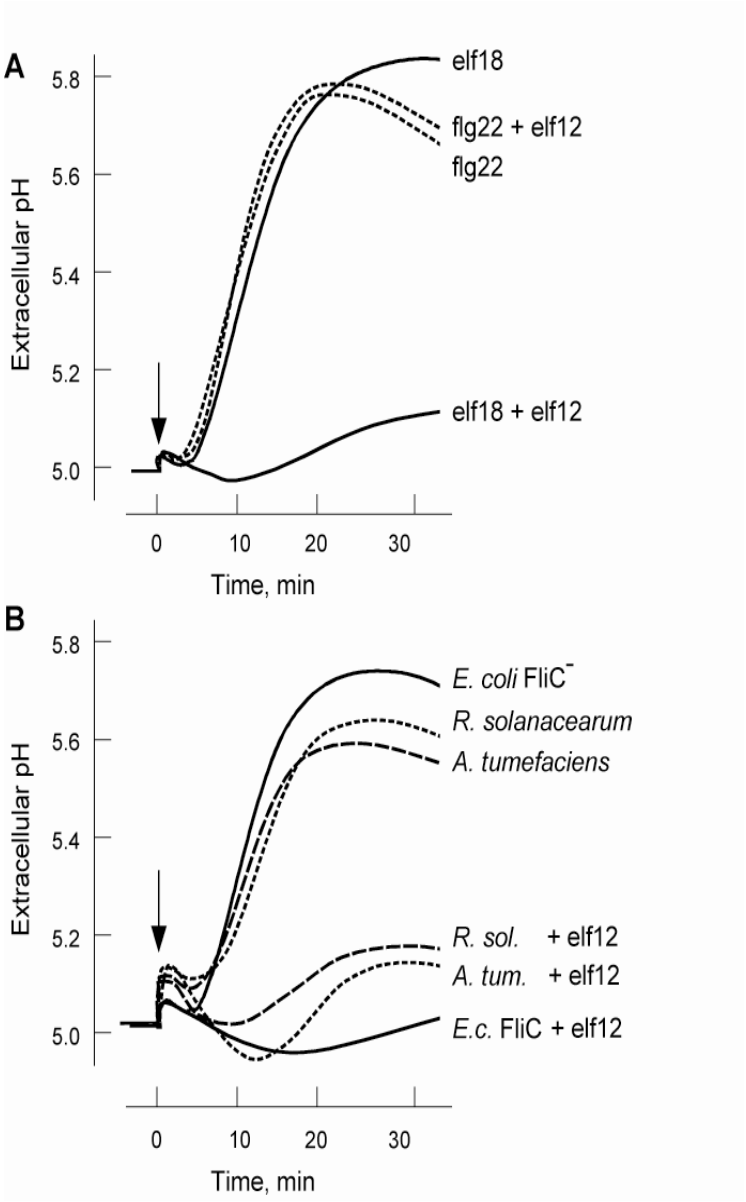

Fig. 6: Antagonistic activity of elf12 for EF-Tu related elicitors
(A) Alkalinization induced by 1 nM flg22 or 0.5 nM elf18 when applied alone or together with 30 nM elf12. (B) Effect of 30 nM elf12 on the alkalinization induced by the cell-free supernatant from living E. coli FliC- or crude bacterial extracts from *R. solanacearum* and *A. tumefaciens*.

1.3.6 EF-Tu-induced defense responses in Arabidopsis and other plant species

Production of reactive oxygen species (oxidative burst) and increased biosynthesis of the stress hormone ethylene are symptomatic for plants attacked by pathogens or treated with elicitors (Lamb and Dixon, 1997). Leaf tissues of all *Arabidopsis* accessions tested showed increased biosynthesis of ethylene after treatment with EF-Tu peptides (Fig. 7A, data not shown for accessions Tu-1, Cal-0, Si-0, Kil-0, Berkeley, Pog-0, Cvi-0, Nd-0, Kä-0, Can-0, Kas-1, Ct-1, Be-0, and C24). Similarly, leaf tissue from other *Brassicaceae* like *Brassica alboglabra, Brassica oleracea* and *Sinapis alba* also responded to the EF-Tu peptides. In contrast, all plants tested so far that do not belong to the family of *Brassicaceae* showed no response to treatment with EF-Tu-peptides. Besides the examples shown in Figure 7A, this includes potato, cucumber, sunflower, soybean and *Yucca alifoli*, all of which showed enhanced ethylene biosynthesis when challenged with flg22 as a positive control (data not shown).

Arabidopsis accession Ws-0 carries a mutation in the flagellin receptor FLS2 and shows no response to flagellin elicitor (Zipfel et al., 2004). Importantly, leaves of this accession showed normal response to EF-Tu elicitors when tested for induction of ethylene (Fig. 7A) but also when assayed for induction of oxidative burst (Fig. 7B). Although the amount of light emitted varied considerably between independent experiments with different plants, induction of an oxidative burst with a clear and significant increase above the straight base line was reproducibly observed with EF-Tu protein, elf18 and elf26 but not with elf12, elf18-A2/A5 and the peptides representing the plastid- or cytoplasmic forms (Fig. 7B).

Induction of the *SIRK / FRK1* gene (At2g19190) has been used in several studies as a molecular marker for induction of defense-related genes during basal defense (Asai et al., 2002; de Torres et al., 2003; Robatzek and Somssich, 2002). In *Arabidopsis* lines Ws-0 and Col-0 made transgenic for the *GUS* gene under the *SIRK* promoter GUS activity was clearly induced at the sites in the leaves that were inoculated by pressure infiltration with 1 µM elf26 (Fig. 8A). After 24 h of treatment clear GUS staining was observed also with crude bacterial extracts from *E. coli* FliC$^-$ or *R. solanacearum* in both lines of transgenic plants whereas flg22 only induced GUS in the Col-0 background expressing a functional FLS2 protein (Fig. 8A). In summary, these results show that *A. thaliana* and other *Brassicaceae* have a highly

sensitive perception system for the N-terminal domain of bacterial EF-Tu, which functions independently of the perception system for flagellin.

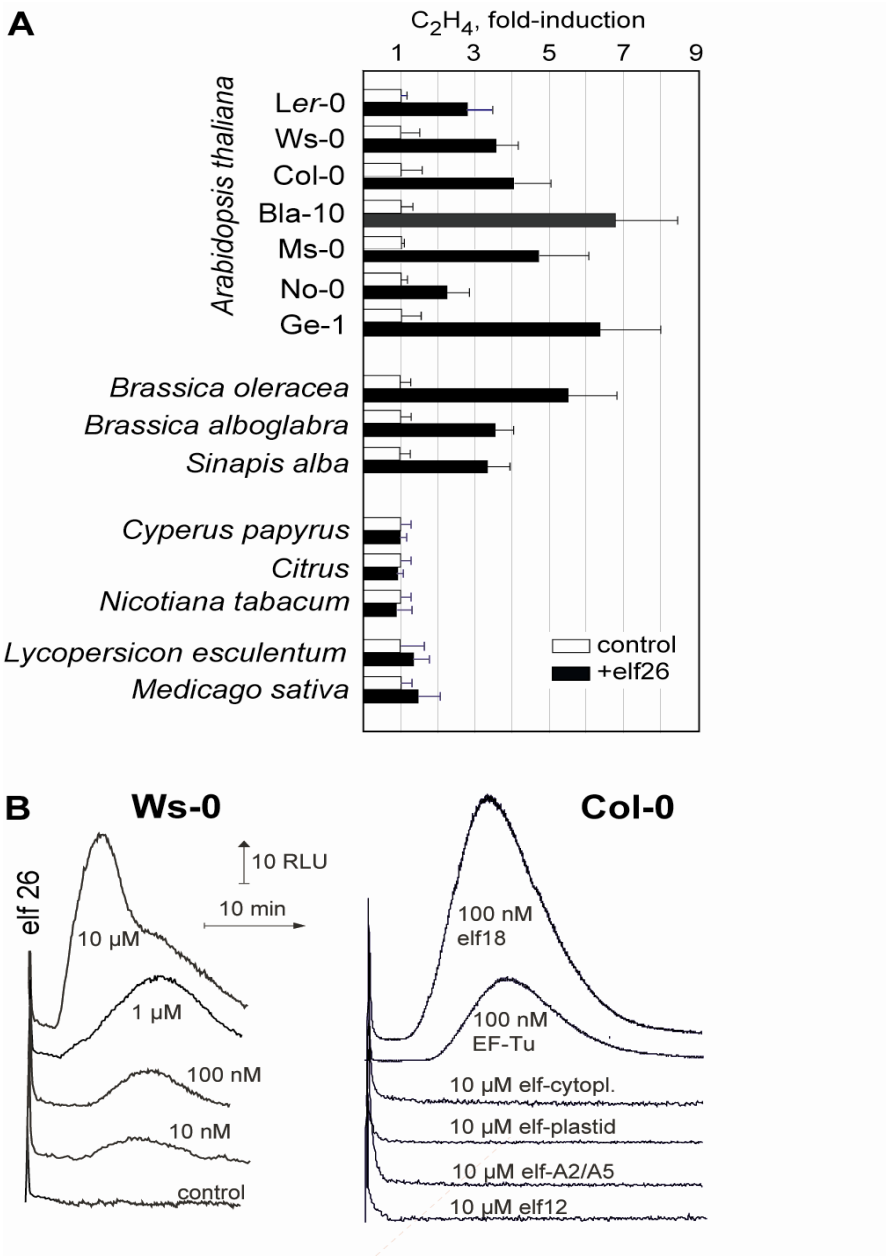

Fig. 7: Induction of elicitor responses in leaf-tissues of different plant species
(A) Induction of ethylene biosynthesis in leaf-tissue. Leaf pieces from various plant species were mock-treated (controls) or treated with 1 µM elf26 and ethylene was measured after 2 h. Results, represented as fold-induction over control, show mean and standard deviation of n=4 replicates. (B) Oxidative Burst in leaf tissues of *Arabidopsis* accessions Ws-0 (left panel) and Col-0 (right panel). Luminescence (relative light units, RLU) of leave slices in a solution with peroxidase and luminol was measured after addition of EF-Tu protein or the peptides indicated. Light emission during the first seconds of the measurements was due to phosphorescence of the green plant tissue.

1.3.7 Induction of resistance

In recent work we found that pretreatment of *Arabidopsis* leaves with the flagellin-derived elicitor flg22 triggered the induction of disease resistance and restricted growth of the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Zipfel et al., 2004). EF-Tu-related elicitors such as elf26 induced a similar effect when infiltrated into leaves 1 day before infection with *Pst* DC3000 (Fig. 8B). In contrast to flg22, elf26 induced this effect also in *fls2-17* mutant plants carrying a mutation in the flagellin receptor FLS2. Although somewhat weaker than the effect of flg22 in the experiment shown, significant, ~20-fold reduction of bacterial growth was observed in four out of four independent experiments. Importantly, no direct effect of elf26 (or flg22) on bacterial growth could be detected on *Pst* DC3000 growing in LB medium supplemented with 10 µM of the peptides, indicating no direct toxic effect of this peptide (data not shown).

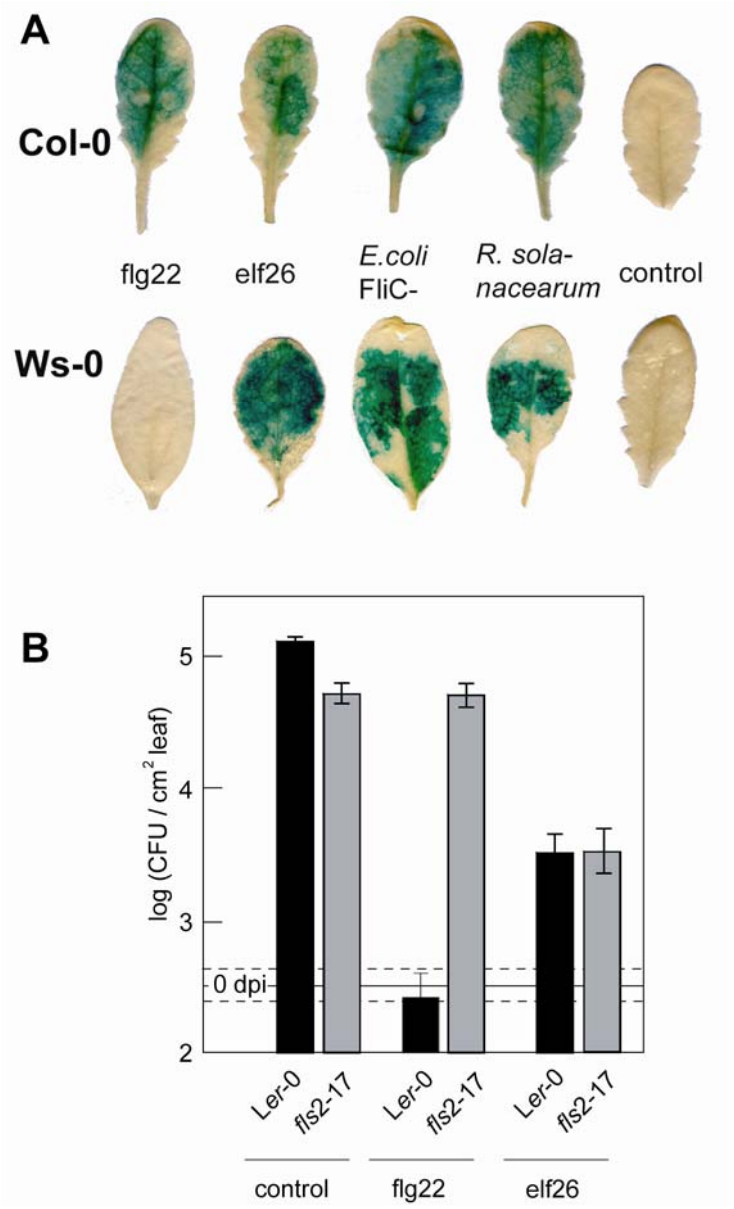

Fig. 8: Induction of defense responses in Arabidopsis

(A) Induction of GUS activity in lines of Ws-0 and Col-0 transgenic for *SIRK*p::*GUS*. Leaves of both lines were pressure infiltrated with 1 □M flg22, 1 □M elf26, crude preparations of *E.coli* FliC$^-$ and *R. solanacearum* (diluted 1:100 in 10 mM $MgCl_2$) or 10 mM $MgCl_2$ (control). After 24 h of treatment leaves were detached from the plants and stained for GUS activity.

(B) Arabidopsis wild-type Ler-0 and fls2-17 plants were either were pretreated for 24 hours with 1 □M flg22, with 1 □M elf26 or with H_2O as a control. These leaves were subsequently infected with 10^5 cfu/ml *Pst* DC3000, and bacterial growth was assessed 2 days post-infection (2 dpi). Results show average and standard error of values obtained from 4 plants with 2 leaves analysed each (n=8). The solid and the dashed lines indicate mean and standard deviation of cfu extractable from leaves at 0 dpi (n=12).

1.4 Discussion

The novel perception system described in this report exhibits high sensitivity and selectivity for peptides with the core structure 'acetyl-xKxKFxR', a motif that is highly characteristic and unique for EF-Tu's from bacteria. EF-Tu binds aminoacyl-transfer RNAs (all except fMet-tRNA and selenocysteine-tRNA) and catalyses the delivery of the amino acids to the nascent peptide chain in the ribosome in a GTP-dependent process. With ~100,000 molecules/cell, EF-Tu amounts to 5 - 9 % of total bacterial cell protein and thus is one of the most abundant proteins in bacteria. Due to its essential role in protein biosynthesis the EF-Tu protein has been studied extensively at the biochemical and structural level (Kawashima et al., 1996; Krab and Parmeggiani, 1998; Rodnina and Wintermeyer, 2001).

Perception of EF-Tu by plant cells exhibits characteristics resembling the perception of flagellin (Felix et al., 1999) and cold shock protein (Felix and Boller, 2003), two general elicitors studied previously. In all three cases, elicitor-activity could be attributed to a highly conserved epitope comprising a single stretch of 15 to 20 amino acid residues of the respective protein. Synthetic peptides representing the genuine amino acid sequences of these domains display activity at subnanomolar concentrations. Truncating flagellin and EF-Tu peptides at their C-termini leads to elicitor–inactive forms that specifically antagonize elicitor-activity of flagellin (Bauer et al., 2001; Meindl et al., 2000) and EF-Tu (Fig. 6), respectively. Functionally, these elicitors can be divided in a part responsible for binding and a part required for activation of the receptor. As postulated for flagellin perception (Meindl et al., 2000), perception of EF-Tu appears to involve two consecutive steps according to the address-message concept, a concept originally put forward to explain functioning of peptide hormones in animals (Schwyzer, 1987). Although sharing common characteristics the perception systems for flagellin and EF-Tu obviously involve different receptors, since perception of flagellin requires the receptor kinase FLS2 (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004), while EF-Tu is also active in plants carrying mutations in FLS2 (Fig. 7 and 8).

In our ongoing work we further compare perception of EF-Tu and flagellin in *Arabidopsis* in more detail (Kunze et al., in preparation). Results emerging demonstrate a high-affinity binding site specific for EF-Tu that clearly differs from the one for the flagellin elicitor. After this initial step of perception, however, EF-Tu- and flagellin-derived elicitors induce the same elements of signal transmission, including

activation of a MAPK, and the same set of responses with similar kinetics (data not shown). Thus, we hypothesize that perception of EF-Tu occurs via an EF-Tu-receptor that functions in a manner very similar to the receptor for flagellin.

EF-Tu is among the most slowly evolving proteins known (Lathe and Bork, 2001). The first 300 hits obtained by a BLAST analysis with the N-terminus of *E. coli* EF-Tu in the non-redundant GenBank database covered bacterial EF-Tu sequences from many different species and diverse taxons (data not shown). Based on our results with the Ala-substitutions and other sequence variations of the elf peptides (Fig. 5) one can classify at least ~140 of these genes to encode EF-Tu's with full elicitor-activity in *Arabidopsis*. This list includes the EF-Tu's from the plant pathogens *Erwinia carotovora*, *Ralstonia solanacearum* and *Agrobacterium tumefaciens*. In contrast, there were ~70 hits encoding genes with modifications at positions relevant for elicitor-activity, and these EF-Tu's are probably less active. With our current, limited knowledge on the exact sequence requirements for a fully active structure, the remaining ~90 sequences cannot be classified.. Overall, however, the structure rendering full elicitor-activity to the N-terminus of EF-Tu is present in many bacterial species and this highly conserved epitope can be regarded as a PAMP. Interestingly, the EF-Tu's from some of the bacterial species pathogenic to plants, such as Pseudomonas *syringae* pv tomato DC3000 and *Xylella fastidiosa,* exhibit reduced activity as elicitors. Although correlative, this provides evidence for the hypothesis of an evolutionary pressure on these pathogens to modify this part of their EF-Tu protein and to avoid recognition by the defense system of the plants. This is reminiscent of the sequence variations observed for the elicitor-active domain in flagellins of bacteria pathogenic to plants. Several of these bacteria carry sequence variations that render them undetectable for the flagellin-detection system of the plant (Felix et al., 1999).

Homology of elongation factors extends through all bacteria but also to elongation factors acting in mitochondria, plastids and the cytoplasma of eukaryotes. Therefore, we considered the possibility that the perception system described here could also recognize the plant's own EF-Tu. If this were true, the EF-Tu released from wounded cells might act as wound-factors signaling 'danger' to neighboring cells. However, peptides representing the N-termini of the elongation factors from the plant cells showed either no or only marginal activity (Fig. 5). Also, as determined in preliminary

experiments, crude extracts from *Arabidopsis* cells seem to contain no EF-Tu-related elicitor activity (data not shown).

The EF-Tu protein has been extensively studied for its essential function in protein translation. Specific molecular interactions and processes have been assigned to many parts of the three domains of the protein (Krab and Parmeggiani, 1998). The function of the N-terminus, however, remains largely unexplained and x-ray crystallography did not reveal a clear structure for the seven amino acids at the N-terminus of the protein (Song et al., 1999). Nevertheless, this part of the protein is equally highly conserved, notably for the basic residues and the phenylalanine found to be relevant also for elicitor-activity, suggesting an essential function also for this part of the EF-Tu (Laurberg et al., 1998). EF-Tu proteins with mutations in the well conserved basic amino acid residues at positions 2, 5 and 7 were found to be impaired in binding of GTP and aminoacyl-tRNA *in vitro*. According to the hypothetical, computer-assisted, model for the N-terminus of EF-Tu protein (Fig. 3D) at least the first 12 amino-acid residues of the N-terminus are surface-exposed and separated from the other domain structures – a suitable target for a chemoperception system such as the one described in this report or as a target for newly designed antibiotics interfering with bacterial protein translation in pharmaceutical research (Krab and Parmeggiani, 1998). Interestingly, a monoclonal antibody highly selective for bacterial EF-Tu and useful to detect bacterial contamination in medical samples has been found to specifically recognize the same N-terminal core structure (Baensch et al., 1998). Whereas the first 12 amino acid residues form a protruding group, residues 13 to 18 appear to reside within the first domain of EF-Tu. This is intriguing with respect to our finding that the elicitor-activity of synthetic peptides crucially depends on a length of >12 amino acid residues. At present, the specific requirements for this C-terminal part are less clear and the mechanism by which the perception system of the plants can interact with this part of EF-Tu remains to be elucidated. Importantly, intact, non-denatured EF-Tu is a highly active elicitor in tissue of intact plants and in cultured cells (Fig. 2D).

It is worth noting that N-terminal acetylation of the synthetic peptides corresponding to the N-terminus of EF-Tu increases their potency by a factor of about 20. EF-Tu is well-known to be N-acetylated in *E. coli* (Laursen et al., 1981). While N-acetylation occurs frequently in eukaryotes, *E. coli* contains merely three N-acetylated proteins in addition to EF-Tu, namely the ribosomal proteins S5, S18, and L7, each of which is

acetylated by a specific N-terminal acetyltransferase (Polevoda and Sherman, 2003). The enzyme responsible for EF-Tu acetylation is still unknown, and it is equally unknown whether this modification has any functional significance. In view of the observation that PAMPs represent particularly conserved structures of a whole class of microbes, we predict, however, that N-terminal acetylation of EF-Tu is functionally important, and we want to point out that our finding reveals a surprisingly neglected field in the biochemistry of prokaryotes.

The elicitor-active epitopes of the bacterial proteins we identified as general elicitors are not freely accessible for receptors residing in the plasmamembrane of plant cells. EF-Tu and Csp are considered to be in the cytoplasm and the flg22-epitope faces the inside of the bacterial flagellum, a supramolecular structure that cannot penetrate the plant cell wall. Interestingly, TLR5 receptor of animal innate immunity also recognizes an epitope of flagellin that faces the inside of the intact flagellum (Smith et al., 2003), and other PAMPs stimulating the innate immune response in animals include cytoplasmic components such as the heat shock protein HSP60 and bacterial DNA (Takeda and Akira, 2003). Although phagocytic cells appear to play an important role, the process leading to release of these non-accessible PAMPs from the bacteria is not fully understood. The release of PAMPs in plants could be based on bacterial export systems activated in the course of the infection process, or it could result from plant processes causing a leakiness of the infecting bacteria. Recently, we observed that *Arabidopsis* plants mutated in the flagellin receptor gene *FLS2* show enhanced susceptibility to infection by *Pseudomonas syringae* pv *tomato* (Zipfel et al., 2004). This provides a functional proof for such a release mechanism at least for the flagellin elicitor. In the initial experiments of this work, at least part of the EF-Tu-related elicitor activity was detectable in the cell-free supernatant of *E.coli* cells (Fig. 1). A transfer of this cytoplasmic protein to the periplasm has previously been observed in *E. coli* cells after osmotic downshock or growth in media containing low amounts of carbohydrates, nitrogen and phosphate (Berrier et al., 2000). Similar conditions of low osmolarity and low nutrient content might prevail for bacteria invading the apoplast of plants (Hancock and Huisman, 1981). Recently, EF-Tu was located at the surface of *Mycoplasma pneumoniae* where it contributes to the binding of these bacteria to host surfaces (Dallo et al., 2002). Similarly, EF-Tu was found to localize to the surface of Lactobacillus *johnsonii* where it appears to mediate the attachment of these probiotic bacteria to human intestinal cells (Granato et al., 2004). Most

interestingly, in this very recent report EF-Tu was also observed to act as a stimulator of a pro-inflammatory response in the presence of soluble CD14. This opens the possibility that EF-Tu, similar to flagellin, might act as a PAMP for the innate immune system of both, animals and plants. It will be interesting to test whether animals have a perception system specific for the N-terminus of EF-Tu as well or whether they recognize another part of this bacterial 'hall-mark' protein.

Treatment of plants with crude bacterial extracts induces defense responses and leads to induced resistance (Jakobek et al., 1993; Zipfel et al., 2004). While bacterial flagellin might be the inducing-factor prevailing in many of these bacterial preparations this induction occurs also in the absence of elicitor-active flagellin (Pfund et al., 2004), and it also occurs in plant-hosts lacking functional flagellin perception (Zipfel et al., 2004). The results presented in this work identify EF-Tu as such a novel factor capable of triggering innate immune responses and induced resistance in *Arabidopsis* plants.

Material and Methods

Peptides were synthesized by F. Fischer (Friedrich Miescher-Institute, Basel, Switzerland) or obtained from Peptron (Daejeon, South-Korea). Peptides were dissolved in H_2O (stock solutions of 1 to 10 mM) and diluted in a solution containing 1 mg/ml BSA and 0.1 M NaCl. Pronase (Calbiochem) and sequencing grade trypsin, endoprotease Arg-C, endoprotease Asp-N, endoprotease Lys-C and endoprotease Glu-C (Roche) were used as recommended by the suppliers.

Bacteria and preparation of bacterial extracts

E.coli GI826 was obtained from Invitrogen and grown in LB Medium at 37°C on a rotary shaker. *Agrobacterium tumefaciens* (strain C58 T), *Sinorhizobium meliloti* and Ralstonia *solanacearum* (from DSM GmbH, Braunschweig, BRD) were grown in King's B broth at 26°C on a rotary shaker. Bacteria were harvested by centrifugation, washed and resuspended in H_2O (~20-30 % cells (fresh weight) / volume). Crude bacterial extracts were prepared by boiling the bacterial suspensions for 5 to 10 min or, in the case of *A. tumefaciens,* by 3 cycles of freezing and thawing and subsequent incubation in lysis buffer (50 mM Tris-HCl pH 8, 0.2 mg/ml lysozyme) for 1 h at 37°C, and removing of bacterial debris by centrifugation.
For elicitor purification from *E. coli* GI826, the extract obtained after lysis of bacteria with lysozyme was treated with DNAse (100 units/ml, RQ1 Promega) for 1 h at 37°C. Proteins were precipitated with 80% acetone, resolubilized in 20 mM Tris-HCl (pH 7.5) and fractionated over a MonoQ anion-exchange column (Pharmacia Biotech) equilibrated with the same buffer. Fractions with elicitor activity were pooled and separated by SDS-PAGE. The gel was stained with Coomassie brilliant blue, dried, and cut into 2 mm segments. These slices were placed in 0.1 ml H_2O containing 0.1% SDS and pH was adjusted to ~6 with NaOH. After incubation for 1 h at 70°C and 16 h at 37°C supernatants were assayed for alkalinization-inducing activity. Eluates containing activity were treated with trypsin and analyzed for peptide masses by MALDI-TOF (TofSpec 2E, Micromass, Manchester UK).

Cleavage of EF-Tu with CNBr and identification of the active peptide

Purified EF-Tu (0.5 mg) was suspended in 70% formic acid and treated with CNBr (~20 mg/ml) for 48 h at room temperature. The resulting peptides were separated by reversed-phase chromatography on a C8 column (Vydac, 1x250mm, 5µm) at pH 3.5 (0.05 % TFA in H_2O as solvent A and 80 % acetonitrile / 20 % H_2O with 0.05 % TFA as solvent B). The eluate was split for assaying elicitor activity and for ion-spray mass spectrometry (API 300, PE Sciex, Toronto, Canada) using 5500V for ionization and analysis in single quadrupole mode. The peptide masses were calculated using BioSpec Reconstruct. Peptides further digested with trypsin were analysed by MALDI-TOF on a TofSpec 2E (Micromass, Manchester, UK).

Plant cell cultures and alkalinization response

The *Arabidopsis* cell culture (May and Leaver, 1993) was maintained and used for experiments 4-8 days after subculture as described before (Felix et al., 1999). To measure the alkalinization response, 3 ml aliquots of the cell suspensions were placed in open 20 ml vials on a rotary shaker at 150 cycles per min. Using small combined glass electrodes the extracellular pH was either recorded continuously with a pen recorder or measured after 20 to 30 min of treatment, as indicated.

Oxidative burst and ethylene biosynthesis in plant leaves

Fully expanded leaves of 3-6 week old *Arabidopsis* plants grown in the greenhouse were cut into 2 mm slices and floated on H_2O overnight. The release of active oxygen species was measured by a luminol-dependent assay (Keppler et al., 1989). Briefly, slices were transferred to assay tubes (two slices, ~10 mg fresh weight) containing 0.1 ml of H_2O supplied with 20 µM luminol and 1 µg horseradish peroxidase (Fluka). Luminescence was measured in a luminometer (LKB 1250 Wallac, Turku, Finland or TD-20/20, Turner Designs, Sunnyvale, US) for 30 min after addition of elicitor. For assaying ethylene production, leaf slices (~20 mg fresh weight per assay) were transferred to 6 ml glass tubes containing 1 ml H_2O and the elicitor preparation to be tested. The tubes were closed with rubber septa and ethylene accumulating in the free air space was measured by gas chromatography after 2 h incubation.

Induction of GUS activity in Arabidopsis *lines transgenic for* SIRK*p::*GUS

Arabidopsis thaliana Ws-0 and Col-0 plants were transformed with a *SIRK*p::*GUS* construct (Robatzek and Somssich, 2002) using kanamycin resistance as selection marker and *A. tumefaciens*-mediated gene transfer. Fully expanded leaves of the T_3 generation were pressure infiltrated (needle-less syringes) with 1µM peptide solutions, crude bacterial extracts (diluted 1:100) or 10 mM $MgCl_2$ as control. One day later injected leaves were detached and stained for GUS activity wih X-GLUC (5-Bromo-4-chloro-3-indolyl-β-D-glucoronide cyclohexylammonium).

Infection of Arabidopsis *leaves with* Pseudomonas syringae *pv.* tomato

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) was grown at 28°C on King's B plates with 50 mg/l rifampicin. Bacteria were resuspended at $1x10^5$ cfu/ml H_2O, and injected into leaves using a syringe without needle as described before (Zipfel et al., 2004). To count bacteria present in leaves, discs from two different leaves were ground in 10 mM $MgCl_2$ with a glass pestle, thoroughly mixed, serially diluted and plated on NYGA solid medium containing 50 mg/l rifampicin.

Accession numbers

The accession number for EF-Tu protein from E. coli (Laursen et al., 1981) is P02990 (Swissprot); the protein structure of whole, unmodified, Ef-Tu (Song et al., 1999) can be found at **MMDB:** 9879 **PDB:** 1EFC .

Acknowledgements

This work was initiated by G.K., G.F. and T.B. at the Friedrich Miescher-Institute with the support of the Novartis Research Foundation, and completed at the Botanical Institute with the support of the Swiss National Science Foundation. We thank Franz Fischer for the synthesis of various peptides, Daniel Hess and Ragna Sack for mass spectrometrical analysis, Martin Regenass (Friedrich Miescher Institute, Basel) for maintaining the cell cultures, Linda Spremulli (University of North Carolina), Kirill Gromadski and Marina V. Rodnina (University of Witten/Herdecke) for the generous gifts of purified EF-Tu.

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2 Manuscript in preparation:

The two bacterial PAMPs flagellin and EF-Tu are sensed by two separate receptors, but induce the same set of defense responses in *Arabidopsis*

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Running Title: Similarities and differences in *A. thaliana* EF-Tu and Flagellin PAMP perception

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The abbreviations used are: EF-Tu, elongation factor Tu; IC_{50}/EC_{50}, concentration required to induce a half-maximal binding competition / alkalinization response; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; LRR, leucine rich repeat; CSP, cold shock protein; elfNN, peptide representing the acetylated NN amino acid residues of the N-terminus of EF-Tu; elf-^{125}I, elf26-^{125}I-Tyr-Cys; flg22, peptide representing the conserved domain of flagellin sufficient to induce plant defense responses; ^{125}I-flg, ^{125}I-Tyr-flg22; MAPK, mitogen activated protein kinase; TLR, Toll-like receptor; PRR, Pattern recognition receptor; PAMP, pathogen associated molecular pattern.

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2.1 Abstract

Plants are able to detect microbial invasions by sensing pathogen-associated molecular pattern (PAMPs). In previous work we identified defined epitopes of flagellin and EF-Tu as two bacterial PAMPs that activate defense responses in *Arabidopsis*. Whereas the receptor kinase FLS2 has been identified as the pattern recognition receptors (PRR) for flagellin, the receptor site for EF-Tu remains unknown. Here, we characterize a specific, high-affinity binding site that shows all the characteristics expected for a receptor site for EF-Tu. While perceived by two distinct receptors, flagellin and EF-Tu trigger a common MAP kinase based signaling pathway, and both PAMPs induce a nearly congruent set of ~1000 genes within 30-60 min of treatment. The *FLS2* gene itself is induced by both PAMPs and the receptor binding sites for flagellin increase after treatment with EF-Tu while binding sites for EF-Tu accumulate after treatment with flagellin. Co-stimulation with saturating concentrations of both PAMPs does not result in clear additive or synergistic stimulation. Together, these data suggest that perception of different PAMPs operates with parallel chemosensory detection systems to enhance sensitivity of pathogen detection and trigger a common defense response.

2.2 Introduction

To detect attacking microbial pathogens, plants seem to have neither sensory 'organs' nor specialized sensing cells that can circulate throughout the plant tissues. Thus, detection of the pathogens or the injury caused by them must occur locally in a cell autonomous manner, either in some or all of the cells in a given tissue. Plants have an array of perception systems that base on the products of resistance (R) genes which, whether direct or indirect, sense the products of corresponding Avr genes from the pathogens. Besides these patterns specific for particular races of pathogens, plants also sense presence of microbes with perception systems for molecular patterns characteristic for whole groups or classes of microorganisms. Perception of these "general elicitors" or pathogen-associated molecular patterns (PAMPs) (Medzhitov, R. and Janeway, C. A. Jr. 2002) shows striking homology to activators of the innate immune response in animals (Jones, D. A. and Takemoto, D. 2004, Nürnberger, T. et al. 2004). PAMPs for which plants have been reported to have a perception system include structures characteristic for oomycetes like the ß-glucan from the cell wall, the pep13 epitope conserved in cell wall transglutaminase (Brunner, F. et al. 2002) and secreted elicitin-proteins (Huitema, E. et al. 2005) (Jones, D. A. and Takemoto, D. 2004), and structures signaling presence of true fungi like the cell wall components chitin and glucan, and the fungal sterol ergosterol (Jones, D. A. and Takemoto, D. 2004). Similarly, plants have been reported to recognize structures characteristic for bacteria like lipopolysachharides (Gross, A. et al. 2005, Zeidler, D. et al. 2004) and bacterial cold-shock protein (CSP) (Felix, G. and Boller, T. 2003), flagellin (Felix, G. et al. 1999) and EF-Tu (Kunze, G. et al. 2004). Some of these PAMPs seem to be perceived by only limited number of plant species, whereas others trigger defense responses in wide range of higher plants. Typically, however, any given plant seems to have perception systems for several PAMPs signaling the same class of microorganisms. In our previous work with the bacterial PAMPs flagellin, CSP and EF-Tu this was observed for the perception system for flagellin which is present in many different species while perception of CSP seemed to be restricted to *Solanaceae* and that of EF-Tu to *Brassicaceae*. The domains of these bacterial proteins that get recognized by the pattern recognition systems of the plants could be narrowed to small epitopes comprising single stretches of ~20 amino acid residues. In *Arabidopsis,* the PAMP-activity of flagellin and EF-Tu can be fully mimicked by the synthetic peptide flg22 and peptides representing the acetylated N-

terminus of EF-Tu with at least the first 18 amino acids as in elf18, respectively. Perception of flagellin has been studied in detail and was shown to occur via the receptor FLS2 (Gómez-Gómez, L. and Boller, T. 2000). In contrast, much less is known on perception of EF-Tu and no receptor for this PAMP has been identified so far. Here, we characterized a high-affinity binding site for EF-Tu that shows the qualities expected for a receptor site and studied more thoroughly the responses induced by this perception system. Clearly, perception of EF-Tu occurs at a receptor site distinct from FLS2. However, after the initial steps of perception EF-Tu and flagellin induce the same broad set of defense responses at similar strength and with similar kinetics. Most notably, a nearly congruent set of ~1000 genes shows rapid changes of expression after treatment with both PAMPs. In *Arabidopsis* these two perception systems appear to work in parallel and one can now address the role of their interaction and cooperation for plant defense.

2.3 Results

2.3.1 *Arabidopsis* cells have a high-affinity binding site specific for EF-Tu

In previous experiments we have used suspension cultured *Arabidopsis* cells to establish the structure-activity relationship for various EF-Tu-derived peptides (Kunze, G. et al. 2004). The sensitivity and specificity of these cells for the acetylated N-terminus of the EF-Tu suggested that perception occurs via a receptor site specific for this novel PAMP. In order to probe for this perception site we used an elf26-derivative prolonged at its C-terminus by the amino acid residues Tyr and Cys. This peptide, either iodinated at its single Tyr or not, exhibited the same specific activity as non-modified elf26 when tested for responses in *Arabidopsis* cells (data not shown). Elf26-Tyr-Cys labeled with ^{125}Iodine (elf-^{125}I) was used to follow kinetics of binding to intact cells of *Arabidopsis* (Fig. 1*A*). Binding reached a maximum within the first 25 min and then remained stable for at least 3 h (value shown for 120 min only). Non-specific binding, binding of radiolabel in the presence of a 10 µM excess of non-labeled elf26, stayed low throughout the experiment (Fig. 1A). Adding a 10 µM excess of non-labeled elf26 25 min after addition of elf-^{125}I did not result in detectable displacement of radioligand, indicating essentially non-reversible binding of

radioligand. Since these experiments were performed at 4°C this non-reversibility is probably not due to an uptake process.

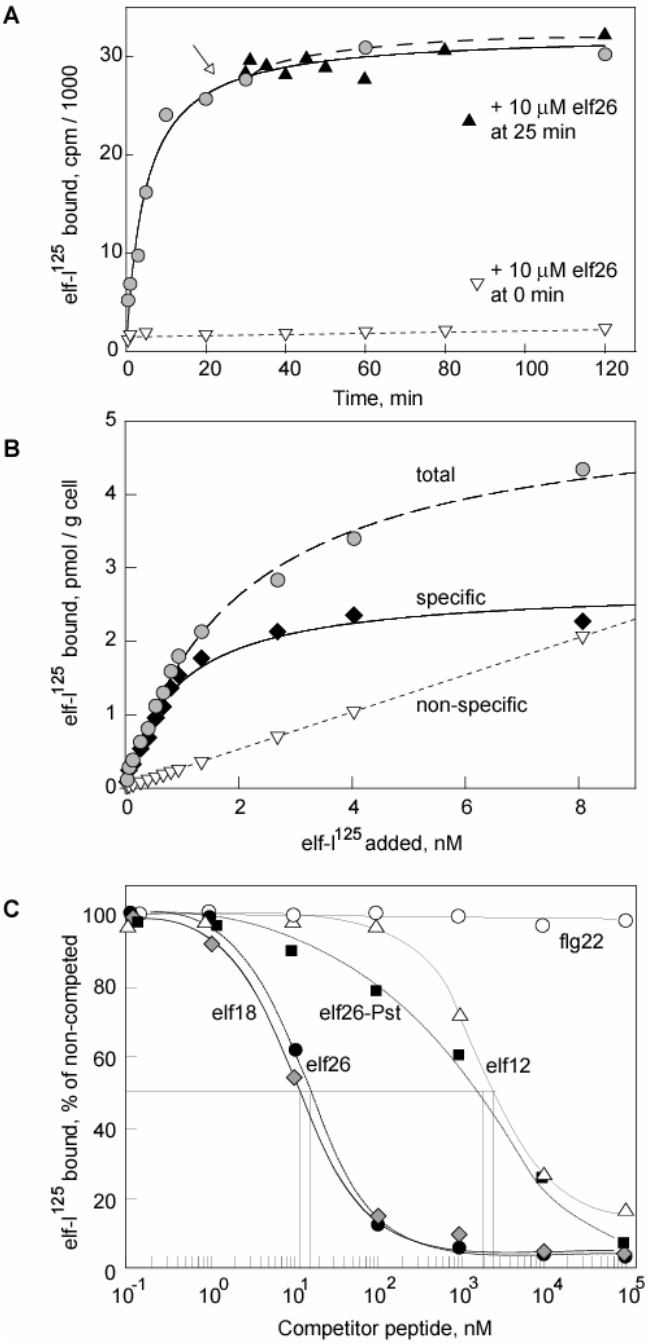

Figure 1: High-affinity binding sites specific for EF-Tu on intact cells of *Arabidopsis*

(A) Binding kinetics of the radiolabeled elf26-derivative elf-^{125}I to intact cells of *Arabidopsis.* Aliquots of 0.1 ml cell suspension (25 mg, fresh weight) were incubated with 0.3 nM elf-^{125}I in the absence (shaded circles) or presence of 10 µM elf26-Tyr-Cys added at *t* = 0 min (open triangles) or at 25 min (solid triangles). Radioactivity retained on the cells was measured by γ-counting after washing the cells with cold binding buffer. Kinetics of binding was reproducible in four independent series of experiments with different batches of cells.

(B) Saturation of Binding

Aliquots of 0.1 ml cell suspension were incubated in with different amounts of elf-^{125}I (specific activity diluted to 200 Ci/mmol with non-radioactive elf26-Tyr-Cys) at 4°C for 25 min in the absence (total binding, shaded circles) or in the presence of 10 µM Tyr-elf26 (non-specific binding, open triangles). Specific binding (closed diamonds) was determined by subtracting non-specific

binding from total binding. The values for specific binding fitted to rectangular hyperbola (solid line) resulted in a B_{max} of 2.1 pmol/g cells and a K_d of 0.8 nM. Values for saturation were reproduced in an independent saturation assay with a different batch of cells.

(C) Specificity of elf-^{125}I binding
Binding assays with elf-^{125}I (0.6 nM) and various concentrations of unlabeled flg22 and elf-derived peptides elf18, elf26, elf26-PsT and elf12. Results were obtained with different batches of Arabidopsis cells and are presented as percentage of specific binding in the absence of competitor. Total binding for these different batches was between 8,000 and 12,000 cpm, and non-specific binding was between 150 and 300 cpm. Competition of binding was tested for all competitors at least twice in independent assays.

The affinity and the number of EF-Tu binding sites on intact cells were determined by saturation curves with increasing concentrations of labeled elf-^{125}I (Fig 1 B). The values for specific binding accurately fitted to a rectangular hyperbola with an apparent K_d of 0.8 nM and B_{max} corresponding to 2.1 pmol of binding sites per g of cells. Since the *Arabidopsis* cells used in these assays contain about 4 x 10^4 cells / mg fresh weight (Bauer, Z. et al. 2001) one can estimate ~3 x 10^4 receptor sites/cell. The specificity of binding was tested in competitive binding assays with different EF-Tu-derived peptides and the structurally unrelated flg22-peptide (Fig 1 C). Most effective competition resulting in 50 % inhibition of radioligand binding at concentrations of ~10 nM (IC_{50} values) was observed for elf26 and elf18. Whereas these two peptides are fully active as agonists, the shorter peptide elf12 exerts an antagonistic effect (Kunze, G. et al. 2004), and this peptide also competed binding, albeit with an IC_{50} of ~3000 nM. The peptide elf26-Pst, representing the N-terminus of EF-Tu of the plant pathogen *Pseudomonas syringae* pv *tomato*, is a weaker agonist than elf18, and is also acts as a less efficient as competitor in binding assays (IC_{50} of ~2000 nM, Fig. 1C).

2.3.2 Affinity crosslinking of elf-^{125}I specifically labels a polypeptide of ~150 kD

Covalent chemical affinity crosslinking of labeled ligands to their binding sites has been successfully used to characterize receptor binding sites for phytosulfokine in carrot and rice (Matsubayashi, Y. et al. 2002), for the wound hormone systemin in *Lycopersicon peruvianum* (Scheer, J. M. and Ryan, C. A. 2002) and for bacterial flagellin in *Arabidopsis* cells (Chinchilla, D 2005). In experiments with intact cells of

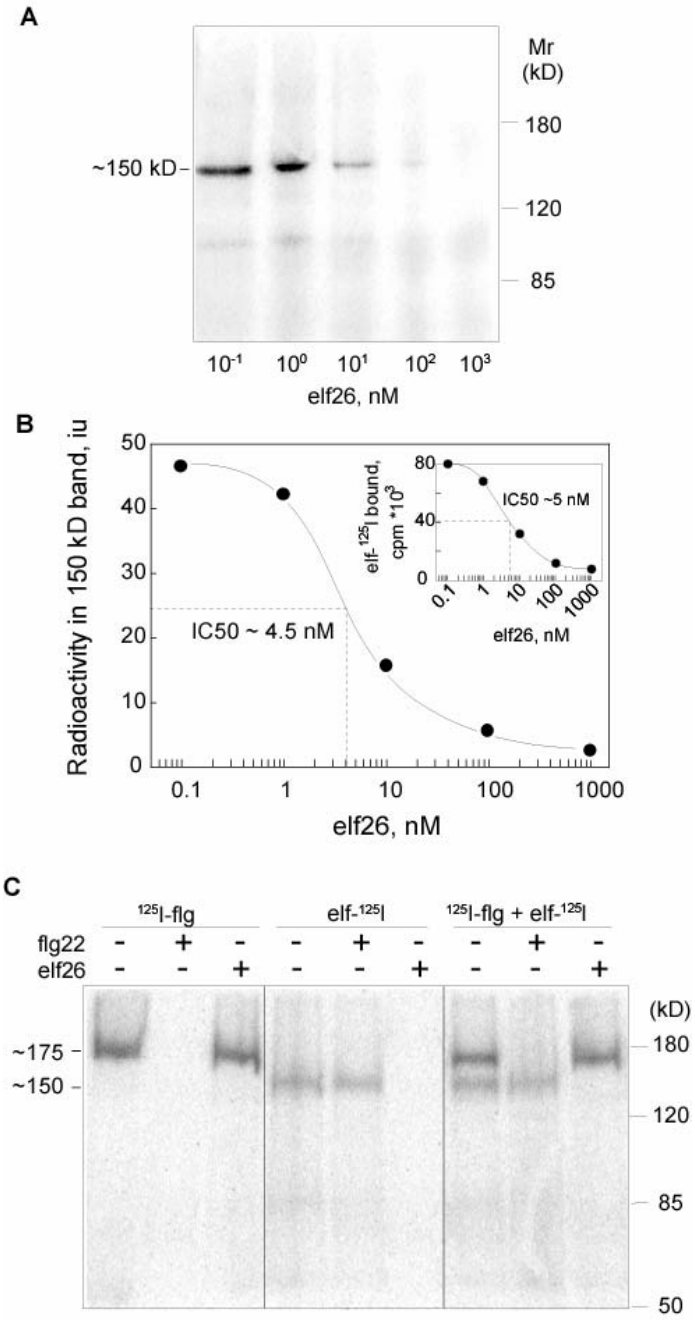

Fig. 2: Chemical crosslinking of elf-^{125}I and ^{125}I-flg to *Arabidopsis* cells.

(A) Aliquots of *Arabidopsis* cells were incubated with elf-^{125}I in presence of different concentrations of unlabeled elf26. After binding, chemical crosslinking was initiated by the addition of EGS. Radiolabeled proteins were analyzed after separation by SDS-PAGE with a Phosphor Imager. Numbers and dashes at right denote positions and molecular masses (Mr) in kilodaltons (kD) of standard proteins. Equal loading with proteins was checked by Coomassie-staining of the gel (not shown).

(B) Quantitative analysis of radioactivity in the band migrating at 150 kD band in (A).
Inset, elf-^{125}I bound to cells after washing in a parallel experiment with the same batch of cells. Dashed lines indicate IC50-value for inhibition of labeling by elf26.

(C) Aliquots of *Arabidopsis* cells were supplied with ^{125}I-flg and/or elf-^{125}I either alone, or with an excess of unlabeled flg22 or elf18 peptide as indicated. Crosslinking was performed in presence of 2.5 mM EGS.

Arabidopsis, we reproducibly observed specific crosslinking of elf-^{125}I to a single polypeptide migrating with an apparent molecular mass of ~150 kD on SDS-PAGE (Fig 2A). Addition of unlabeled elf26 to the assays suppressed labeling of this band in a dose-dependent manner with 50 % reduction (IC_{50}) of labeling at ~4.5 nM (Fig. 2A and 2B). This is in accordance with the IC_{50} value for elf26 in competitive binding assays (Fig1. C, 2B inset). However, in contrast to these binding assays, crosslinking was performed in the presence of bound and unbound ligand, thus demonstrating a high selectivity for the crosslinking of the 150 kD protein with elf26. Labeling of the150 kD protein was dependent on presence of crosslinker (data not shown) and only <1 % of the label bound to the cells was found in the 150 kD protein. This low efficiency might be explained by the tri-molecular chemical reaction needed to link the radioligand to the 150 kD and hydrolytic inactivation of crosslinker occurring under aqueous conditions.

In order to compare the binding sites for EF-Tu with those for flagellin, we performed crosslinking assays with double labeling using elf-^{125}I and ^{125}I-flg on the same cells. Clearly, the 150 kD polypeptide labeled by elf-^{125}I is different from the 175 kD band labeled by ^{125}I-flg (Fig. 2C), which was previously identified as the FLS2 protein (Chinchilla, D 2005). In summary, EF-Tu interacts specifically with a high-affinity binding site on a single ~150 kD polypeptide in *Arabidopsis* cells.

2.3.3 Induction of extracellular alkalinization and MBP kinase activity

Rapid extracellular alkalinization in cultured plant cells has been used to characterize structure-activity relationship for flagellin- or EF-Tu-derived peptides (Felix, G. et al. 1999, Kunze, G. et al. 2004). When directly compared at saturating doses of 100 nM of the peptides (Fig. 3A), flg22 induced alkalinization with a lag of only few seconds while extracellular pH in after treatment with elf18 started to rise after an apparent lag of ~70 s. In this batch of the cell culture flg22 reproducibly induced a slightly higher pH increase than elf18 (Fig.3 A and B), and a combined treatment with flg22 and elf18 resulted in a response that was not significantly different from the treatment with flg22 alone. In other batches of the *Arabidopsis* cell culture the relative strength of the two peptides can occur reversed and elf18 caused a somewhat larger pH increase than flg22 (data not shown). However, the lag phase for EF-Tu-derived peptides was always longer than that for flg22, and concomitant treatment with both

peptides never led to significant increase above the response obtained with the stronger of the stimuli alone (data not shown). An additive effect of the two peptides was observed only at very low, non-saturating doses of the elicitors Fig. 3B).

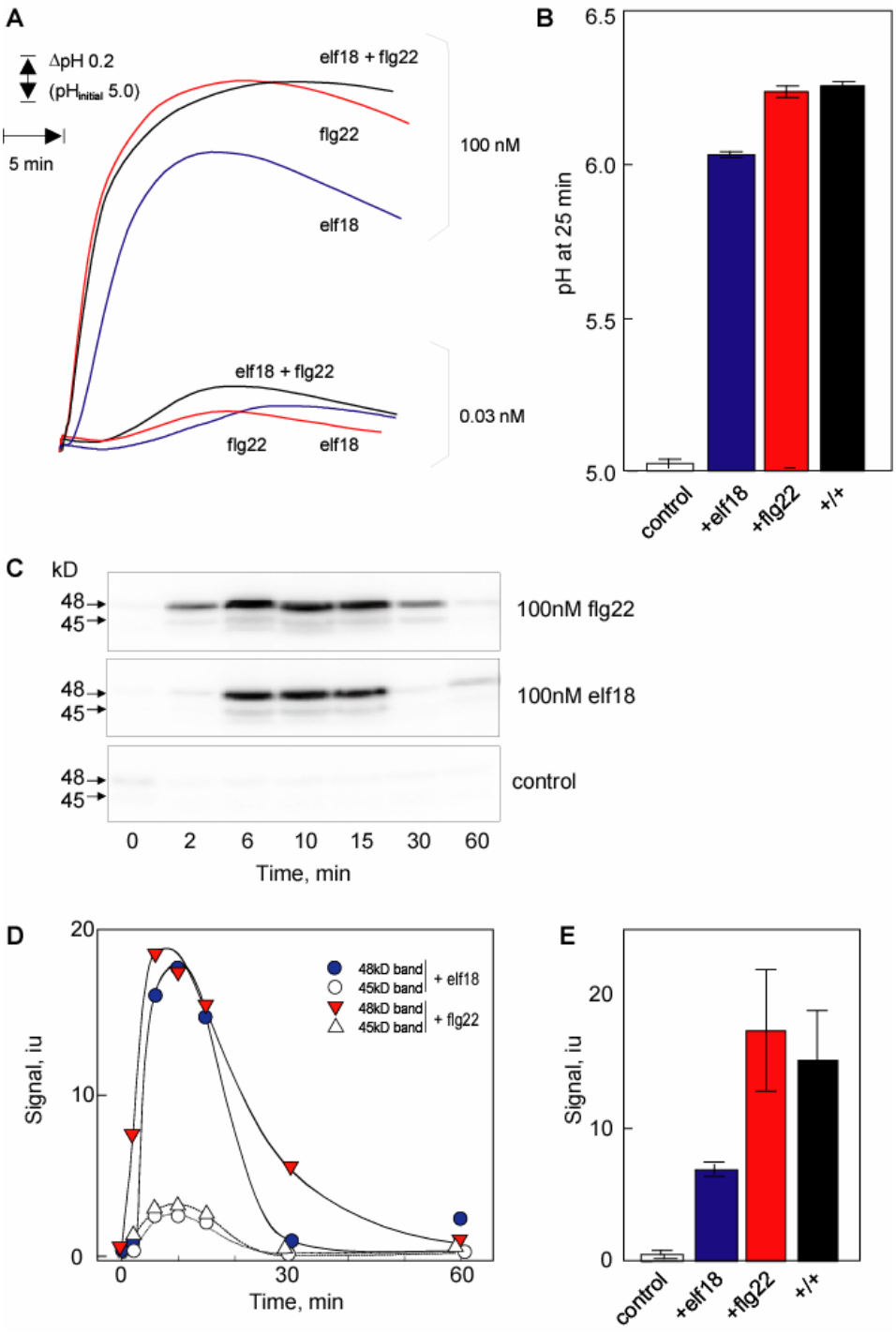

Fig. 3: Induction of medium alkalinization and MBP kinase activities after stimulation with EF-Tu and flagellin

(A) Extracellular pH in cells treated with 0.03 nM or 100 nM of flg22 or elf18 as indicated.

(B) Extracellular pH in cells treated for 25 min with 100 nM of flg22, 100 nM elf18 or both peptides as indicated. Bars and error bars indicate means and standard deviations of 4 replicates.

(C) MBP kinase activity in cells treated with 100 nM flg22, 100 nM elf18, or without peptide as a control. Arrows mark bands with kinase activity migrating at 48 kD and 45 kD. None of the proteins migrating with >50 kD or <40 kD on the SDS-PAGE showed labeling and these parts of the gels are not shown.
(D) Quantitative analysis of MBP kinase activity in (C). Values show radioactivity in polypeptides at 48k Da and 45 kD, as indicated.

(E) Quantitative analysis of MBP kinase activity after 10 min treatment with 100 nM 100 nM, 100 nM flg22 or a combination of both peptides. Bars and error bars indicate means and standard deviations of the integrated values for thet 48 kD band from 3 replicate treatments. Gels were loaded with equal amounts of protein. Loading gels with double amounts of extracts doubled incorporation of label, indicating linearity of the assay (data not shown)

Rapid activation of MBP kinases has been reported as early signaling events in plants treated with several pathogen- and wound-related stress signals like harpin, elicitin, systemin or flg22 (Asai, T. et al. 2002, Droillard, M. et al. 2000, Lee, J. et al. 2001, Meindl, T. et al. 1998, Nühse, T. S. et al. 2000, Romeis, T. et al. 1999). Similarly, treatment with 100 nM elf18 led to a strong, transient activation in two MBP kinases migrating with apparent molecular masses of ~48 kD and ~45 kD on SDS-PAGE (Fig. 3C and D). The same two kinases were likewise induced in cells treated with flg22 (Fig. 3C). As observed above for medium alkalinization, the overall kinetics of induction appear to be similar for both stimuli, but induction by flg22 was slightly faster than by elf18. The effect of a combined treatment with both peptides on MBP kinase activity was tested by treatment of cells for 10 min (Fig. 3E). At this time point, close to the maximal activation (Fig 3C) observed after treatment with both peptides, there was no additive effect detectable for co-treatment with both PAMPs, suggesting that the two stimuli activate MBP kinases belonging physically to the same pool within the cells.

2.3.4 Changes in the transcriptome of *Arabidopsis* seedlings after treatment with flagellin or EF-Tu

The whole genome array ATH1 (Affymetrix) is a powerful tool to analyze changes in gene expression of the >22000 genes of *Arabidopsis* in a single experimental setup and with a high reproducibility (Hennig, L. et al. 2003, Redman, Julia C. et al. 2004). Previously, we have described changes in mRNA levels of ~1000 genes in response to treatment of *Arabidopsis* seedlings with flg22 for 30 min (Zipfel, C. et al. 2004). Using the same experimental conditions fro growth and treatment, *Arabidopsis* seedlings were treated with EF-Tu-derived peptides for 30 or 60 min. In untreated

control seedlings, nearly 13'000 of the 23'000 genes showed significant signal (signal value >100) and these genes were analyzed for changes induced by treatment with 1 µM elf26 for 30 min and 60 min (Fig. 4A). Compared to untreated controls and applying a threshold-filter of 2-fold, treatment with 1 µM elf26 caused 427 genes to be up-regulated after 30 min and this number further increased to 866 after 60 min, respectively (Fig. 4A and Supplementary Table 1). All but 7 of the genes with increased mRNA levels after 30 min were still induced after 60 min. A >2-fold decrease of mRNA levels was observed for 7 genes after 30 min and 83 genes after 60 min, respectively. In further series of experiments with seedlings of *fls2-17*, carrying a mutation in the flagellin receptor gene *FLS2*, treatment with 1 µM of the minimal peptide elf18 caused changes in the same set of genes (Supplementary Table 1). In contrast, treatment with elf12, elicitor-inactive in all other bioassays, stimulated >2-fold accumulation in only 49 genes after 60 min. Higher levels for some of them probably reflects statistical noise but 30 of these genes were found induced in parallel by elf18 in the *fls2-17* seedlings. However, these genes form a small group that is induced by elf18 in *fls2-17* but not by elf26 in wildtype seedlings. Thus, rather than stimulation by an EF-Tu-related mechanism, this indicates induction by an unknown stimulus or disturbance during treatment of the *fls2-17* seedlings with elf12 and elf18.

The set of genes induced by elf26 or elf18 showed a striking overlap with the set induced by 30 min of treatment with flg22 described previously (Zipfel, C. et al. 2004) (Fig. 4B). In particular, 624 out of the 648 genes induced >2-fold by flg22 were also induced after 30 and/or 60 min treatment with EF-Tu. Most of the 24 genes induced only in the experiment with flg22 ranked close to the 2-fold threshold value. Conversely, the list combining the 30 min and 60 min treatments of EF-Tu-induced genes is somewhat bigger than the one for flagellin but this difference can be accounted for by difference in time of induction. In summary, no clear evidence was obtained for genes induced in a flagellin-specific or EF-Tu specific manner (Chapter 3).

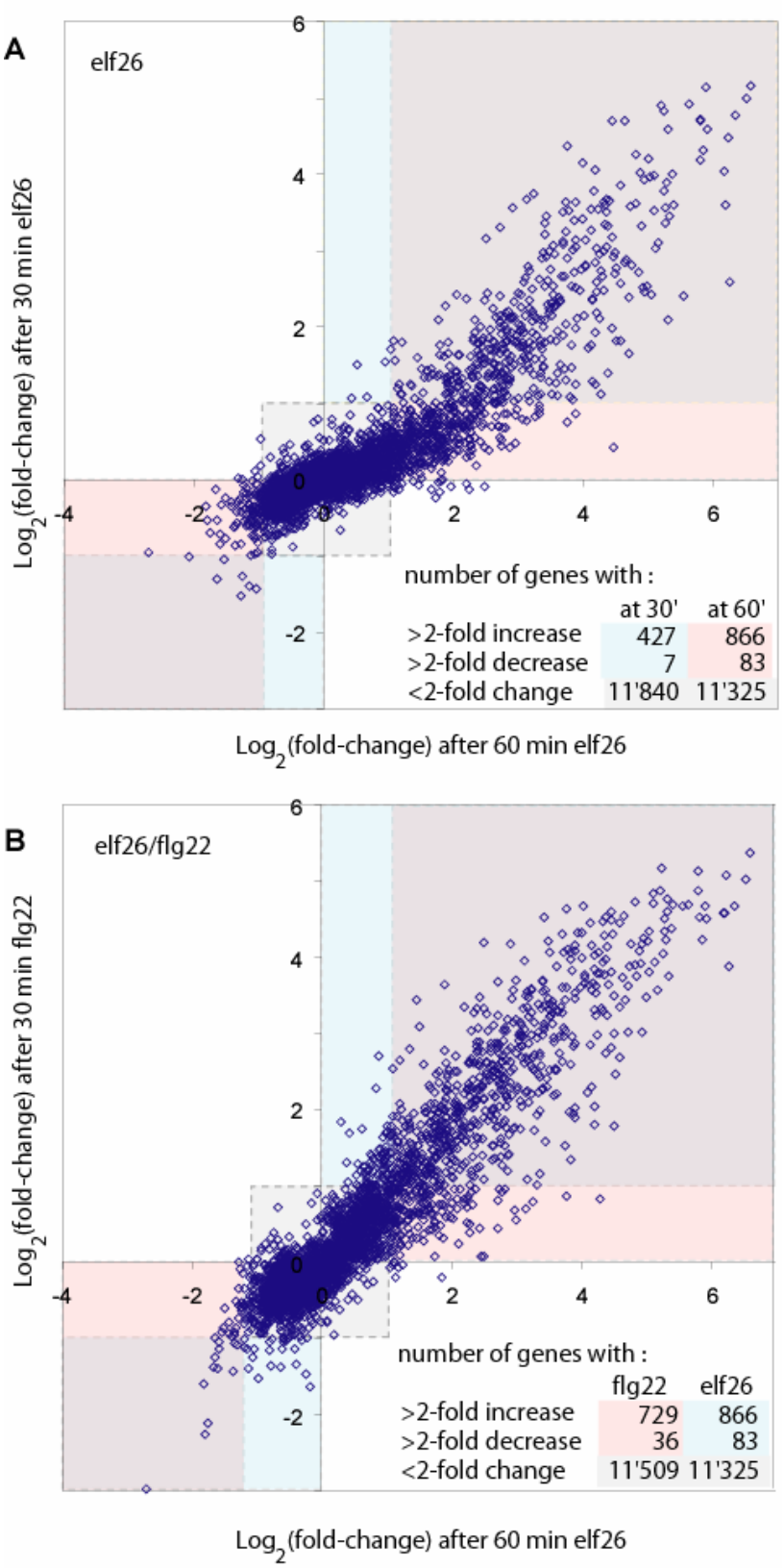

Fig. 4 Summary of experiments based on Affymetrix ATH1 Gene array

(A) Fold-induction (log base 2) over control after 60 min (x-axis) versus 30 min (y-axis) calculated for the 12'000 genes with significant expression in control seedlings. Note: the vast majority (>90%) of values show changes below 2-fold and group the origin of the axis (shaded in grey).

(B) Comparison of changes after treatment with elf26 for 60 min (x-axis) with those previously described for flg22 after 30 min (y-axis, values from (Zipfel, C. et al. 2004)).

2.3.5 EF-Tu and flagellin inhibit growth of *Arabidopsis* seedlings in a qualitative different way

Treatment of young *Arabidopsis* with flg22 has an inhibitory effect on seedling growth (Gómez-Gómez, L. and Boller, T. 2000). Similarly, elicitor-active EF-Tu derived peptides cause strong inhibition of seedling growth. In comparison to flg22, elf18 induced a stronger reduction of shoot growth but had a somewhat weaker effect on root development (Fig. 5A). Shoots of seedlings treated for 10 d with 1 µM elf18, either alone or in combination with 1 µM flg22, showed strong curling and necrotic browning (Fig. 5A, close up). Shoots of seedlings treated with flg22 alone showed less growth inhibition and stayed green. In dose-response experiments half-maximal growth inhibition was observed at ~6 nM with elf18 and at ~20 nM with flg22, respectively (Fig. 5B). In relation to the weight at the beginning of the experiment, elf18 caused a nearly complete stop in fresh weight increase. Interestingly, while strongly affecting branching of the root system, elf18 had only little effect on the elongation of the primary root (Fig. 5A and Fig. 5B, right panel). Treatment with flg22 had a smaller effect on overall fresh weight increase but caused a stronger effect on root elongation. Seedlings treated with a combination of the peptides under saturating conditions showed combined effects of elf18 and flg22 with browning of shoots and strongly reduced root elongation (Fig. 5A and 5C). When analysed for binding sites, the shoot parts of the seedlings were found to have 2-3 pmol sites / g tissue for flagellin and EF-Tu while the root parts had a similar number of sites for flagellin but only <0.2 pmol / g tissue for EF-Tu.

As far as tested, the structural requirements of EF-Tu derived elicitors for induction of growth inhibition paralleled the ones observed for induction of medium alkalinization. In particular, growth inhibition could also be observed with intact, purified EF-Tu protein but never with the truncated peptide elf12. Growth inhibition was observed with all accessions of *Arabidopsis* tested, as well as in *fls* mutants which are insensitive to treatment with flg22 (data not shown).

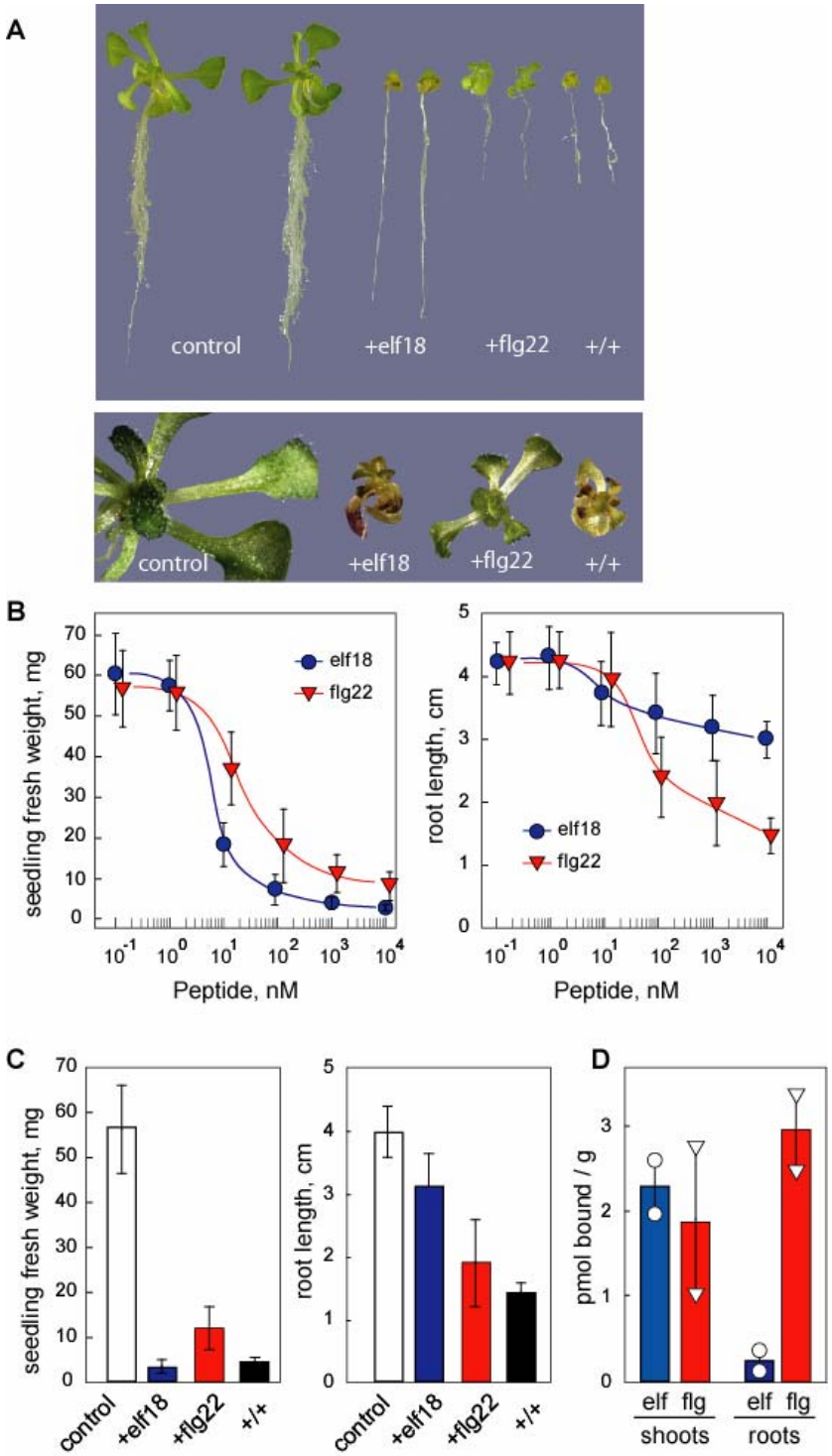

Fig. 5 Effects of flg22 and elf18 on seedling development

(A) Col-0 seedlings incubated for 10 d in the absence of peptide (control) or in the presence of 1µM flg22, 1µM elf18 or a combination of the two peptides (+/+). Photos show intact seedlings (upper panel) and close up of the shoot parts (lower panel) for representative examples after the different treatments. Seedlings at the beginning of the treatment had a fresh weight of 5 mg.

(B) Dose-response relationship for the effects of elf18 and flg22 on fresh weight (left panel) and root length (right panel). Values represent mean and standard deviation of n=12 seedlings.

(C) Fresh weight of plantlets (including roots, left panel) and root length (right panel) after 10 d of treatment with 1 µM of the peptides indicated. Values represent mean and standard deviation of n=12 seedlings.

(D) Specific binding of elf-^{125}I and ^{125}I-flg in crude extracts of shoots and roots. Symbols and bars indicate values and means of 2 replicate measurements.

2.3.6 Effects of combined treatments with EF-Tu and flagellin

In cultured *Arabidopsis* cells EF-Tu and flagellin stimulate the same type of responses with about the same strength, as demonstrated above for the alkalinization response or the triggering of the MAP kinase activity (Fig. 3A and 3C). As expected for independent stimuli, combined treatments with elf18 and flg22 at low, non-saturating concentrations resulted in approximately additive effects (data not shown). However, treatment with saturating doses showed no clear additive effect indicating that maximal response can be triggered by a single stimulus alone. Pre-treatment of *Arabidopsis* leaves with either EF-Tu peptide or flagellin has been found to restrict growth of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Kunze, G. et al. 2004). Since both peptides did not show direct inhibitory or toxic effects on bacterial growth this was interpreted as a consequence of induced resistance in the host tissue. In order to test for an additive effect of flagellin and EF-Tu *Arabidopsis* leaves were pressure infiltrated with solutions containing 100 nM of either one or both peptides (Fig. 6). In leaves pretreated for one day, growth restriction with a combined treatment with both peptides was not more efficient than pretreatment with one of the peptides alone (Fig. 6). Prolonging the time of pretreatment to two days abolished induction of resistance in all of the treatments, indicating that induction of resistance is a transient process. In contrast, addition of the peptides concomitantly with the bacteria induced resistance and restricted bacterial growth by >10-fold. The slightly stronger effect visible in the example shown in Fig. 6 was statistically not significant and could not be reproducibly detected in repetitions of this experiment. Addition of flg22 20 h after inoculation with bacteria had no significant effect while the peptide elf18 still caused a significant decrease of living bacteria extractable from the leaves 4 days after inoculation (Fig 6).

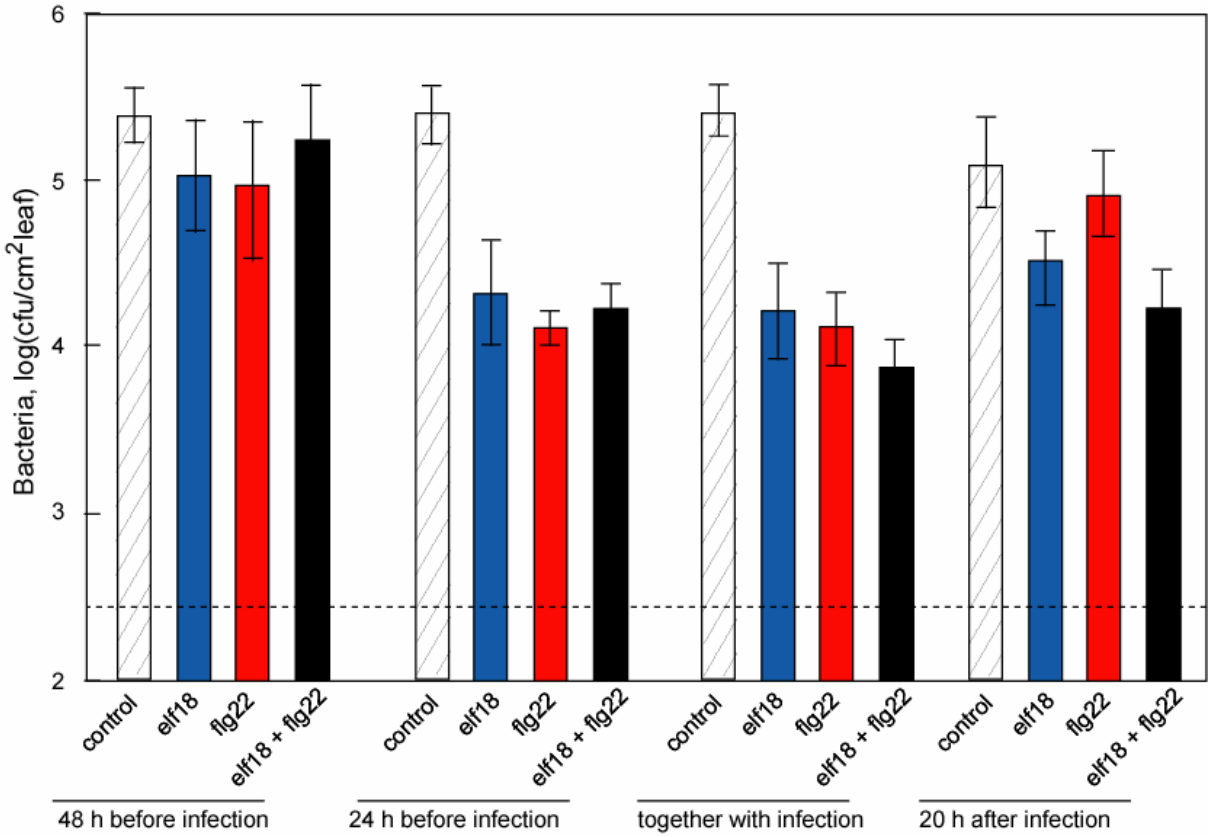

Fig. 6 EF-Tu and flagellin limits growth of *Pseudomonas syringae* pv *tomato* (Pst) DC3000 in *Arabidopsis*

Leaves of *Arabidopsis* plants, 5 weeks old, were pressure infiltrated with 100 nM flg22, 100 nM elf26, or with both peptides either before (48 h or 24 h), concomitantly or after (20 h) with the Pst DC3000 bacteria (10^5 cfu/ml) as indicated. Bacterial growth was assessed 4 days post-infection (4 dpi). Results show average and standard error of values obtained from three plants with two leaves analyzed each (n=6). The dashed horizontal line indicates cfu extractable from leaves immediately after inoculation (0 dpi).

2.3.7 Induction of receptor sites for EF-Tu and flagellin

Among the genes that are rapidly induced in *Arabidopsis* seedlings after treatment with flagellin or EF-Tu are many that encode elements thought to be involved in perception and signal transmission of these external stimuli (Zipfel, C. et al. 2004). In particular, induction occurs on a high percentage of the 610 genes encoding RLKs in *Arabidopsis* (Shiu, S. H. et al. 2004). In the Affymetrix experiments described above, 262 of the 610 RLKs showed significant expression levels and between 16 % (30 min stimulation with elf26) and 40 % (60 min stimulation with elf18) of these were induced

by EF-Tu and flagellin, respectively (Supplementary Table 2, Chapter 3). The flagellin receptor FLS2 is one of the RLKs found induced after treatment with flagellin and EF-Tu. When tested for number of flagellin binding sites present in seedlings after treatment with elf18, binding activity significantly increased within 1h and reached a maximum of >2-fold higher binding within 4 to 6 h of treatment (Fig. 7A). Using a reciprocal approach, pretreatment with flg22 and measuring binding activity for EF-Tu, showed an analogous increase in binding sites for elf-^{125}I with similar kinetics and a >2-fold maximal increase after ~7h of pretreatment (Fig. 7B). A similar, ~2-fold increase within 3 to 6 h for both binding sites was observed also in cultured *Arabidopsis* cells (data not shown), thus corroborating the finding that stimulation with one PAMP signal increases the number of its own receptor site but also the number of receptor sites for other PAMPs. The non-reversible interaction of flagellin and EF-Tu with their corresponding binding sites on intact cells impedes accurate determination of flagellin binding sites after flagellin treatment and EF-Tu binding sites after EF-Tu treatment, respectively.

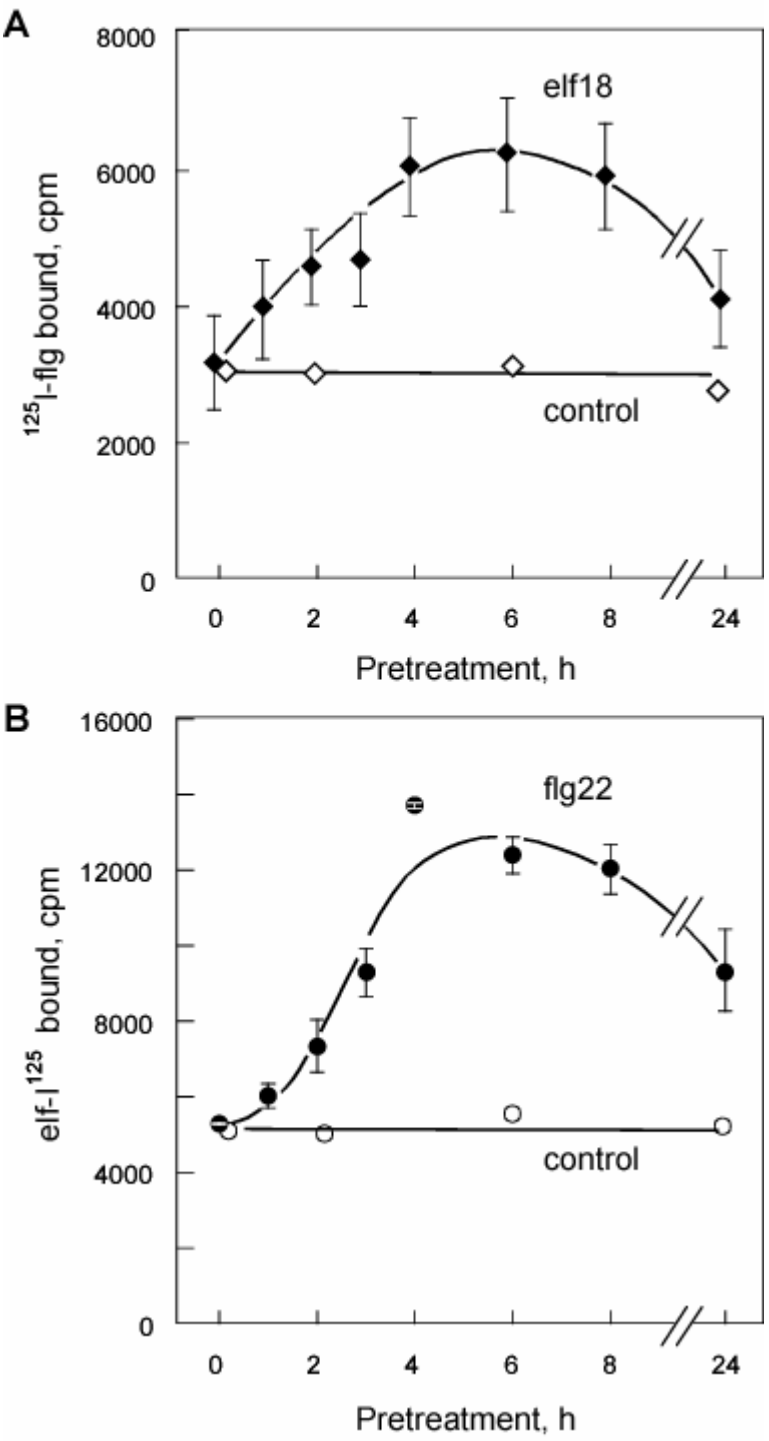

Fig. 7 Number of binding sites in plants treated with flg22 or elf18.

A) Binding activity for ^{125}I-flg in crude plant extracts of Col-0 seedlings pretreated with 1 µM elf18 for different times. Values show specific binding determined by subtraction of non-specific binding from total binding. Non-specific binding, radiolabel bound in the presence of 10 µM non-labeled flg22, was at ~800 +/- 50 cpm in all samples. Symbols and bars represent mean and standard deviation of n=3 replicate plant extracts.

B) elf-^{125}I binding activity in crude plant extracts of seedlings pretreated with 1 µM flg22 for different times. Non-specific binding was at ~3000 cpm in all samples. Symbols and bars represent mean and standard deviation of n=3 replicate plant extracts.

2.4 Discussion

Flagellin and Elongation factor Tu are distinct bacterial proteins with different localization and functions in bacterial cells. Flagellin is the protein monomer that builds up the flagellum, and flagellum-based motility is an important virulence factor for bacterial pathogens (Hatterman, D. R. and Ries, S. M. 1989, Ramos, H. C. et al. 2004, Vande Broek, A. and Vanderleyden, J. 1995). Elongation factor Tu, in contrast, is of fundamental importance for protein translation. As such, it is not surprising that EF-Tu is one of the most conserved proteins and also the most abundant protein in bacteria (Jeppesen, M. G. et al. 2005). The epitopes of these proteins which are recognized by the pattern recognition systems of *Arabidopsis* are restricted to defined regions of ~20 amino acid residues, and the PAMP-activity of these domains can be fully mimicked by the synthetic peptides flg22 and elf18, respectively. Interestingly, both of these PAMP epitopes are not freely exposed to the bacterial cell surface. Flg22 forms a structure called 'spike' that faces the inner core of the flagellum (Yonekura, K. et al. 2003). Similarly, the epitope of flagellin that acts as PAMP in mammals, although different from flg22, also faces the inside of the flagellum (Smith, K. D. et al. 2003). EF-Tu is primarily located in the cytoplasma but recent reports detected this protein also in the secretome (Watt, S. A. et al. 2005) or associated with the bacterial surface (Dallo, S. F. et al. 2002). Recently, EF-Tu has been reported to activate pro-inflammatory responses in human cells (Granato, D. et al. 2004), indicating that EF-Tu might act as PAMP also in the innate immune system of mammals.

Interaction of EF-Tu with its presumptive receptor shares many characteristics known from the interaction of flg22 with its receptor FLS2 (Bauer, Z. et al. 2001, Chinchilla, D 2005). These similarities include a surface exposed binding site with a clear specificity for biological active ligands and with a high-affinity that results in essentially non-reversible binding of ligands. For both perception systems we found truncated peptides that have no activity as agonists but act as specific, competitive antagonists. The existence of these antagonists suggests that receptor-ligand interaction in both cases follows the concept of address-message involving two consecutive steps to activate of the receptor (Bauer, Z. et al. 2001, Kunze, G. et al. 2004). However, these two PAMPs are recognized through distinct, independent perception systems, as evident from work with mutants of *FLS2* and clearly

demonstrated in chemical cross-linking experiments in which the two ligands specifically label two different proteins of high mw (Fig. 2C).

Whereas the receptor sites for these bacterial PAMPs are distinct, they both trigger convergent signaling pathways and common responses. These responses include early events like medium alkalinization in cultured cells or oxidative burst in leaf tissue, but also identical later responses like changes in mRNA levels and induction of resistance. A slight difference of the two perception systems exists with respect to the kinetics of receptor activation. In cultured cells of *Arabidopsis* PAMP-induced responses occur in a synchronized manner and allow resolution of kinetics to few seconds. Using saturating concentrations of the PAMPs, the lag phase for flg22-induced alkalinization and activation of MAP kinase is always somewhat shorter than that for elf18. A further difference is evident for the effect of the two peptides on growth of young *Arabidopsis* seedlings with EF-Tu having a stronger effect on leaf development and flagellin more strongly suppressing root elongation. So far, the biological mechanisms underlying PAMP-induced growth inhibition are not known and differences in peptide-stability or their capacity to penetrate into the seedlings might cause the differences in the growth effect. However, the reciprocal character of the differences observed rather hints at a different distribution of the susceptibility towards the two PAMPs in different tissues. Indeed, using a coarse separation in shoot and root, receptor binding sites for flagellin were detectable in roots and shoots while those for EF-Tu-specific sites were detectable only in shoots.

Although there might be a tissue-specific expression, indicated by the fact that ATH1 transcriptome analysis with flg22 treated root tissues, resulted in an up-regulation of FLS2 (our unpublished data), whereas elf-binding studies on roots could not detect a measurable amount of present binding sites for the unknown elf-receptor (Fig 3E). The perception systems for flagellin and EF-Tu are concurrently present and functional in mature leaf tissue and also cultured cells of *Arabidopsis*. Apart from these two PAMPs, LPS has been reported to act as a PAMP in *Arabidopsis* (Bedini, E. et al. 2005, Zeidler, D. et al. 2004), and one can assume further bacterial structures to be identified as PAMPs in future, considering the amount of potential receptors encoded in the genome of Arabidopsis (The, Arabidopsis Genome, I 2000). The apparent redundancy in the chemosensory system to detect bacteria opens questions on the interplay and functional integration of the individual detection systems. In our experiments with concomitant application of flagellin and EF-Tu we

could observe additive effects with peptides added at low, non-saturating doses. Additive effects have been reported for combined treatment with weakly acting elicitor preparations of LPS and PGN (Nürnberger, T. et al. 2004). In contrast, saturating doses of either PAMP stimulated a nearly full response and no clear additive or synergistic effect was detected. A strongly cooperative or synergistic interaction would resemble the logic of a Boolean [and], with response only in the presence of two or more stimuli. This type of signal integration might increase safety for severe decisions such as turning on programmed cell death or a hypersensitivity response. Such a mechanism of signal integration has been postulated in the “danger signal theory” for induction of immune response (septic shock?) in animals (Matzinger, P. 2002). Signal integration corresponding to a Boolean [or], in contrast, rather increases sensitivity and ensures detection of a broader spectrum of bacteria. In particular, it renders more hurdles for pathogens to hide from the recognition systems of the host plants.

Leucine-Rich Repeat Receptor Kinases are believed to represent the homologue of the animal PRRs in plants (Torii, Keiko U. 2004). Various PRR are involved in the detection of PAMPs in the innate immunity system in mammals and drosophila (Fraser, I. P. et al. 2004, Pasare, C. and Medzhitov, R. 2004), but, the interaction between the microbial ligand and a corresponding receptor has been investigated so far only to a small extent. FLS2, the flagellin receptor in plants, is the only described example of a PRR in Arabidopsis, but this study demonstrate the existence of at least one additional receptor, that is independent, but linked to FLS2. This is indicated by the change of gene expression, by higher binding sites and an increase of the plants sensitivity towards PAMP perception after elf-treatment.

MAPK cascades have been thought to act as key regulators of gene expression during innate immune response (Lee, J. et al. 2004, Pedley, K. F. and Martin, G. B. 2004). According to this hypothesis, factors that induce the same MAP kinase cascades might also induce the same set of genes. Flagellin has been reported to stimulate MAPK6/MAPK3 (Asai, T. et al. 2002, Nühse, T. S. et al. 2000) and the same MAPK appear to be induced by EF-Tu (Fig. 2). The same, stress related MAPKs have been found stimulated also by living compatible and incompatible bacteria, fungal PAMPs like chitin, and wounding (Cardinale, F. et al. 2000, Droillard, M. J. et al. 2004, Pedley, K. F. and Martin, G. B. 2004). Indeed, in cases where gene chip data are available, there seems to be a considerable overlap of genes induced

by these various stress signals (Mussig, C. et al. 2002) and the ones induced by flagellin and EF-Tu. Similarly , studies with living compatible and incompatible bacteria based on the 8k Arabidopsis Gene Array, show reproducible and significant regulation of up to 25 % of the genes present on this Chip (Tao, Y. et al. 2003).
This indicates that the defense system of plants, at least in a first phase, does not discriminate between signals originating from bacteria, fungi and other stress conditions like wounding. Rather, all these signals appear to be interpreted as general signs for 'danger' and induce a general 'stress/defense syndrome' involving a common set of genes. Differences in this 'stress/defense syndrome' can arise from differences in the strength and the duration of the stress perceived but also from additional, superimposed regulation processes. Most interestingly, pathogenic bacteria have evolved mechanisms to suppress plant immunity by secreting effector-proteins with the bacterial type III secretion mechanism (Espinosa, A. and Alfano, J. R. 2004) (Abramovitch, Robert B. and Martin, Gregory B. 2005).

Work with individual, defined PAMPs as described in this report, should provide an experimental basis to study induction and function of the 'stress/defense syndrome', a basis certainly required to integrate knowledge on the many other processes occurring during interaction of living pathogens with their plant hosts.

Material and Methods

Peptides were synthesized by F. Fischer (Friedrich Miescher-Institute, Basel, Switzerland) or obtained from Peptron (Daejeon, South-Korea). EF-Tu- and flagellin-derived peptides were dissolved in H_2O as stock solutions of 1 to 10 mM and, in the case of flagellin-derived peptides, were diluted in a solution containing 1 mg/ml BSA and 0.1 M NaCl. Tyr-flg22 and elf26-Tyr-Cys were labeled with [^{125}I]iodine at their Tyrosine residues to yield ^{125}I-Tyr-flg22 (^{125}I-flg) and elf26-^{125}I-Tyr-Cys (elf-^{125}I) with specific radioactivity of 2000 Ci/mmol by Anawa Trading SA (Wangen, Switzerland).

Plant cell cultures and alkalinization response

The *Arabidopsis* cell culture (May, M. J. and Leaver, C. J. 1993) was maintained and used for experiments 4-8 days after subculture as described before (Bauer, Z. et al. 2001). To measure the extracellular alkalinization, 2 ml aliquots of the cell suspensions were placed in open 20 ml vials on a rotary shaker at 150 cycles per min. Using small combined glass electrodes the extracellular pH was either recorded continuously with a pen recorder or measured after 20 to 30 min of treatment, as indicated.

Binding Assays

Aliquots of cells or plant homogenates were incubated in 0.1 ml binding buffer consisting of 25 mM MES pH 6.0, 10 mM NaCl and 3 mM $MgCl_2$ for assays with ^{125}I-flg (Bauer, Z. et al. 2001) and 25 mM MES pH 6.0, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM KI, 2 mM KCl and 1 mM DTT for assays with elf-^{125}I, respectively. In standard assays, samples were supplied with 30 fmol of radiolabeled peptides (2000 Ci/mmol) and the peptides used as competitors. Assays to determine the number of binding sites were carried out under conditions close to saturation with radiolabeled peptides diluted to a specific activity of 66 Ci/mmol and a total concentration of 10 nM of the ligands. After incubation for the times indicated, unbound radiolabel was washed off as described before (Bauer, Z. et al. 2001) except that for EF-Tu binding paper filters, (Macherey-Nagel NW713 for cells and Whatman 3 mm CHr for crude extracts) were used. The radioactivity retained on the filters was determined by γ-counting.

Chemical crosslinking

Crosslinking experiments were performed according to Chinchilla et al. (2005). Briefly, after binding of radioligands elf- ^{125}I and/or ^{125}I-flg to intact cells as described above, crosslinking was initiated by addition of 10 µl 25 mM EGS (ethylene glycol bis(succinimidylsuccinate) (Pierce) in dimethylsulfoxide directly to the incubation mixture. After further incubation for 30 min at room temperature the reaction was stopped by addition of 2.5 µl 1 M Tris-HCl (pH 7.5). Samples were solubilized in Laemmli buffer (5 min, 95 °C). Proteins were separated by SDS-PAGE on gels containing 7 % (w/v) acrylamide. Gels were fixed, dried and analyzed using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA).

In-gel MBP protein kinase assays

Samples (0.5 ml) of *Arabidopsis* cell cultures were collected at the time points indicated, mixed with an equal volume of 10% trichloroacetic acid, and frozen in liquid nitrogen. After thawing and ultrasonication, pellets were collected by centrifugation (10 min at 12,000*g*) and washed twice with 80% acetone and 20% Tris–MES buffer (20mM; pH 8.0). Proteins were solubilized from pellets with SDS–sample buffer and one fourth of the preparation was separated by SDS-PAGE on gels containing 12% (w/v) acrylamide and 0.2% (w/v) MBP (Sigma). Proteins in gels were renatured and assayed for kinase activity as described (Suzuki, K. and Shinshi, H. 1995). Radioactivity in dried gels was analyzed and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Growth inhibition experiment

Surface sterilized *Arabidopsis* seeds were germinated for 5 days on a medium solidified with 0.8% agar and containing 1 % (w/v) sucrose and the salts of Murashige-Skoog (MS) (Sigma). For growth inhibition assays, seedlings were pre-treated for two days in medium without agar (two seedlings per

well with 200-400 µl of medium in 24-well-plates) and then incubated for 7 to 14 d in the presence of the test substances as indicated.

Affymetrix ATH1 array

Arabidopsis seeds were germinated and pre-treated as described for growth inhibition assays. Seedlings were treated for 30 or 60 min with the peptide indicated. Incubation was stopped by freezing in liquid nitrogen. RNA was extracted with the Quiagen Plant RNA Extraction Kit. RNA was analysed by Affymetrix Microarray procedure and Silicon Genetics Software (Gene Spring Vers.5.1) as described before (Zipfel, C. et al. 2004).

Infection of Arabidopsis leaves with Pseudomonas syringae pv. tomato

Pseudomonas syringae pv. *tomato* DC3000 (Pst DC3000) was grown at 28°C on King's B plates with 50 mg/l rifampicin. Bacteria were re-suspended at $1x10^5$ cfu/ml H_2O, and injected into leaves using a 1 ml syringe without needle as described before (Zipfel, C. et al. 2004). To count bacteria present in leaves, discs from two different leaves were pooled and grounded in 10 mM $MgCl_2$ with a glass pestle, thoroughly mixed, serially diluted and plated on NYGA solid medium containing 50 mg/l rifampicin.

Acknowledgements
We like to thank H. Angliker and E. Oeakley (FMI, Basel) for Affymetrix analysis, V. Mandak for maintaining the cell cultures.

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3 Additional experiments and Results

3.1 Affymetrix gene chip array ATH1

Classification of induced genes after elf26 treatment

To examine effects toward the change in the expression pattern of genes, the use of DNA microarrays has become a powerful and commonly used research tool (Hennig, L. et al. 2003). The whole genome array (ATH1) from Affymetrix offers the possibility to analyze more than 22000 genes for transcriptional differences at a single experimental setup with high reproducibility (Redman, Julia C. et al. 2004).

Experimental Setup:

Arabidopsis seedlings (Ler-0) were grown for 6 days on solid MS-10 Media and afterwards transferred in liquid MS-10 media. After 10 days (in total) the seedlings are treated with peptide elf26 for 30 and 60 minutes. Process was stopped by freezing with liquid nitrogen. RNA was purified according to Quiagen Plant RNAeasy Kit und proceeded according to the FMI Affymetrix facility procedure (done by Herbert Angeliker, FMI). Chips were analyzed with SiliconGenetics Genespring 5.1.

Intensity values used for the analysis are median values derived from Chip replicates (with the exception of elf12-treatments, where no replicates have been performed). For classification of elf-induced genes values and annotations from Raw-Data with a threshold filter of 2-fold were taken. To statistically analyze different independent peptide treatments (e.g. elf and flg22 experiment series in different Arabidopsis ecotypes) a 1-way ANOVA approach in combination with a Tukey-post hoc test (ANOVA Dataset) has been performed.

The results of all experiments are summarized in Table 3.1 (Detailed data, including signal values and fold-changes for >12000 genes, can be found in the supplementary info of Chapter 2 / Appendix)

Table 3.1: Summary for all ATH1 experiments performed in different ecotypes of *Arabidopsis thaliana* (Ler-0, FLS2). Rate of identity based on the 729/649 genes induced by flg22 in wild type (Ler-0, Experiment by C. Zipfel) from Raw Data-set and ANOVA Dataset, respectively.

	Time	30	60	30	60	30	60		30	30
	Ecotype	wt	wt	FLS2	FLS2	FLS2	FLS2		wt	FLS2
	Peptide	Elf26	Elf26	Elf18	Elf18	Elf12	Elf12		Flg22	Flg22
RAW-Dataset	**Up-regulated genes**	**427**	**864**	**672**	**907**	**52**	**49**		**729**	**2**
	Genes present in: flg22 wt treated (30 min) list	420	669	591	598	14	9		729	1
	Representation in % of flg22 induced genes	57.61	91.77	81.07	82.03	1.92	1.23		100%	0.14
	Down-regulated genes	7	83	79	224	18	69		36	1
Anova-Dataset	**Up-regulated genes**	**422**	**736**	**630**	**761**	**45**	**45**		**649**	**2**
	Genes present in: flg22 wt treated (30 min) list	418	619	563	568	14	9		X	1
	Representation in % of flg22 induced genes	64,35	95.38	86.75	87.52	2.16	1.39		100%	0.15
	Down-regulated genes	9	30	51	102	10	10		16	1

3.2 Classification of genes induced by elf26-treatment in Ler-0

Exemplary chosen experimental setup:

EF-Tu (elf26) treatment 30 minutes in wild type (Ler-0)

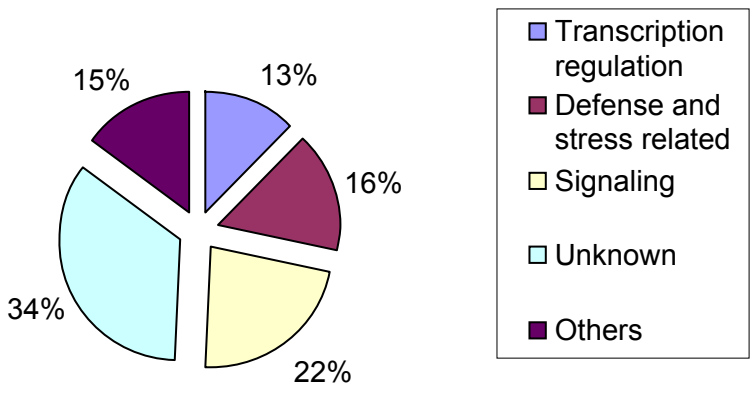

Fig. 3.1: Classification overview for induced genes after elf26 (30 minutes) treatment in wild type Ler-0

The experiment results in a coverage of 51% induced genes, that can be classified to be involved either in transcriptional regulation, defense and stress related, or to act as signaling components. For a high percentage (34%) the functions are unknown and 15% can not directly linked to one of the three major groups used for this classification.

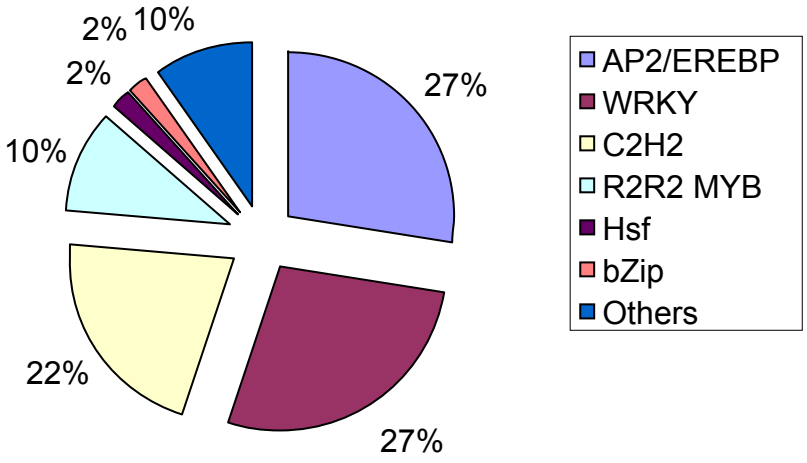

Fig. 3.2: Classification for genes thought to be involved in transcription regulation

Analyzing the 13% of genes that play a role in transcriptional regulation in more detail, one can find high representation of three classes: AP2/EREBP, WRKY and C_2H_2-transcription factors. In this regard the WRKY transcription factors (WRKYs) are an interesting family of plant-specific transcriptional regulators containing more than 70 members. They belong to the zinc-finger-type class and are implicated in the regulation of plant processes during pathogen defense, wound response and senescence (Eulgem, T. et al. 2000, Eulgem, T. 2005). The participation of WRKY-transcription factors in signaling events after PAMP elicitation has been demonstrated previously in parsley cells, treated with Pep25 (Pep25). In this study WRKY1, 2 and 3 have been characterized and a potential role in a signal transduction pathway leading to PR1 gene activation has been proposed (Rushton, P. J. et al. 1996).

38 putitative WRKYs are expressed in at least one single experiments, representing 53% of all family-members. 28% of all known WRKY-transcription factors (55% of all expressed WRKYs) are up-regulated upon treatment with flg22 or elf-peptides (Table 3.2). Among these were previously described ones like AtWRKY6 (Robatzek, S. and Somssich, I. E. 2002) and AtWRKY22. Over-expressed AtWRKY22 and AtWRKY29 have been demonstrated to support enhanced resistance to bacterial and fungal pathogens (Asai, T. et al. 2002). Interestingly AtWRKY29 was not found either to be expressed or induced in all our experiments (compare ((Navarro, L. et al. 2004)). This might be due to different experimental setups that have been used. In particular, for their analysis Asai et. al chose protoplasts as material, and the Affymetrix-data published by Navarro et. al was generated with the Arabidopsis 8k Gene Chip.

EF-Tu treated seedlings react similar in activation of WRKY-transcription factors like induced upon flg22 elicitation, with partly different kinetics (Chapter 2). Four additionally induced WRKY-transcription factors, which are not regulated upon flg22 treatment, could be identified after elf18 (60 minutes) treatment, but only with relatively low fold changes. AtWRKY30 and AtWRKY53 show high fold changes up to 53-fold (elf18 treatment in FLS2 for 60 minutes) underlining the importance and indicating the fast regulation of this transcription factors. The elicitor-inactive elf12 peptides are not capable to activate any WRKY transcription factors.

Table 3.2: 21 out of 74 WRKY-transcription factors are affected by peptide treatment (found by ATH1 analysis) (Fold changes >2 are defined as up-regulated and marked in red, < 0.5 as down-regulated and marked in yellow) (I = induced, NC = No change, D = decrease)

											FOLD-CHANGE							
ecotype		wt	FLS2	wt		FLS2					wt	FLS2	wt		FLS2			
Peptide		flg		elf		elf18		elf12			flg		elf		elf18		elf12	
time, min		30	30	30	60	30	60	30	60		30	30	30	60	30	60	30	60
AT5G24110	AtWRKY30	I	NC	I	I	I	I	NC	NC		34	0.61	22.4	75.2	34.8	53.3	*1.08*	*0.91*
AT4G23810	AtWRKY53	I	D	I	I	I	I	NC	NC		29.5	0.49	29.9	37.0	48.2	19.8	*0.95*	*0.79*
AT1G80840	AtWRKY40	I	NC	I	I	I	I	NC	NC		26.5	0.56	19.2	28.4	23.6	12.5	*1.58*	*0.98*
AT4G01250	AtWRKY22	I	NC	I	I	I	I	NC	NC		19.2	0.87	15.2	30.9	32.1	26.1	*1.2*	*1.69*
AT2G46400	AtWRKY46	I	NC	I	I	I	I	NC	NC		18.6	0.71	7.99	11.2	10.7	5.25	*1.6*	*0.93*
AT2G38470	AtWRKY33	I	NC	I	I	I	I	NC	NC		15.6	0.81	11.3	20.7	12.5	10	*1.24*	*1.1*
AT4G31550	AtWRKY11	I	NC	I	I	I	I	NC	NC		12.3	0.96	6.75	15.8	11.5	10.5	*1.28*	*1.14*
AT4G31800	AtWRKY18	I	NC	I	I	I	NC	NC	NC		8.42	1.11	3.46	5.27	4.36	1.37	*1.59*	*0.82*
AT3G56400	AtWRKY70	I	NC	NC	NC	I	NC	NC	NC		6.53	0.9	1.7	1.83	2.34	0.71	*1.54*	*0.92*
AT2G24570	AtWRKY17	I	NC	I	I	I	I	NC	NC		5.97	0.83	2.45	6.58	5.26	8.94	*1.32*	*1.19*
AT4G18170	AtWRKY28	I	NC	I	I	I	I	NC	NC		5.71	0.97	3.85	12.2	6.14	6.45	*1.27*	*1.17*
AT5G49520	AtWRKY48	I	NC	I	I	I	I	NC	NC		4.88	1.14	2.81	10.5	7.26	16.6	*1.28*	*1.03*
AT2G23320	AtWRKY15	I	NC	I	I	I	I	NC	NC		3.9	0.99	2.01	3.93	2.63	3.24	*1.12*	*1.15*
AT1G62300	AtWRKY6	I	NC	NC	I	I	I	NC	NC		3.51	0.87	1.95	5.32	4.43	12.7	*1.09*	*1.07*
AT2G30250	AtWRKY25	I	NC	NC	I	I	I	NC	NC		2.6	1.23	1.35	3.34	2.17	4.66	*1.32*	*1.24*
AT5G15130	AtWRKY72	I	NC	NC	I	NC	I	NC	NC		2.25	1.21	1.64	2.44	1.84	3.07	*1.51*	*1.06*
AT4G24240	AtWRKY7	NC	NC	NC	NC	NC	I	NC	NC		1.97	1.07	1.15	1.55	1.44	2.56	*1.16*	*1.16*
AT2G40740	AtWRKY55	NC	NC	NC	NC	NC	I	NC	NC		1.02	1.16	0.98	1.61	1.32	2.23	*1.01*	*0.91*
AT4G01720	AtWRKY47	NC	NC	NC	NC	NC	I	NC	NC		1.16	1.07	1.10	1.35	1.70	2.65	*1.12*	*1.42*
AT4G24240	AtWRKY7	NC	NC	NC	NC	NC	I	NC	NC		1.97	1.07	1.15	1.55	1.44	2.56	*1.16*	*1.16*
AT5G13080	AtWRKY75	NC	NC	NC	I	I	I	NC	NC		1.05	0.84	1.63	5.46	2.46	4.7	*0.93*	*0.86*

64% of the annotated genes can be classified as resistance genes or related to the production of reactive oxygen species (ROS). This has been demonstrated in various studies to be of importance for the activation of plant defense mechanisms towards PAMP-treatment (Nürnberger, T. et al. 2004) (Laloi, C. et al. 2004).

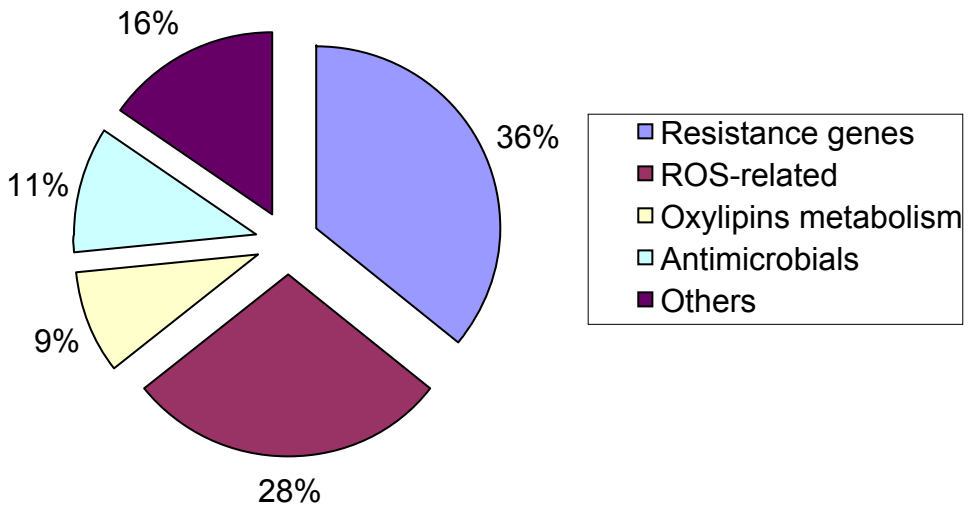

Fig 3.3: Classification for genes annotated as to be involved in defense and stress related reactions

Furthermore the group of kinases is representing more than half of the genes that have been found to likely play a role in PAMP signaling (Fig. 3.1).

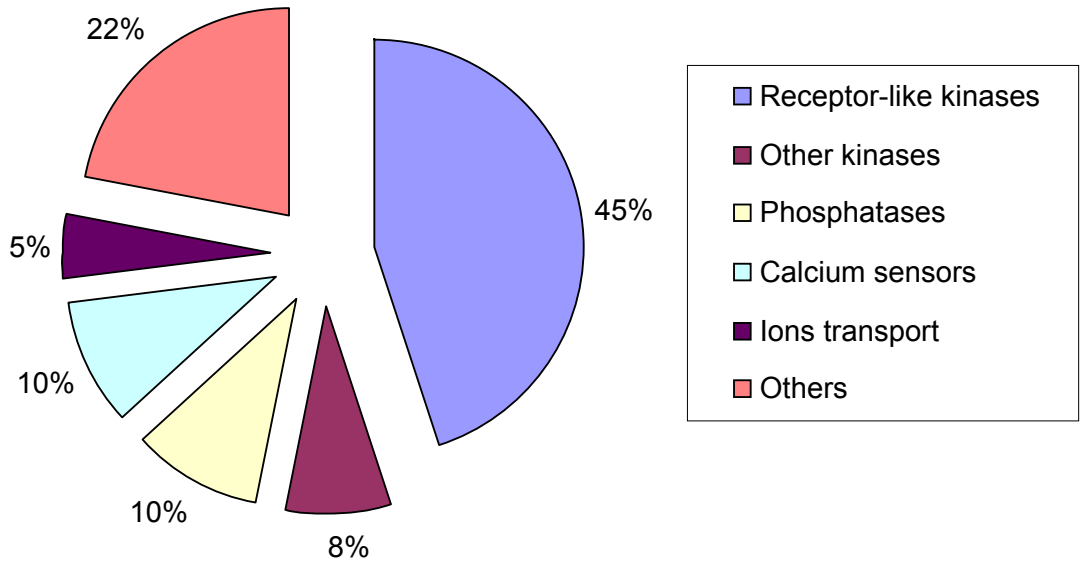

Fig. 3.4: Classification for genes thought to be involved in signaling mechanisms

The division into sub-families for further characterization of this group results in an obvious overrepresentation of up-regulated Receptor-like Kinases (RLKs, 64%). The MAP kinases that have been shown to be an integrative part of signaling after perception of potential pathogenic molecules (Cardinale, F. et al. 2000, Lee, J. et al. 2004) are represented with 5%.

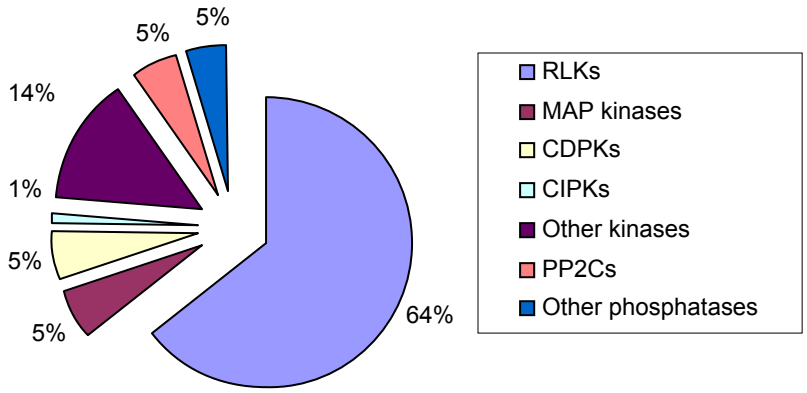

Fig. 3.5: Classification of Kinases and Phosphatases induced after elf26 treatment

The RLKs form a large gene family with >600 members in *Arabidopsis thaliana* genome. RLKs are transmembrane anchored proteins, containing a diverse N-terminal extracellular domain, a single membrane-spanning domain and a C-terminal intracellular kinase domain. A similar structure can be found in the receptor tyrosine kinases (RTKs) and receptor serine/threonine kinases (RSKs) in animals. One group of RLKs lacks signal peptides or transmembrane regions and is classified as receptor-like cytoplasmatic kinases (RLCKs) that are also highly represented in the up-regulated kinases (FIG 3.6) (Cock, J. M. et al. 2002).

They have been classified by (Shiu, S. H. and Bleecker, A. B. 2003) according to the (>20) structures found in their extracellular domains (Table 3.3). The amount of present RLKs in plants indicates their involvement in the perception of various signals. RLKs have been found to play a role in development, growth, symbiosis and plant defense (Godiard, L. et al. 2003, Torii, K. U. 2004, Wang, Xiaofeng et al. 2005).

A majority of the RLKs contain LRRs as extracellular domains. The LRRs are participating in direct protein-protein interactions (Dievart, A. and Clark, S. E. 2004) and are believed to interact or bind ligands directly. The LRRs have been found in the animal field to be of importance during pathogen recognition, e.g. Drosophila Toll and mammalian TLR (Cock, J. M. et al. 2002, Viala, J. et al. 2004b)

The flagellin receptor FLS2, as the only known PRR in Arabidopsis involved in PAMP perception belongs to the LRR-RLK family (LRRXII). The elf-sensing in Arabidopsis was shown to be independent from FLS2 (Chapter 2). Because the characteristics of EF-Tu perception and the induced defense responses are nearly flg22-congruent, this particular group was of special interest, because the unknown EF-Tu receptor might be of a similar type. Interestingly flg22 it-self is able to increase the *FLS2* transcript level upon treatment (Table 3.3: Tree order number 550, marked in grey). The fact that elf-peptides have also an effect on FLS2 led to a successful reversed genetic approach (Appendix 4) by testing a *RLK*-mutant collection for defects in PAMP perception in order to identify the EF-Tu receptor. Furthermore LRR-RLKs are highly represented among all induced (up to 18%) genes and therefore classified in more details (based on the classification of Shiu and Bleecker): (see also Table 3.3)

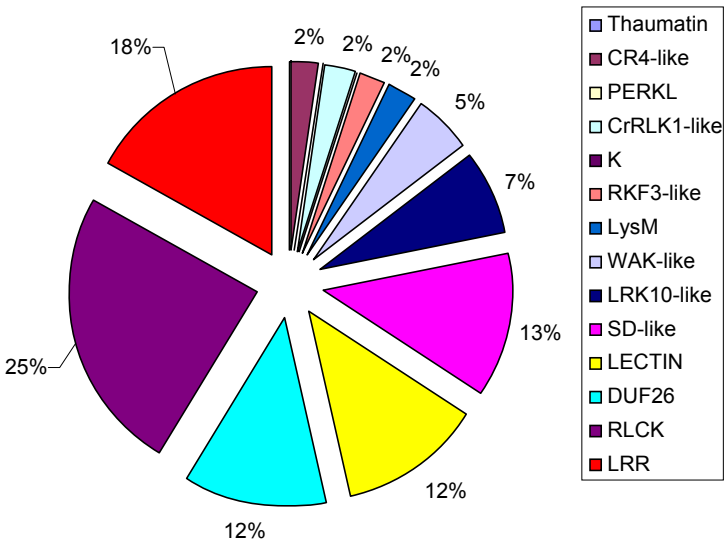

Fig.3.6: Classification of RLKs, induced after elf26 treatment

Briefly, the RLCKs and the LRR-receptor kinase family are induced to a high extent (43%). After 30 minutes elf26-treatment the percentage of activated LRR-RLKs is

around 18%, whereas already 27% of the up-regulated RLKs belongs to the LRR-RLK class after flg22-treamtment (Fig.3.7). According to the differences in kinetics between the both perception systems (Chapter 2) 25% up-regulated LRRs were found after 60 minutes elf26-treatment (data not shown). The effect on the RLCKs after EF-Tu treatment seemed to be higher than after flg22 elicitation (elf26: 25%, flg22: 19%) and it is tempting to speculate if this effect is EF-Tu dependent.

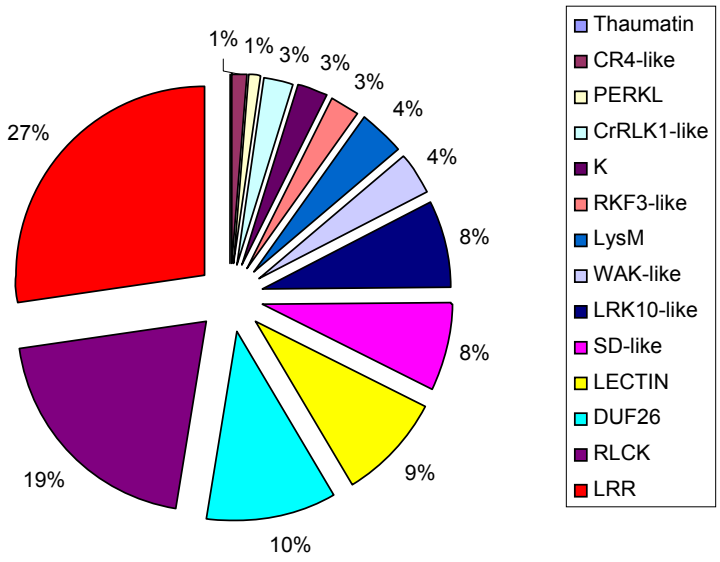

Fig.3.7: Classification of induced RLKs after flg22 treatment

In direct comparison of RLKs induced by elf26 (Fig 3.6) and flg22 (Fig 3.7) the similar distribution after both peptide stimuli can be seen. More than 40% of the genes found to be induced belong to the LRR- or RLK-family, suggesting that the potential EF-Tu receptor is included in this group (Chapter 2, Appendix 4).

Table 3.3: Summary of expressed RLKs (sorted after Shiu, S.H. and Bleecker, A.B.2003) and fold-changes upon Peptide-treatment in wild type (Ler-0) and *fls2* mutant plants; Fold-change of genes that are induced upon treatment are marked in red (>2 fold) or yellow (<0.5) when gene shows decrease.

				2.5		flg22		elf26		elf18		elf12	
				0.5		30 Min	30 Min	30 Min	60 Min	30 Min	60 min	30 min	60 min
Tree Order*	Gene name†	Exons ‡	ECD§	Subfamily¶	Locus**	Ler	FLS2	Ler	Ler	FLS2	FLS2	FLS2	FLS2
1	AT2G28930	6	K	RLCKVII		0.90	1.11	0.94	1.16	0.83	1.86	1.19	1.00
2	AT1G07570	6	K	RLCKVII	APK1	0.98	1.12	0.96	1.02	1.14	1.70	1.11	0.95
3	AT5G02290	6	K	RLCKVII	NAK	5.68	0.92	2.57	5.82	3.43	4.18	1.20	1.11
4	AT2G39660	6	K	RLCKVII		3.93	1.06	1.64	3.48	2.77	5.13	1.04	1.33
5	AT3G55450	6	K	RLCKVII		1.80	0.94	1.19	1.98	1.46	2.64	1.16	1.11
7	AT5G15080	6	K	RLCKVII		0.86	0.94	0.93	0.83	1.03	0.92	0.93	0.97
8	AT3G28690	6	K	RLCKVII		0.97	1.05	0.98	0.98	0.94	0.86	1.08	1.05
9	AT1G14370	6	K	RLCKVII	APK2a	5.17	0.94	2.07	5.75	3.52	5.20	1.19	1.27
10	AT2G02800	6	K	RLCKVII		1.35	1.04	1.19	1.48	1.12	1.38	1.19	1.09
14	AT2G17220	6	K	RLCKVII		2.89	1.07	1.95	3.80	2.55	3.96	1.13	1.09
15	AT1G76360	5	K	RLCKVII		3.07	1.00	1.82	3.11	1.65	1.94	1.13	0.99
16	AT4G35600	6	K	RLCKVII		3.25	1.00	1.62	3.25	2.78	3.61	0.97	0.99
18	AT2G05940	4	K	RLCKVII		3.53	0.93	2.48	3.72	3.46	2.90	1.07	1.03
20	AT2G07180	5	K	RLCKVII		1.18	1.04	1.12	1.38	1.09	1.61	1.09	1.23
21	AT5G01020	5	K	RLCKVII		0.75	1.04	0.97	0.68	0.88	0.75	1.02	1.09
25	AT3G09830	4	K	RLCKVII		6.71	0.94	3.53	7.91	6.34	7.32	1.31	1.10
26	AT5G03320	4	K	RLCKVII		1.47	1.02	1.05	2.28	1.46	2.66	1.27	1.07
30	AT5G47070	4	K	RLCKVII		7.82	0.96	4.08	8.24	6.34	4.90	0.97	1.19
32	AT5G13160	5	K	RLCKVII	PBS1	1.27	0.97	1.10	1.26	1.09	1.39	1.02	1.05
33	AT5G02800	6	K	RLCKVII		1.15	1.05	1.02	1.27	0.98	1.54	1.05	1.10
35	AT1G07870	4	K	RLCKVII		1.42	1.00	1.11	1.18	1.52	1.31	1.13	1.00
42	AT1G20650	6	K	RLCKVII		0.80	0.92	1.06	0.84	0.64	0.42	0.96	0.99
48	AT3G24550	8	TK	PERKL		1.45	1.04	1.34	1.79	1.70	1.64	1.18	1.08
50	AT1G52290	8	TK	PERKL		0.92	0.95	1.05	0.95	0.92	0.62	1.08	0.90
55	AT4G32710	8	TK	PERKL		0.79	0.80	1.05	0.88	0.96	0.80	1.12	1.18
60	AT1G26150	8	TK	PERKL		1.03	0.87	0.95	0.83	0.88	0.62	0.92	1.06
61	AT1G68690	8	TK	PERKL		5.73	0.97	1.77	6.26	3.68	6.02	1.24	0.99
62	AT5G38560	7	TK	PERKL		1.45	0.93	1.03	1.14	1.04	1.16	1.13	1.17
64	AT3G13690	9	K	PERKL		0.77	0.87	0.90	0.74	0.81	0.72	0.93	0.83
65	AT5G56790	8	K	PERKL		0.89	1.02	0.89	0.81	0.92	0.78	0.96	0.95
66	AT2G30740	7	K	RLCKVIII		1.39	1.08	1.13	1.45	1.57	1.61	1.14	1.08
70	AT3G59350	5	K	RLCKVIII		3.54	1.21	2.02	5.15	3.54	6.25	1.32	1.50
71	AT2G47060	7	K	RLCKVIII		5.68	0.88	2.87	6.21	4.01	4.67	1.25	1.20
72	AT3G62220	7	K	RLCKVIII		1.21	1.04	1.07	1.29	1.27	1.26	1.10	1.27
73	AT1G48210	7	K	RLCKVIII		1.15	1.04	1.00	0.94	1.04	0.97	0.83	0.93
78	AT2G20300	3	K	Extensin		1.10	0.99	1.14	1.08	0.98	0.91	0.99	1.03
79	AT4G02010	7	EXT	Extensin		0.70	0.95	0.89	0.66	0.76	0.65	1.05	0.96
81	AT3G58690	6	K	Extensin		0.94	0.93	0.96	0.78	0.94	0.80	1.02	1.05
84	AT3G51990	1	SK	CR4L		0.94	0.96	1.02	0.95	0.80	0.99	0.93	0.94
89	AT5G47850	1	CR4L	CR4L		5.09	0.92	3.55	7.49	9.46	3.96	0.98	1.32
90	AT3G55950	1	CR4L	CR4L		1.70	0.84	1.38	3.96	2.00	4.07	0.84	0.96
93	AT3G14350	15	LRR6	LRR V		1.00	1.07	1.08	0.99	1.02	1.09	0.90	0.89
94	AT4G22130	6	K	LRR V		0.88	1.09	0.87	0.69	0.92	0.79	1.02	1.03
95	AT4G03390	15	LRR5	LRR V		1.01	1.05	0.94	0.71	1.08	0.83	0.95	1.02
97	AT1G11140	7	K	LRR V		0.84	0.87	1.00	0.86	0.96	0.84	1.04	1.03
102	AT1G21590	10	K	RLCK VI		1.10	0.98	0.86	1.02	0.76	0.79	0.74	0.91

Tree Order*	Gene name†	Exons ‡	ECD§	Subfamily¶	Locus**	flg22		elf26		elf18		elf12	
						30 Min	30 Min	30 Min	60 Min	30 Min	60 min	30 min	60 min
						Ler	FLS2	Ler	Ler	FLS2	FLS2	FLS2	FLS2
103	AT5G63940	10	K	RLCK VI		0.90	1.10	0.89	0.90	0.98	0.92	1.10	1.18
105	AT2G16750	6	SK	RLCK VI		0.73	1.11	0.85	0.78	1.09	0.86	1.32	1.17
111	AT5G10520	8	K	RLCK VI		1.14	1.22	0.93	1.35	1.17	1.55	1.26	1.12
113	AT2G18890	7	K	RLCK VI		0.84	1.03	0.95	0.64	0.78	0.68	1.03	0.93
116	AT3G59110	7	K	TAKL		0.76	0.97	1.12	0.91	0.91	0.65	1.14	1.13
119	AT3G17420	5	K	TAKL		1.36	1.03	0.94	0.97	1.20	0.96	0.99	0.83
120	AT5G18500	7	SK	TAKL		0.95	0.98	1.03	1.15	1.24	1.09	1.22	1.11
121	AT4G01330	6	K	TAKL		1.12	1.05	1.19	0.97	1.21	0.80	1.10	1.48
122	AT1G01540	7	SK	TAKL	TAK1	0.94	1.02	1.11	1.11	1.04	1.00	1.12	1.07
123	AT4G02630	1	TK	TAKL		0.70	0.87	0.80	0.65	0.92	0.48	1.00	0.86
124	AT4G34500	6	K	TAKL		1.07	1.14	0.94	0.95	0.91	0.80	1.01	0.88
128	AT5G39020	1	CrRLK1L	CrRLK1L-2		7.67	0.84	4.94	11.93	5.90	7.42	1.00	1.08
129	AT5G39030	1	CrRLK1L	CrRLK1L-2		1.89	0.99	1.85	2.92	1.99	2.21	1.12	1.66
131	AT5G38240	3	LRKL	LRK10L-2		2.05	1.02	1.52	2.87	2.08	2.76	1.06	0.96
133	AT1G66920	2	LRKL	LRK10L-2		2.15	0.98	1.22	1.91	1.46	2.71	0.94	0.87
139	AT4G18250	3	THN	Thaumatin		1.48	0.92	1.65	4.05	3.20	3.12	1.03	0.79
141	AT5G60900	4	SD	SD-2	RLK4	1.95	0.87	2.19	2.28	2.64	1.99	0.77	1.28
143	AT2G19130	1	SD	SD-2		3.99	0.94	2.23	5.72	3.26	6.05	1.06	1.13
145	AT4G32300	2	SD	SD-2		1.57	1.13	1.04	1.67	1.17	2.69	0.98	1.14
146	AT1G34300	1	SD	SD-2		1.25	1.01	1.03	1.37	1.06	1.67	1.07	0.85
147	AT5G35370	2	SD	SD-2		1.59	1.10	1.30	2.03	1.64	1.67	1.07	1.02
148	AT5G20050	1	SK	N.A.		1.85	1.00	1.25	3.12	1.86	4.71	1.05	1.15
149	AT1G34210	11	LRR4	LRR II		0.84	1.21	1.03	0.65	0.85	0.63	0.87	0.85
150	AT1G71830	11	LRR3	LRR II		0.97	0.99	1.05	0.91	1.04	0.98	0.91	1.04
151	AT4G33430	9	TK	LRR II		1.80	1.04	1.10	1.90	1.69	2.62	1.05	1.19
152	AT2G13790	8	LRR4	LRR II		4.78	1.05	1.81	5.36	3.75	5.37	0.99	0.90
155	AT5G10290	10	LRR4	LRR II		1.08	1.01	1.03	0.90	0.95	0.85	1.03	1.01
156	AT5G63710	10	LRR4	LRR II		0.90	1.01	0.86	0.71	0.83	0.80	1.05	1.12
157	AT2G23950	10	LRR4	LRR II		1.00	0.89	0.89	0.84	0.75	0.83	1.04	1.01
158	AT4G30520	9	LRR3	LRR II		0.93	1.08	0.94	0.71	0.86	0.89	0.98	0.93
159	AT3G25560	9	LRR4	LRR II		0.85	1.07	0.93	0.84	0.63	0.69	0.98	0.86
160	AT5G16000	9	LRR4	LRR II		0.75	1.00	0.94	0.62	0.69	0.61	0.90	1.16
161	AT1G60800	11	LRR3	LRR II		1.01	0.94	0.97	0.75	0.74	0.56	0.95	0.96
163	AT1G11050	1	RKF3L	RKF3L	RKF3	7.44	1.06	3.54	5.40	4.44	1.99	1.31	1.22
164	AT2G48010	1	RKF3L	RKF3L		2.32	1.21	1.75	1.65	1.68	1.00	1.16	0.91
169	AT1G16130	3	WAKL	WAKL		7.30	0.88	3.06	9.02	6.97	7.14	0.96	1.15
171	AT1G79670	3	WAKL	WAKL		2.00	1.07	1.40	2.95	1.92	3.05	0.94	1.04
176	AT1G69730	3	WAKL	WAKL		0.98	0.63	1.00	1.21	1.14	1.08	1.06	0.98
177	AT1G79680	3	WAKL	WAKL		3.44	0.83	7.87	22.67	6.24	10.51	0.93	1.11
178	AT1G16260	3	WAKL	WAKL		1.38	0.98	1.33	1.38	0.98	1.24	1.16	1.19
181	AT1G21270	3	WAKL	WAKL	WAK2	0.85	0.99	1.06	1.01	1.04	0.98	1.12	1.31
184	AT1G21250	3	WAKL	WAKL	WAK1	1.01	1.32	0.83	0.84	1.12	0.88	1.14	1.02
188	AT1G25390	4	LRKL	LRK10L-1		3.19	0.98	1.82	3.35	2.77	3.45	1.10	1.06
189	AT1G18390	3	LRKL	LRK10L-1		4.53	0.99	2.49	7.11	4.72	7.54	1.50	1.41
190	AT5G38210	6	LRKL	LRK10L-1		4.94	0.96	2.31	5.39	5.25	3.83	0.93	0.99
191	AT1G66880	5	LRKL	LRK10L-1		5.26	1.02	2.01	4.99	3.95	6.02	1.49	1.28
192	AT2G23450	3	WAKL	WAKL		1.78	1.05	1.14	1.76	1.67	2.23	1.05	1.21
195	AT3G46290	1	CrRLK1L	CrRLK1L-1		0.85	0.93	0.89	1.08	1.02	1.40	1.01	0.98

Tree Order*	Gene name†	Exons ‡	ECD§	Subfamily¶	Locus**	flg22		elf26		elf18		elf12	
						30 Min	30 Min	30 Min	60 Min	30 Min	60 min	30 min	60 min
						Ler	FLS2	Ler	Ler	FLS2	FLS2	FLS2	FLS2
197	AT5G54380	1	CrRLK1L	CrRLK1L-1		1.19	1.01	1.17	1.18	1.30	0.95	1.20	1.07
205	AT3G51550	1	K	CrRLK1L-1	AAK1	1.16	1.02	1.12	1.16	1.19	1.26	1.20	1.03
206	AT2G39360	1	CrRLK1L	CrRLK1L-1		1.88	0.97	1.44	1.91	1.92	2.29	1.28	1.11
207	AT2G23200	1	CrRLK1L	CrRLK1L-1		1.22	0.99	1.03	1.69	1.11	1.72	1.03	1.24
208	AT5G38990	1	CrRLK1L	CrRLK1L-1		2.20	1.05	1.40	2.24	1.90	2.98	1.12	1.23
211	AT5G01950	17	LRR9	LRR VIII-1		1.74	1.01	1.08	1.70	1.65	2.07	1.04	0.97
214	AT5G49760	19	LRR9	LRR VIII-1		1.15	1.02	1.06	1.46	1.19	1.45	1.01	0.99
218	AT4G00330	6	K	RLCK IV		1.79	1.12	1.22	1.79	1.52	1.83	1.17	1.07
220	AT2G11520	5	K	RLCK IV		1.86	1.00	1.37	2.10	1.68	1.73	0.99	0.98
221	AT2G37050	16	LRR2	LRR I		0.95	1.16	1.00	0.82	1.03	0.93	1.02	1.10
222	AT1G67720	15	LRR2	LRR I		1.23	1.10	1.12	1.11	1.13	0.99	1.10	1.00
224	AT5G15730	6	K	LRR I		2.44	1.08	1.18	2.56	1.99	2.47	1.05	0.91
245	AT1G51850	13	LRR3	LRR I		2.52	1.15	1.44	3.74	4.31	6.28	1.43	1.02
252	AT1G51800	12	LRR3	LRR I		4.61	1.18	2.01	6.18	4.05	10.08	1.18	0.81
256	AT1G51890	13	LRR3	LRR I		2.13	1.25	2.15	8.90	4.48	9.19	1.34	1.03
265	AT2G19190	13	LRR3	LRR I		1.36	0.93	1.41	5.37	2.36	17.05	1.00	0.89
270	AT1G51790	5	LRR3	LRR I		2.26	0.91	1.29	3.78	2.87	8.04	1.14	1.00
271	AT1G80640	9	K	RLCK X		0.77	1.15	1.03	0.85	0.74	0.73	0.85	0.97
278	AT4G25390	1	TK	RLCK XI		2.19	0.94	1.39	2.49	1.88	2.29	1.08	0.90
279	AT2G45590	1	TK	RLCK XI		0.79	1.01	0.92	0.68	0.71	0.67	1.00	1.16
280	AT1G80870	1	TK	RLCK XI		0.79	1.13	0.82	0.59	0.72	0.56	0.76	0.88
281	AT1G66150	2	LRR10	LRR IX	TMK1	0.81	1.01	0.96	0.84	0.81	0.66	1.06	1.18
282	AT2G01820	2	LRR10	LRR IX		0.87	0.96	0.88	0.77	0.92	0.84	0.98	1.05
284	AT3G23750	2	LRR10	LRR IX		1.08	1.01	1.22	1.09	1.26	0.95	1.04	0.99
285	AT3G21630	10	LysM	LysM		2.72	0.88	1.38	3.45	1.93	3.42	1.21	1.13
286	AT1G51940	11	LysM	LysM		0.76	0.89	1.17	0.89	0.93	0.68	1.15	1.29
287	AT1G69270	1	LRR2	N.A.	RPK1	3.00	1.04	1.38	2.39	1.99	2.15	1.08	1.22
288	AT3G02130	1	LRR18	N.A.		2.04	1.05	1.33	1.61	1.58	1.09	0.97	1.09
299	AT5G43020	2	LRR4	LRR III		0.76	0.90	0.86	0.82	0.86	0.71	0.99	1.01
302	AT1G25320	2	LRR7	LRR III		0.90	0.99	0.84	0.70	0.90	0.86	0.87	1.09
307	AT1G67510	2	LRR7	LRR III		0.91	0.95	0.94	0.92	1.17	0.99	0.96	1.00
310	AT5G67280	2	LRR7	LRR III		0.73	0.93	0.88	0.66	0.59	0.39	0.78	0.75
312	AT4G34220	3	LRR7	LRR III		0.74	0.96	0.99	0.63	0.65	0.50	1.07	1.20
313	AT3G08680	2	LRR5	LRR III		0.88	1.03	0.95	0.78	0.83	0.78	1.09	0.87
314	AT5G58300	2	LRR5	LRR III		0.84	0.99	0.92	0.79	0.92	0.76	0.84	1.01
315	AT2G26730	2	LRR5	LRR III		0.68	0.89	0.98	0.70	0.75	0.59	1.01	0.79
316	AT4G23740	3	LRR5	LRR III		0.97	1.02	1.03	0.88	0.83	0.78	1.03	1.00
321	AT5G05160	2	LRR5	LRR III		0.85	0.97	0.96	0.79	0.99	0.87	0.98	0.89
323	AT1G68400	2	LRR5	LRR III		0.87	0.98	0.84	0.76	0.97	0.84	1.00	1.18
324	AT5G16590	3	LRR5	LRR III		0.89	0.93	1.06	0.93	1.01	0.82	1.19	1.10
325	AT3G02880	3	LRR5	LRR III		1.93	0.93	1.27	2.05	1.93	2.64	1.08	0.93
326	AT1G48480	2	LRR5	LRR III	RKL1	0.84	0.92	0.94	0.82	0.74	0.58	0.99	1.10
327	AT3G17840	2	LRR5	LRR III		0.90	0.98	0.93	0.79	0.80	0.65	0.93	0.93
328	AT3G51740	2	LRR1	LRR III		1.30	1.03	1.00	0.83	0.91	0.84	0.92	1.05
332	AT3G24660	2	LRR7	LRR III	TMKL1	0.98	1.01	0.91	0.68	0.88	0.73	0.91	0.81
334	AT5G10020	3	TK	LRR III		0.86	0.97	0.99	0.74	0.73	0.55	0.94	0.97
335	AT2G27060	3	LRR16	LRR III		0.74	0.94	0.99	0.86	0.78	0.65	0.72	0.86
336	AT5G13290	2	K	N.A.		0.81	0.90	1.01	0.79	0.79	0.77	1.12	0.98

Tree Order*	Gene name†	Exons ‡	ECD§	Subfamily¶	Locus**	flg22		elf26		elf18		elf12	
						30 Min	30 Min	30 Min	60 Min	30 Min	60 min	30 min	60 min
						Ler	FLS2	Ler	Ler	FLS2	FLS2	FLS2	FLS2
338	AT5G58150	1	LRR10	LRR VII		1.57	1.18	1.33	1.45	1.04	1.19	0.91	1.03
339	AT5G45800	2	LRR7	LRR VII		0.79	1.00	0.90	0.67	0.90	0.70	1.01	1.04
340	AT4G36180	2	LRR25	LRR VII		0.85	0.89	0.96	0.66	0.81	0.67	0.81	0.82
342	AT5G01890	2	LRR18	LRR VII		0.85	0.96	0.88	0.64	0.83	0.69	0.93	0.83
343	AT3G56370	2	LRR18	LRR VII		0.73	0.90	0.87	0.64	0.88	0.59	0.97	0.96
344	AT3G28040	2	LRR20	LRR VII		0.63	0.94	0.88	0.60	0.61	0.37	0.86	1.01
347	AT5G65700	2	LRR22	LRR XI		0.97	1.03	0.87	0.75	0.95	0.93	0.94	1.05
348	AT3G49670	2	LRR22	LRR XI		0.60	1.01	0.84	0.57	0.63	0.58	0.79	0.94
349	AT1G75820	2	LRR21	LRR XI	CLV1	0.92	0.89	1.01	0.79	0.88	0.88	1.04	1.05
350	AT4G20270	2	LRR22	LRR XI		0.75	0.91	0.96	0.59	0.85	0.79	0.97	0.99
352	AT5G63930	2	LRR25	LRR XI		0.90	0.82	0.85	0.84	0.96	1.12	1.03	1.22
355	AT3G24240	2	LRR4	LRR XI		0.97	0.91	0.90	0.75	0.91	0.97	0.87	1.15
357	AT5G56040	3	LRR21	LRR XI		0.56	0.97	0.91	0.53	0.76	0.60	1.12	1.15
360	AT1G08590	1	LRR21	LRR XI		0.89	1.13	0.96	0.86	1.19	0.90	1.02	1.04
361	AT5G61480	2	LRR21	LRR XI		0.91	0.99	0.92	0.63	0.84	0.69	0.92	0.90
362	AT4G28490	2	LRR21	LRR XI	HAESA	1.24	1.01	0.90	1.10	0.85	1.43	1.23	1.06
363	AT1G28440	2	LRR22	LRR XI		0.79	0.95	0.90	0.69	0.83	0.65	0.87	0.94
364	AT5G65710	3	LRR20	LRR XI		0.77	1.18	0.96	0.75	0.71	0.53	0.89	0.80
365	AT1G72180	3	LRR20	LRR XI		0.80	0.97	1.04	0.72	0.75	0.52	0.99	0.89
366	AT5G49660	2	LRR20	LRR XI		0.96	1.07	0.95	0.73	0.89	0.90	1.05	1.26
367	AT4G20140	3	LRR32	LRR XI		1.08	0.97	0.96	0.91	1.12	1.56	1.02	0.97
369	AT1G09970	4	LRR19	LRR XI		1.61	1.09	1.03	1.46	1.43	2.03	1.09	1.00
371	AT5G25930	2	LRR22	LRR XI		11.68	0.74	3.50	8.97	6.25	6.51	1.04	1.08
372	AT1G73080	2	LRR26	LRR XI		3.11	1.07	2.22	3.85	2.60	3.27	1.12	1.23
374	AT2G31880	1	LRR5	LRR XI		12.11	0.94	5.64	9.29	7.64	5.72	1.13	1.17
375	AT1G31420	13	LRR5	LRR XIII		0.82	0.99	0.97	0.75	0.74	0.63	0.88	0.93
376	AT2G35620	12	LRR4	LRR XIII		0.87	1.16	0.94	0.76	0.88	0.70	1.16	1.05
379	AT5G07180	26	LRR20	LRR XIII		0.68	0.97	0.92	0.93	0.73	0.67	1.05	0.75
380	AT5G62230	27	LRR19	LRR XIII		0.91	0.83	0.98	0.92	0.91	0.81	0.86	0.90
381	AT2G26330	27	LRR20	LRR XIII	ERECTA	0.88	0.99	0.92	0.83	0.81	0.78	1.00	0.98
382	AT2G41820	2	LRR17	LRR X		0.68	1.00	0.95	0.73	0.66	0.63	0.82	0.81
383	AT1G34420	2	LRR10	LRR X		2.46	1.08	1.81	4.63	2.79	2.64	0.97	0.98
385	AT1G27190	1	LRR4	LRR X		0.81	0.94	1.06	0.81	1.00	0.83	0.98	0.84
386	AT3G28450	1	LRR5	LRR X		3.27	1.10	2.20	4.00	2.96	4.37	1.17	1.07
387	AT5G48380	2	LRR4	LRR X		4.98	1.07	1.91	3.82	3.23	4.40	0.94	1.17
389	AT3G13380	1	LRR23	LRR X		1.25	1.03	1.16	1.31	1.38	1.44	1.18	1.20
390	AT4G39400	1	LRR24	LRR X	BRI1	0.86	1.03	1.11	0.81	0.97	0.67	1.12	1.26
391	AT2G01950	1	LRR21	LRR X		0.94	0.93	0.98	0.70	0.89	0.73	0.95	1.12
393	AT5G53890	1	LRR20	LRR X		0.82	1.08	0.77	0.63	0.78	0.58	0.82	0.90
394	AT2G02220	1	LRR17	LRR X		4.20	0.88	1.62	4.76	4.07	8.02	1.24	1.17
395	AT1G72300	1	LRR19	LRR X		0.95	0.98	1.13	1.25	1.22	1.57	1.21	1.22
397	AT1G74360	1	LRR20	LRR X		4.07	1.04	2.40	14.80	8.16	18.69	1.37	0.97
401	AT4G23190	8	DUF26	DUF26	RLK3	12.35	1.27	5.25	26.31	10.90	20.80	1.41	0.96
402	AT4G23300	8	DUF26	DUF26		0.94	0.99	0.90	0.68	0.98	0.89	0.86	1.17
405	AT4G23150	8	DUF26	DUF26		1.20	0.75	1.17	1.66	1.59	5.95	0.89	1.15
406	AT4G23180	6	DUF26	DUF26		7.92	0.93	4.64	9.36	7.43	7.54	0.99	1.21
408	AT4G23280	7	DUF26	DUF26		1.64	0.76	1.45	4.73	2.73	5.71	1.02	1.11
410	AT4G23130	8	DUF26	DUF26		1.60	1.46	1.11	1.62	1.66	2.14	1.10	1.24

						flg22		elf26		elf18		elf12	
Tree Order*	Gene name†	Exons ‡	ECD§	Subfamily¶	Locus**	30 Min	30 Min	30 Min	60 Min	30 Min	60 min	30 min	60 min
						Ler	FLS2	Ler	Ler	FLS2	FLS2	FLS2	FLS2
412	AT4G23270	7	DUF26	DUF26		3.44	0.87	1.31	3.52	2.41	4.62	1.07	0.91
417	AT4G23260	7	DUF26	DUF26		1.19	0.80	1.03	1.46	1.54	2.69	1.18	1.13
420	AT4G23290	8	DUF26	DUF26		0.87	0.98	0.95	0.71	0.90	0.76	0.90	1.05
421	AT4G23320	9	DUF26	DUF26		1.94	1.14	2.07	4.37	2.99	3.40	0.92	0.88
422	AT4G23210	7	DUF26	DUF26		2.89	0.86	1.50	3.97	3.04	7.32	1.43	1.12
423	AT4G00970	6	K	DUF26		6.73	0.99	5.02	12.63	3.66	2.36	0.94	0.90
425	AT4G21400	6	DUF26	DUF26		1.55	1.10	1.24	2.78	1.63	3.17	1.04	0.78
426	AT4G21410	8	DUF26	DUF26		1.57	0.98	0.85	1.53	1.57	2.75	1.18	1.10
431	AT4G04540	7	DUF26	DUF26		3.79	1.00	1.63	6.05	4.03	4.78	1.07	1.00
433	AT4G27300	6	SD	SD-1		2.01	1.12	1.21	3.67	1.26	2.38	0.94	0.80
440	AT4G21380	7	SD	SD-1	ARK3	1.10	0.74	0.85	1.50	1.45	2.67	0.95	0.90
444	AT1G11330	7	SD	SD-1		1.37	1.30	0.87	1.05	1.13	1.05	1.12	1.03
446	AT4G21390	6	SD	SD-1		13.95	1.15	3.95	21.83	6.75	11.26	1.21	0.97
448	AT1G67520	7	SD	SD-1		2.39	0.77	1.91	5.36	3.96	4.54	1.33	1.20
459	AT1G61370	7	SD	SD-1		3.68	1.06	2.02	6.29	3.87	6.44	1.01	0.94
460	AT1G11280	7	SD	SD-1		0.97	0.97	0.98	0.89	0.91	0.86	1.05	1.12
461	AT1G61380	7	SD	SD-1		1.83	0.96	1.08	2.19	1.41	2.84	1.10	0.94
462	AT1G61360	7	SD	SD-1		7.34	1.06	4.31	14.93	6.93	11.78	1.17	1.25
464	AT4G11900	7	SD	SD-1		1.64	1.00	1.39	1.87	1.09	1.02	0.94	1.12
466	AT1G53440	23	LRR9	LRR VIII-2		2.40	0.98	1.22	2.49	1.62	1.93	1.11	1.13
467	AT1G53430	23	LRR6	LRR VIII-2		3.32	1.06	1.65	3.82	2.26	3.13	1.14	0.98
468	AT1G07650	25	TK	LRR VIII-2		1.59	1.06	1.18	1.98	1.39	2.01	1.06	1.08
470	AT3G14840	23	LRR9	LRR VIII-2		1.39	1.06	1.08	1.23	1.36	1.76	0.96	1.04
474	AT1G29750	24	LRR9	LRR VIII-2		1.16	0.97	0.91	0.90	0.74	0.88	0.90	0.81
475	AT3G09010	6	K	LRR VIII-2		1.59	1.04	1.19	3.59	2.23	3.57	1.28	1.04
476	AT1G16670	6	K	LRR VIII-2		3.43	0.98	1.60	4.49	2.74	3.50	1.23	1.12
479	AT1G56120	23	LRR7	LRR VIII-2		2.65	0.90	1.11	2.01	2.03	2.52	1.12	1.25
481	AT1G70740	5	K	DUF26		7.05	0.94	3.26	6.02	5.00	2.11	1.05	0.88
483	AT1G70530	8	DUF26	DUF26		2.29	0.92	1.47	3.35	1.47	4.43	1.01	1.00
485	AT1G70520	7	DUF26	DUF26		2.78	1.05	1.42	3.56	2.20	6.10	1.40	1.07
489	AT4G11890	6	K	DUF26		1.78	0.92	1.24	4.59	2.41	8.92	0.85	1.24
490	AT2G45340	4	LRR6	LRR IV		0.79	0.97	0.85	0.44	0.74	0.45	0.82	0.84
491	AT5G51560	4	LRR6	LRR IV		0.78	0.96	0.92	0.75	0.83	0.71	1.03	1.02
495	AT3G59700	1	LEC	L-Lectin	LecRK1	2.29	0.92	2.49	7.22	4.66	10.90	1.12	1.08
502	AT2G37710	1	LEC	L-Lectin	LRK1	1.84	1.01	1.05	1.53	1.58	3.70	1.06	0.83
503	AT3G53810	1	LEC	L-Lectin		3.90	0.89	1.91	3.02	3.72	3.90	1.18	0.97
504	AT4G02410	1	LEC	L-Lectin		7.08	0.93	5.27	6.57	10.12	2.97	1.06	1.02
507	AT1G15530	1	LEC	L-Lectin		1.16	1.00	1.28	2.31	1.39	2.83	1.20	1.24
508	AT5G01550	1	LEC	L-Lectin		2.20	1.05	1.73	4.93	4.62	10.28	0.96	1.14
509	AT5G01560	1	LEC	L-Lectin		2.87	1.07	2.01	6.12	5.38	6.64	0.85	1.16
510	AT5G01540	1	LEC	L-Lectin		10.11	0.84	6.36	19.79	10.93	16.91	1.12	0.98
520	AT5G60300	1	LEC	L-Lectin		0.91	0.97	1.00	0.92	0.97	0.76	0.98	0.80
522	AT5G60270	1	LEC	L-Lectin		1.92	0.92	1.50	2.54	2.00	2.53	1.21	0.95
527	AT4G04960	1	LEC	L-Lectin		1.46	0.92	1.45	2.00	1.91	1.85	1.17	1.07
533	AT5G65600	1	LEC	L-Lectin		1.93	0.94	1.43	6.66	2.20	6.21	1.11	1.01
535	AT2G32800	1	K	L-Lectin		2.97	0.91	2.21	6.73	5.47	8.96	1.22	1.22
542	AT1G52310	5	CLECT	C-Lectin		0.72	1.01	0.88	0.75	0.82	0.90	0.95	1.14
545	AT3G47570	2	LRR21	LRR XII		2.69	0.93	1.64	2.54	1.95	1.73	1.07	0.93

						flg22		elf26		elf18		elf12	
Tree Order*	Gene name†	Exons ‡	ECD§	Subfamily¶	Locus**	30 Min	30 Min	30 Min	60 Min	30 Min	60 min	30 min	60 min
						Ler	FLS2	Ler	Ler	FLS2	FLS2	FLS2	FLS2
546	AT5G20480	2	LRR21	LRR XII		2.21	1.10	1.39	2.48	1.93	2.40	0.88	0.91
550	AT5G46330	2	LRR28	LRR XII	FLS2	3.22	1.32	1.62	4.49	2.85	5.01	0.95	0.95
551	AT4G08850	2	LRR24	LRR XII		3.90	1.00	1.43	3.03	2.90	4.36	1.17	1.03
567	AT5G61560	9	KU	RLCK IX		7.74	0.79	2.15	9.90	4.59	9.11	0.87	0.74
569	AT2G45910	8	KU	RLCK IX		1.00	1.14	1.18	1.18	1.06	1.34	1.05	1.20
570	AT3G49060	14	KU	RLCK IX		0.84	1.03	0.83	1.05	0.84	1.23	0.90	0.91
576	AT4G18640	12	LRR3	LRR VI		1.12	0.89	1.03	0.82	1.22	1.09	1.20	1.03
578	AT2G40270	2	K	RLCK I		6.86	1.07	2.55	3.70	3.68	2.25	1.23	1.05
579	AT3G56050	6	K	RLCK I		1.89	1.01	1.34	1.78	1.29	1.57	1.01	1.07
580	AT1G63430	14	LRR5	LRR VI		0.77	0.93	0.89	0.71	0.90	0.77	1.15	1.06
581	AT5G41180	13	LRR4	LRR VI		1.68	0.97	1.05	1.64	1.48	2.22	1.12	1.02
584	AT3G03770	6	LRR8	LRR VI		0.88	0.93	0.99	0.95	0.73	0.54	0.87	0.72
585	AT5G14210	6	LRR10	LRR VI		0.90	0.95	1.01	0.94	0.81	0.94	0.97	1.14
586	AT5G63410	6	LRR6	LRR VI		1.44	1.08	1.27	1.60	1.11	1.23	0.89	1.07
588	AT4G35230	9	K	RLCK II		0.86	0.99	0.98	0.91	1.10	1.14	1.06	1.04
589	AT5G59010	10	K	RLCK II		0.89	1.03	1.02	0.92	0.91	0.80	0.86	1.09
594	AT5G25440	6	K	RLCK II		4.09	1.33	2.15	6.23	3.37	5.80	1.06	1.11
596	AT3G57710	1	K	RLCK III		0.76	1.06	0.72	0.76	0.70	0.92	0.83	0.91
598	AT1G67470	1	K	RLCK III		2.02	1.11	1.42	1.60	1.93	1.54	1.29	1.30
600	AT2G25790	2	LRR21	N.A.		1.10	1.05	0.98	0.80	0.98	0.85	1.10	0.94
602	AT2G41890	1	SD	SD-3		1.56	0.96	1.29	1.52	1.13	0.79	1.07	0.82
604	AT2G33580	1	LysM	LysM		16.68	0.77	5.82	14.22	14.32	7.97	1.28	1.25
605	AT2G23770	1	LysM	LysM		3.19	0.92	1.35	7.35	1.90	5.52	1.14	1.06
606	AT4G39270	4	LRR10	N.A.		1.25	0.93	1.06	1.22	1.12	1.57	1.09	1.06

3.2.1 Genes that are induced upon elf12-treatment

Elf12 has been shown to act as competitive agonist (Chapter 1) (Kunze, G. et al. 2004)for elf18 and to still have the capability to compete in Binding assays (Chapter 2). 104 genes (out of 1407 genes, ANOVA based data) show regulation upon elf12 peptide treatment (Table 3.4). Because of not existing replicates and to avoid the analysis of statistical noise, the ANOVA-Data has been used to evaluate this expression changes for this analysis.

Around 56 genes appear to be affected by elf12-treatment in Arabidopsis *fls2* mutant plants (45 up- and 11 down-regulated). The values derived for the 60 minutes-experiment show up-regulation in 43 genes, whereas 46 genes are down-regulated. Interestingly the regulated genes in both cases are not completely identical – only 39 genes show congruent expression changes. Furthermore only 24 out of these 104 genes (23%) were found induced in parallel by flg22 elicitation and none showed significant fold-changes when *fls2* was treated with flg22. Partly the activated genes after elf12-treatment overlaps with results from elf18 treatment that was performed simultaneously, but only to less extent with elf26 derived values (previous experiment). Although no clear determination or classification of affected particular gene-families could be detected, suggesting a more spontaneously regulation of this genes. Together our findings indicate an unknown stimulus (e.g. variances in light, temperature and experimental process) that might have caused differences during treatment of the seedlings in this particular approach and seem not to be related for EF-Tu treatment.

Table 3.4: Summary of genes showing regulation after elf12-treatment compared to treatments with flg22 and elf26/18. Fold-change of up-regulated Genes is marked in red (> 2fold), down-regulated genes are marked in yellow. Table is sorted after flg22-results in Ler-0

	flg22		elf26		elf18		elf12		
	30 Min	30 Min	30 Min	60 Min	30 Min	60 min	30 min	60 min	
Systematic	Ler	FLS2	Ler	Ler	FLS2	FLS2	FLS2	FLS2	Description
AT2G39030	0.50	0.69	0.62	0.66	0.42	0.38	0.37	0.38	unknown protein ; supported by cDNA: gi_15451161_gb_AY054661.1_
AT5G24660	0.47	1.11	0.85	0.41	0.22	0.15	0.53	0.34	putative protein similar to unknown protein (emb CAB62461.1)
AT5G20790	0.35	1.31	1.45	0.51	0.40	0.43	0.77	0.37	putative protein predicted protein, Arabidopsis thaliana;
AT4G22780	8.22	0.87	3.61	5.86	8.51	8.90	2.97	2.45	Translation factor EF-1 alpha - like protein translation factor EF-1 alpha genfamily, Arabidopsis
AT3G02550	2.25	1.22	0.95	2.77	3.50	3.77	3.45	3.27	Unknown protein; supported by full-length cDNA: Ceres:20907.
AT4G24110	9.92	1.03	4.98	11.39	6.35	4.57	3.99	3.28	putative protein predicted proteins, Arabidopsis thaliana
AT3G23170	5.80	1.02	3.51	2.12	7.80	4.86	4.09	2.72	Unknown protein; supported by full-length cDNA: Ceres:92314.
AT1G76650	13.29	0.92	6.97	16.49	13.90	10.31	6.16	4.93	putative calmodulin similar to calmodulin GB:CAA56517 [Leishmania tarentolae];
AT5G62520	3.25	1.00	1.65	2.14	15.72	18.65	12.15	11.26	putative protein similar to unknown protein (pir||T07711)
AT5G64260	2.57	1.07	2.20	2.90	3.17	2.42	2.04	1.60	phi-1-like protein ;supported by full-length cDNA: Ceres:37357.
AT5G45340	10.93	0.89	12.47	13.38	11.94	5.28	2.08	1.56	cytochrome P450
AT1G66180	2.98	1.19	1.93	2.10	2.85	1.18	2.09	1.18	Unknown protein ;supported by full-length cDNA: Ceres:99625.
AT1G18570	12.16	0.69	7.50	13.58	9.58	6.48	2.09	1.97	myb factor, putative similar to myb factor GI:1946266 from [Oryza sativa];
AT4G08950	3.33	0.97	3.10	3.48	4.10	1.90	2.12	1.06	putative phi-1-like phosphate-induced protein ;supported by full-length cDNA: Ceres:3552.
AT5G57560	12.57	0.74	11.70	17.36	15.62	9.85	2.31	0.91	TCH4 protein (gb|AAA92363.1) ; supported by cDNA: gi_14194112_gb_AF367262.1_AF367262
AT4G25810	3.61	0.90	3.51	7.08	9.82	7.55	2.71	1.31	xyloglucan endo-1,4-beta-D-glucanase (XTR-6) ;
AT1G35140	12.56	0.75	12.58	21.24	21.92	10.97	3.82	1.10	phosphate-induced (phi-1) protein,
AT1G76690	2.06	1.06	1.13	1.85	0.74	1.14	0.65	0.36	12-oxophytodienoate reductase (OPR2) GB:AAC78441 [Arabidopsis thaliana]
AT2G32190	14.82	0.81	2.63	12.27	6.83	16.53	0.96	0.48	Unknown protein; supported by full-length cDNA: Ceres: 40344.
AT1G28480	2.89	0.99	3.30	6.27	6.45	2.48	0.98	0.44	glutaredoxin, putative similar to glutaredoxin GI:2244924 from [Arabidopsis thaliana];
AT1G69930	4.65	0.82	2.85	8.85	8.62	7.63	1.12	0.40	putative glutathione transferase similar to glutathione transferase GB:CAA09188
AT5G46710	3.18	0.93	3.14	3.76	3.43	2.06	1.68	2.42	putative protein similar to unknown protein (pir||T05076)
AT3G03020	5.35	1.14	2.20	6.88	3.86	6.77	1.74	2.03	Unknown protein ;supported by full-length cDNA: Ceres:35949.
AT3G50930	19.52	0.72	10.11	20.29	13.91	10.08	1.97	2.10	BCS1 protein-like protein functional mitochondrial ubiquinol-cytochrome c reductase complex
AT5G48850	0.74	1.23	1.73	0.94	0.13	0.11	0.22	0.10	putative protein similar to unknown protein (gb|AAC72543.1)
	1.04	1.10	0.89	1.04	1.02	1.08	0.34	0.40	hypothetical protein
AT2G07696	0.87	1.15	1.09	1.07	1.12	1.17	0.37	0.38	hypothetical protein
AT5G24780	0.83	0.54	0.83	0.72	0.41	0.57	0.39	0.31	vegetative storage protein Vsp1 ;supported by full-length cDNA: Ceres:32606.
AT4G15210	0.87	1.03	1.43	1.07	0.53	0.65	0.44	0.26	beta-amylase ; supported by cDNA: gi_166601_gb_M73467.1_ATHAMYB
AT3G28740	0.67	1.10	1.04	0.60	0.18	0.17	0.46	0.10	cytochrome P450
AT1G52400	0.53	0.71	0.49	0.35	0.50	0.62	0.47	0.31	beta-glucosidase, from [Arabidopsis thaliana];
AT1G52000	0.68	0.77	0.60	0.46	0.65	0.60	0.49	0.60	myrosinase binding protein, GI:1711295 from [Brassica napus]
	1.07	1.30	0.98	0.98	1.08	1.19	0.49	0.55	hypothetical protein

	1.30	1.14	1.04	1.10	0.99	1.16	0.50	0.74	large subunit of riblose-1,5-bisphosphate carboxylase/oxygenase
AT1G03610	1.99	0.99	1.33	1.78	2.81	3.70	2.18	2.08	unknown protein similar to hypothetical protein GB:AAD11584;
AT3G23150	0.62	1.27	0.75	0.74	1.63	1.92	2.18	2.43	ethylene receptor, putative (ETR2) [Lycopersicon esculentum],
AT2G41730	1.83	1.15	1.38	2.99	3.19	4.05	2.25	3.31	hypothetical protein predicted by genefinder
AT1G43800	1.04	1.25	1.02	0.91	2.00	3.14	2.31	4.21	stearoyl acyl carrier protein desaturase, GB: AAD28287 GI:4704824 from [Lupinus luteus];
AT5G39110	0.85	1.35	1.02	1.13	1.18	1.50	2.38	2.01	germin -like protein germin -like protein GLP6, Arabidopsis thaliana, EMBL:ATU75194
AT4G39675	1.18	1.08	1.08	1.10	1.93	2.49	2.38	2.13	Expressed protein ; supported by full-length cDNA: Ceres: 14423.
AT2G16060	1.26	1.47	0.95	1.40	2.36	3.73	2.43	3.11	class 1 non-symbiotic hemoglobin (AHB1) identical to GP:2581783:U94998;
AT1G19530	1.20	1.16	1.09	1.36	1.57	4.64	2.54	4.38	unknown protein ;supported by full-length cDNA: Ceres:39579.
AT1G77120	1.01	1.11	0.93	0.76	1.72	2.91	2.56	3.66	alcohol dehydrogenase GI:469467 from (Arabidopsis thaliana);
AT4G10270	1.41	1.17	0.98	1.24	2.53	2.57	3.02	3.06	probable wound-induced protein wound-induced protein, Lycopersicon esculentum, PIR2:S19773;
AT5G15120	1.39	1.06	1.01	1.34	2.82	4.32	3.12	3.63	putative protein predicted proteins, Arabidopsis thaliana and Drosophila melanogaster
AT5G39890	1.05	1.22	0.90	1.04	2.65	4.66	3.25	4.61	putative protein hypothetical protein F8M21.10 - Arabidopsis thaliana, PIR:T49947;
AT4G27450	1.15	1.16	1.08	1.05	2.73	4.58	3.48	5.24	putative protein stem-specific protein - Nicotiana tabacum,PID:g20037;
AT5G42200	1.00	0.93	1.07	0.95	2.09	1.79	3.50	3.11	putative protein similar to unknown protein (gb|AAF16660.1);
AT3G27220	0.79	1.03	0.82	0.80	2.84	3.55	3.51	4.09	unknown protein ; supported by cDNA: gi_15081754_gb_AY048270.1_
AT4G37240	1.44	0.97	1.56	1.31	1.94	0.96	3.61	2.06	putative protein ;supported by full-length cDNA: Ceres:16131.
AT4G33560	0.80	1.05	0.89	1.06	3.41	6.19	3.76	5.28	putative protein ;supported by full-length cDNA: Ceres:17194.
AT2G34390	1.26	1.03	0.94	0.97	3.83	9.95	3.84	4.86	putative aquaporin (plasma membrane intrinsic protein)
AT3G10040	1.17	1.57	0.98	1.36	4.09	4.11	4.09	3.64	unknown protein predicted by genscan, est match
AT1G33055	1.39	1.00	0.91	1.29	4.75	5.00	4.83	5.06	Expressed protein ; supported by cDNA: gi_13877526_gb_AF370464.1_AF370464
AT3G29970	0.89	0.87	1.04	1.22	5.82	22.03	5.23	9.34	gene_id:K17E7.15~unknown protein
AT5G66985	0.87	1.26	1.07	1.07	4.26	6.73	5.54	7.41	Expressed protein ; supported by full-length cDNA: Ceres: 4410.
AT4G26460	1.11	1.00	1.09	1.24	4.16	9.68	6.45	8.61	hypothetical protein
AT4G33070	1.01	1.07	1.04	0.94	5.46	9.13	8.44	11.33	pyruvate decarboxylase-1 (Pdc1)
AT2G17850	1.02	0.97	1.08	1.18	13.11	27.68	8.63	14.38	senescence-associated protein contains similarity to ketoconazole resistant protein GI:928938
AT5G10040	0.93	1.02	1.01	0.95	5.03	21.19	8.67	10.39	hypothetical protein
AT4G22490	0.94	1.18	0.82	0.96	0.91	1.38	2.07	1.45	RCc3- like protein RCc3 protein, Oryza sativa, PIR2:S53012;
AT1G10140	0.90	0.93	1.09	1.40	1.66	2.36	2.10	1.67	unknown protein similar to EST gb|AA598098;supported by full-length cDNA: Ceres:23916.
AT3G13310	0.72	0.98	0.72	0.82	1.35	1.46	2.13	1.84	DnaJ protein, putative contains Pfam profile: PF00226 DnaJ domain;
AT4G19750	0.76	0.92	0.69	0.78	1.11	1.12	2.27	0.85	chitinase - like protein chitinase / lysozyme PZ precursor, Nicotiana tabacum, PIR2:S51591
AT5G19890	1.09	0.88	0.93	0.67	1.47	1.37	2.30	1.38	peroxidase ATP N ;supported by full-length cDNA: Ceres:40493.
AT4G21990	1.06	1.19	1.00	1.08	0.53	0.32	0.51	0.32	PRH26 protein ;supported by full-length cDNA: Ceres:36866.
AT3G55970	0.74	0.85	0.51	0.46	0.39	0.28	0.54	0.26	leucoanthocyanidin dioxygenase -like protein leucoanthocyanidin dioxygenase, PIR:S33144
AT3G26830	1.27	1.10	0.71	1.32	0.75	1.02	0.54	0.33	putative cytochrome P450 similar to cytochrome P450 71B2 GB:O65788 [Arabidopsis thaliana]
AT3G49620	1.06	1.23	0.91	1.51	0.65	1.69	0.56	0.44	putative protein SRG1 protein - Arabidopsis thaliana, PIR:S44261
AT1G73260	0.93	0.79	0.83	0.78	0.39	0.47	0.56	0.41	putative trypsin inhibitor similar to trypsin inhibitor propeptide
AT3G48580	1.02	1.20	0.52	0.50	0.56	0.33	0.57	0.27	endoxyloglucan transferase-like protein EXGT1 (endoxyloglucan transferase) - Pisum sativum,

AT4G04610	0.89	1.10	0.96	0.98	0.51	0.38	0.59	0.47	5-adenylylsulfate reductase ;supported by full-length cDNA: Ceres:40330.
AT3G28220	0.53	0.58	0.55	0.39	0.61	0.85	0.61	0.35	unknown protein
AT2G29350	0.77	0.79	0.51	0.57	0.70	0.43	0.63	0.47	putative tropinone reductase ;supported by full-length cDNA: Ceres:14555.
AT1G52890	1.10	0.80	0.90	0.52	0.96	0.55	0.64	0.42	NAM-like protein similar to NAM (no apical meristem) GB:CAA63101 from [Petunia x hybrida]
AT5G20150	0.53	1.05	1.33	0.65	0.41	0.77	0.66	0.45	ids4-like protein ids-4 protein - Hordeum vulgare, PIR:T05905;
AT1G52410	0.64	0.82	0.57	0.50	0.55	0.48	0.69	0.43	myosin-like protein contains Pfam profile: PF00658 Poly-adenylate binding protein,
AT3G21670	0.79	0.93	1.03	0.81	0.52	0.44	0.71	0.50	nitrate transporter identical to nitrate transporter GB:CAB38706 [Arabidopsis thaliana]
AT3G17790	0.73	1.03	1.01	0.69	0.47	0.41	0.73	0.42	acid phosphatase type 5 identical to GB:CAB63938 from [Arabidopsis thaliana];
AT1G19670	0.53	1.02	0.76	0.52	0.40	0.34	0.73	0.48	unknown protein contains similarity to chlorophyllase GI:7415999 from [Chenopodium album];
AT2G43510	0.75	0.84	0.85	0.76	0.81	0.48	0.73	0.40	putative trypsin inhibitor ; supported by cDNA: gi_15292710_gb_AY050789.1_
AT3G02040	0.82	0.99	1.35	0.83	0.46	0.84	0.76	0.40	hypothetical protein predicted by genefinder;supported by full-length cDNA: Ceres:40090.
AT1G73010	1.89	0.92	2.30	2.22	0.77	1.27	0.82	0.28	hypothetical protein predicted by genemark.hmm;
AT1G10585	0.63	1.65	0.69	0.56	0.51	0.42	0.83	0.35	Expressed protein ; supported by cDNA: gi_15293050_gb_AY050959.1_
AT4G38420	1.19	1.00	1.10	1.40	3.59	3.95	0.84	0.48	putative pectinesterase pectinesterase - Lycopersicon esculentum, PID:e312172
AT4G01870	2.00	1.14	1.21	1.57	0.75	1.19	0.84	0.43	predicted protein of unknown function similar to bacterial tolB proteins
AT2G38940	0.85	1.06	1.02	0.83	0.52	0.56	0.85	0.45	phosphate transporter (AtPT2) identical to GB:U62331
AT1G17710	0.70	1.01	1.32	1.15	0.60	0.67	0.96	0.43	hypothetical protein similar to putative phosphatase GI:3218467 from [Gallus gallus]
AT5G17220	0.86	0.98	0.63	0.64	0.74	0.31	0.97	0.41	glutathione S-transferase-like protein
AT1G03495	0.77	1.26	0.60	0.53	0.72	0.36	0.98	0.42	hypothetical protein similar to Anthocyanin 5-aromatic acyltransferase GB:BAA74428
AT4G14090	0.67	0.99	0.56	0.41	0.72	0.28	0.99	0.45	glucosyltransferase like protein
	1.03	1.01	1.46	0.96	0.83	0.84	1.01	0.50	Arabidopsis thaliana /REF=M65137 /DEF=5S ribosomal RNA gene, complete cds /LEN=497
AT5G22570	1.04	0.96	1.10	0.98	1.61	0.39	1.33	0.47	putative protein contains similarity to DNA-binding protein
AT4G39210	1.03	1.17	0.38	0.45	1.18	0.64	1.35	0.49	glucose-1-phosphate adenylyltransferase (APL3)
AT5G53870	1.12	0.85	0.53	0.57	0.94	0.39	1.47	0.34	putative protein contains similarity to phytocyanin/early nodulin-like protein
AT5G22555	1.51	0.74	0.76	1.19	0.87	0.94	1.59	0.41	Expressed protein ; supported by full-length cDNA: Ceres: 30518.
AT2G30670	0.79	1.07	0.35	0.41	1.07	0.91	1.99	0.49	putative tropinone reductase
AT1G19540	0.77	1.20	1.04	0.95	0.89	1.20	1.20	2.06	2-hydroxyisoflavone reductase, putative similar to PIR:T08106 from [Betula pendula]
AT3G43190	0.96	1.21	1.03	0.85	1.13	3.19	1.35	3.55	sucrose synthase - (SUCROSE-UDP GLUCOSYLTRANSFERASE),
AT4G35770	1.93	0.79	1.81	3.18	1.24	2.61	1.43	2.59	senescence-associated protein sen1 [Arabidopsis thaliana]
AT1G26270	1.74	1.11	1.17	1.59	2.10	3.11	1.62	2.01	hypothetical protein similar to putative ubiquitin GI:4415931 from [Arabidopsis thaliana];
AT3G24500	1.05	0.98	1.08	1.13	1.43	2.17	1.73	2.62	ethylene-responsive transcriptional coactivato
AT5G44730	0.89	1.05	0.95	0.87	1.41	1.66	1.90	2.18	Dreg-2 like protein
AT1G28330	1.08	1.05	1.07	1.30	1.45	2.00	1.99	2.10	dormancy-associated protein, [Arabidopsis thaliana
AT1G72360	1.37	1.26	1.12	1.17	1.61	3.58	1.99	2.55	putative AP2 domain transcription factor contains Pfam profile: PF00847 AP2 domain

3.3 Identification of mutants impaired in EF-Tu perception or signaling

In addition to the reversed-genetic approach with the LRR-RLK mutant collection (by C. Zipfel, Appendix) and in order to identify the EF-Tu receptor, we attempted a second screen using an activation-tagging TDNA-collection (kindly provided by the laboratory of B. Hohn, FMI) in combination with a growth inhibition assay. This experimental setup was already successful applied by screening for flg22-insensitve mutants and led to the discovery of the FLS2 receptor (Gómez-Gómez, L. and Boller, T. 2000).

3.3.1 Growth-Inhibition Experiments:

This mutant collection contains 146 pools containing 100 independent transgenic lines. For each pool >1500 seedlings were analyzed for their ability to respond to elf-treatment. In one of the tested pools (Pool 120), several (4) plants were clearly insensitive to elicitation with elf-peptides and named *P120A-D*.

The plants were further separated and the inhibition assay was repeated with seeds derived from these plants. Again we found partial insensitivity to elf18 and in addition a 7-fold sensitivity shift in flg22 treated plants (half-maximal growth inhibition, EC_{50}= 120 nM) compared to wild-type seedlings (Col-0) (EC_{50}= 18 nM) (Fig. 3.8). In this dose-response experiment in Col-0 half-maximal growth inhibition was observed at ~1 nM with elf18 and at ~18 nM with flg22, respectively (Fig. 3.8). In relation to the weight of the seedlings before treatment (Fresh weight average of 5,4 mg, representing 20,3% of fresh weight control value at evaluation), elf18 is able to stop nearly completely an increase in fresh weight and cause strong inhibition of seedling growth. Like observed in other experiments (Chapter 2) flg22 causes a smaller effect on overall fresh weight increase effect then elf derived peptides. All repetitions of this growth inhibition experiment with *P120* mutants never resulted in a reduction of fresh weight lower than 60% compared to non treated seedlings. In addition this effect is doses-independent (tested up to 100 µM, data not shown).

Interestingly in two out of four independent experiments we observed an effect when *P120* was treated with flg22. The peptide elf12 has no effect when tested with *P120* (data not shown).

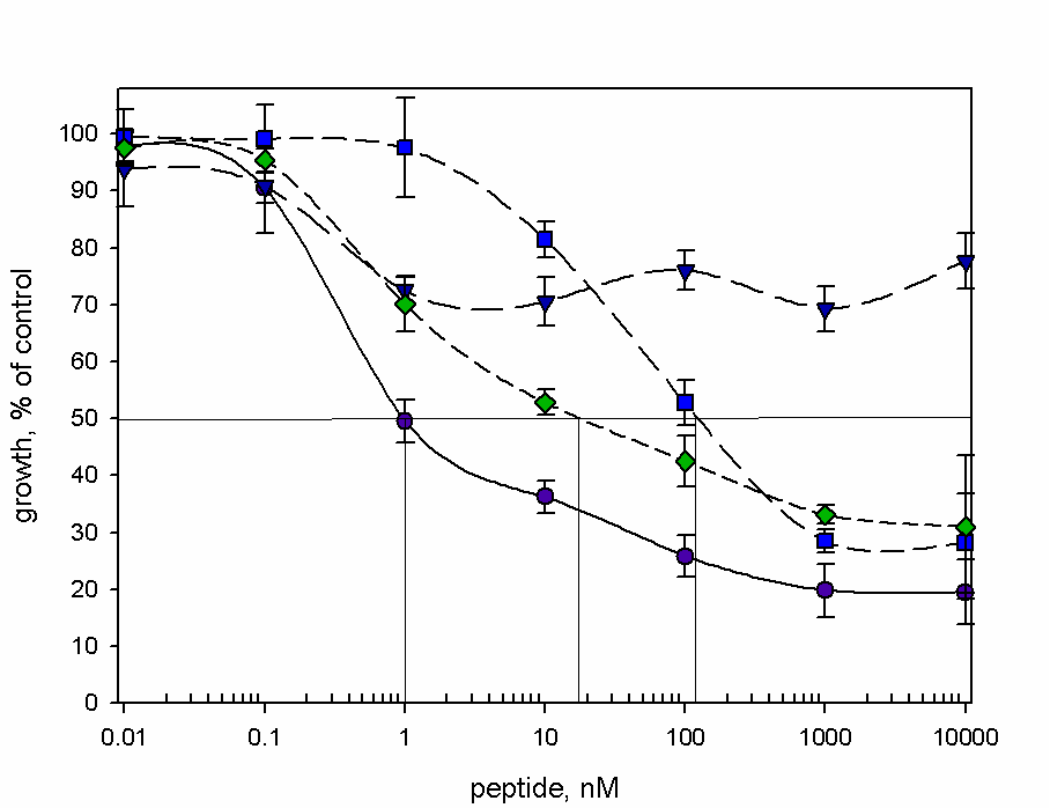

Fig. 3.8: Growth-Inhibition Assay with P120 treated with elf26 (blue triangle), flg22 (blue squares), in comparison to Col-0 treated with elf26 (blue circles) and flg22 (green diamonds). Experiment with similar readout towards P120 has been reproduced at least in three independent experiments.

Our finding suggests that *P120* is still able to sense elf18 and flg22, but the effect is somehow smaller as in wild-type, indicating a possible deficiency in signaling, or due to an alternative existing low-affinity perception system.

3.3.2 Binding analysis

Arabidopsis thaliana has a high-affinity binding site specific for flg22 ((Bauer, Z. et al. 2001)) and EF-Tu (Chapter 2). In order to evaluate if *P120* is impaired in binding both peptides were labeled with ^{125}Iodine and were used to analyze binding specificity (Fig. 3.9).

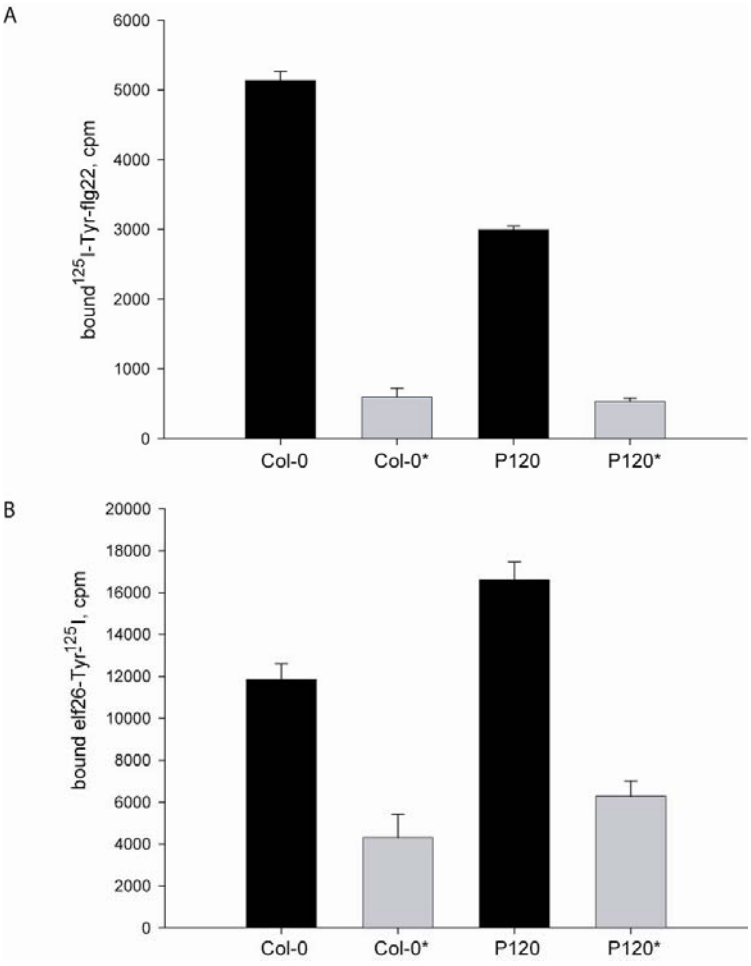

Fig. 3.9: Binding of the radiolabeled peptide-derivatives to plant tissue from *Arabidopsis P120:* Aliquots of 100 µl plant tissue (P1) (20 mg fresh weight) were incubated with radiolabled peptides in the absence (black bars) or presence of 10 µM flg22 or elf18 peptide (grey bars, presence of competitor peptide indicated with (*)) Radioactivity retained on the plant material was measured by γ-counting after washing with binding buffer.
A: ^{125}I-flg binding in comparison to wild type Col-0,
B: elf-^{125}I binding in comparison to wild type Col-0

The detection of specific binding (non specific binding (grey bars Fig 3.9) subtracted from total binding (black bars Fig 3.9) indicates that *P120* is able to bind flg22 and elf26, respectively and therefore we can suggest that both receptors are functional.

3.3.3 Oxidative Burst and ethylene biosynthesis

The production of reactive oxygen species (oxidative burst) and increased biosynthesis of the stress hormone ethylene are common measurable signals from plants attacked by pathogens or elicitor treatment (Lamb, C. and Dixon, R. A. 1997). The leaf tissues of *P120* showed induction of an oxidative burst with a significant increase above the straight base line, when treated with elf18 or flg22 peptide (Fig. 3.10). Repeated elf18-treatment in the same sample (shown for *120D*, Fig 3.10) did not result in a second induction of an oxidative burst. If the first elicitor was elf18 and the second flg22, in both cases induction of oxidative burst occurs (shown exemplary for *120A*, and was also found in reversed experiment) indicating the presesence of two independent receptors. Although the light emission varies between independent experiments with different plant material, the induction of oxidative burst was mostly clear in all *P120* mutants.

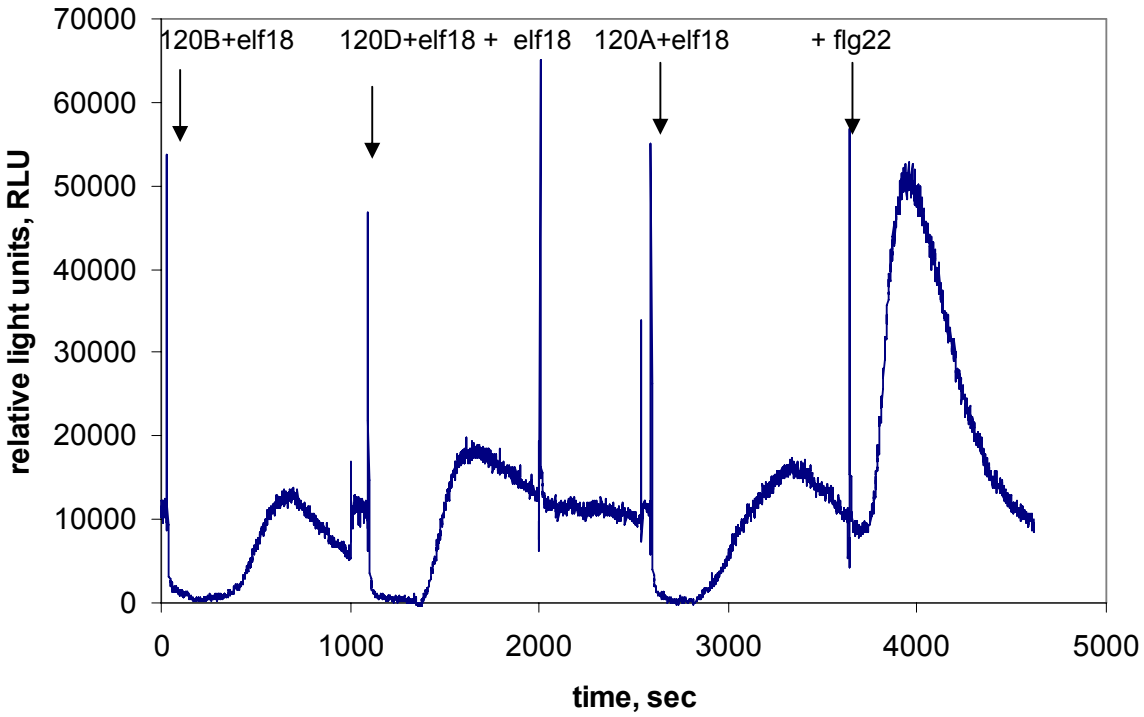

Fig. 3.10: Oxidative burst in leaf tissues of Arabidopsis *P120*. Luminescence (relative light units (RLU)) has been measured in a solution containing peroxidase and luminol and was measured after addition of peptides. Light emissions during first seconds are resulting from phosphorescence of green plant tissue, several independent experiments are combined in this figure, and peptide concentration used was 10µM. Arrows indicate time point of treatment.

Similar results were obtained for the enhancement of ethylene biosynthesis when challenged with elf26 peptide (Fig. 3.11). When flg22 peptide was used as the elicitor, all mutants showed an increase of ethylene biosynthesis (data not shown).

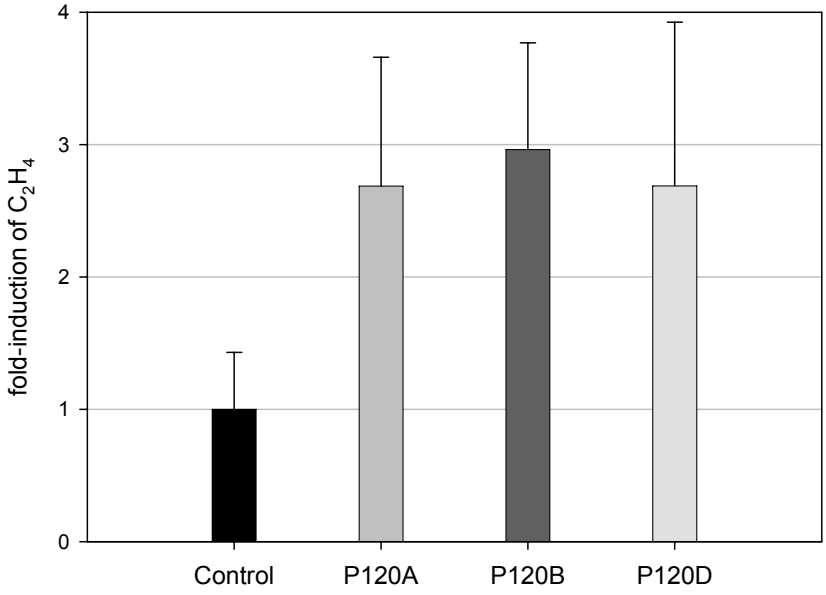

Fig. 3.11: Induction of ethylene biosynthesis in leaf tissues of *P120* mutants. Leaf pieces were not treated (*P120* untreated, control) or treated with 1 µM elf26, and ethylene accumulation was measured after 4 hours. Results represented as fold induction over control, show mean and standard deviation of n=10 replicates

3.3.4 Plasmid-Rescue

To locate the T-DNA insertion the following protocol (modified from O. Fritsch, FMI) was used:

The T-DNA insertion site cloning was achieved by plasmid rescue of the pUC sequence (Fig. 3.12) in pAC102 T-DNA (Fig 3.12, Appendix 2). 0.5 µg of genomic DNA extracted from plant tissues was digested in a (Plant Phyto-Pure Kit, Amersham/Nucleon Biosciences according to manufactures manual) 100 µl-digestion reaction with 50U of *Hind* III and 3 µl RNase. After overnight incubation at 37°C, the DNA was further purified by (i) phenol/chloroform 1/1 (v/v), (ii) chloroform (to remove phenol), (iii) ethanol precipitation (20 min at –20°C) and resuspended in 32 µl of water (37°C, 1 h) and kept at –20°C. For the ligation, three reactions containing 10, 5 and 1 µl of the DNA in a total volume of 100 µl (T4 DNA-ligase, 2U) and incubated

overnight (or 24 h) at 16°C, to allow for different intramolecular ligation conditions. The ligations were precipitated with ethanol (optional: add 1µl glycogen to see the pellet), resuspended in 15 µl of water and 2-5 µl of those were transformed in electrocompetent TOP10 *E*. *coli* (Invitrogene) cells (Growth-Medium: 2YT) and plated on LB plates with ampicillin. Incubation time varies between 12 and 24h. Colonies were analyzed by *Hind* III restriction on plasmid mini-prep (Promega Wizzard Plus SV Minipreps DNA Purification System (Vacuum Protocol)) and positive clones were sequenced with primers rbnos#5 (5'-gatcagattgtcgtttcccgcc), M13rev and RB-53pAC (5'-cccggggatcagattgtcg).

This protocol has been modified according to incubation conditions and amount of restriction enzymes, but sequencing never results in usable sequences that in theory would be blasted against the public *Arabidopsis* genome sequence after checking for and removing vector sequences.

To further exclude that the TDNA-Insertion in *P120* is situated in *EFR1*, found in the reversed-genetic approach (Appendix), we analyzed purified *P120* genomic DNA by PCR with *EFR*-specific primers spanning the complete sequence (Table 3.5, Appendix 3) of *EFR1*.

Table 3.5: Names of used primers, number of start and end nucleotide sequence, and the resulting length after PCR-reaction.

Nr	Forward	Reverse	start	end	length
Primer-Names					
01	NeoI	EFS1-6	1	1935	1935
02	EFS1-1	EFS1-6	340	1935	1595
03	EFS1-2	EFS1-6	761	1935	1174
04	EFS1-3	EFS1-6	1168	1935	767
05	EFS1-4	EFS1-6	1465	1935	470
06	M544334for	EFS1-6	1791	1935	144
07	M544334for	M544334rev	1791	2412	621
08	EFS1-5	M544334rev	2185	2412	227
09	EFS1-5	End	2185	3192	1007
10	Chip1-seq8	End	2486	3192	706
11	Chip1-seq9	End	2849	3192	343

In all PCR-reactions amplification was observed (data not shown), indicating that the TDNA is not located in the sequence of *EFR1* and also not in the EFR-Promoter region (data not shown).

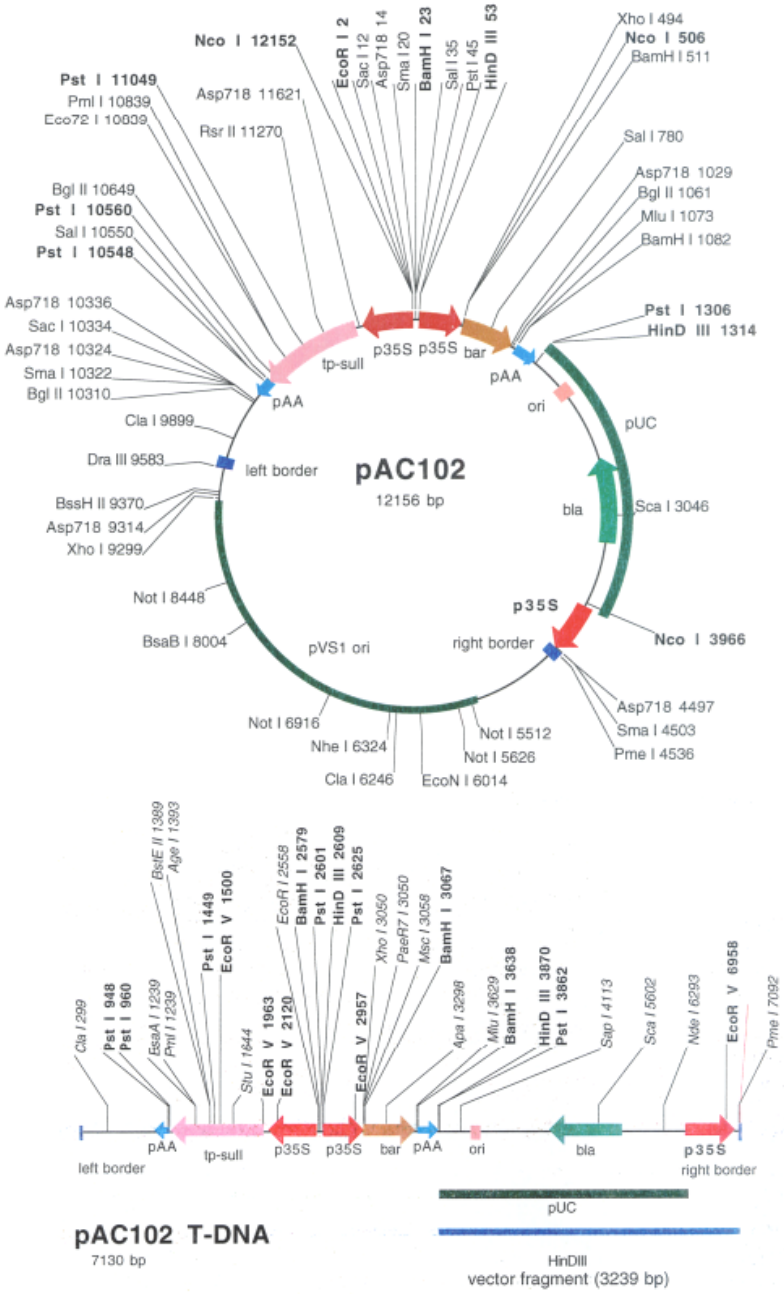

Fig. 3.12: T-DNA region map and pAC102 vector. Single sites are shown. Bold names=multiple sites.

Concluding remark:

The growth inhibition screen resulted in a mutant that showed a weak response upon elf18 treatment compared to similar treated wild type control seedlings. However, all other experiments (Oxidative Burst, Ethylene measurement and binding) suggest that the perception of elf26 (and also flg22) is functional, furthermore the PCR-result exclude a T-DNA-insertion in the *EFR* gene. To characterize this mutant further it is important to detect the localisation of the T-DNA insertion and map the affected gene(s), therefore the procedure of plasmid rescue must be modified to lead to a successful readout.

3.4 Direct toxic effect of peptide elicitors

Growth of plant pathogenic *Pseudomonas syringae* pv. t*omato* DC3000 can be restricted when *Arabidopsis thaliana* leaves were pre-infiltrated with the peptide elicitors flg22 and elf18 (Kunze, G. et al. 2004, Zipfel, C. et al. 2004). In order to address the question if this peptide elicitors have direct toxic effects on bacteria and are able to inhibit bacterial growth, growing bacteria were challenged with 10 µM flg22,10 µM elf18 and with combination of both peptides, respectively. Bacterial growth was monitored by analyzing samples from bacterial media and photometric determination of changes in density by OD_{600}. An over-night culture (KingB-Media, 28°C) of *Pseudomonas syringae* DC3000 was taken as start material. An aliquot of bacteria was transferred into fresh media and the first measurement was performed after 1 hour.

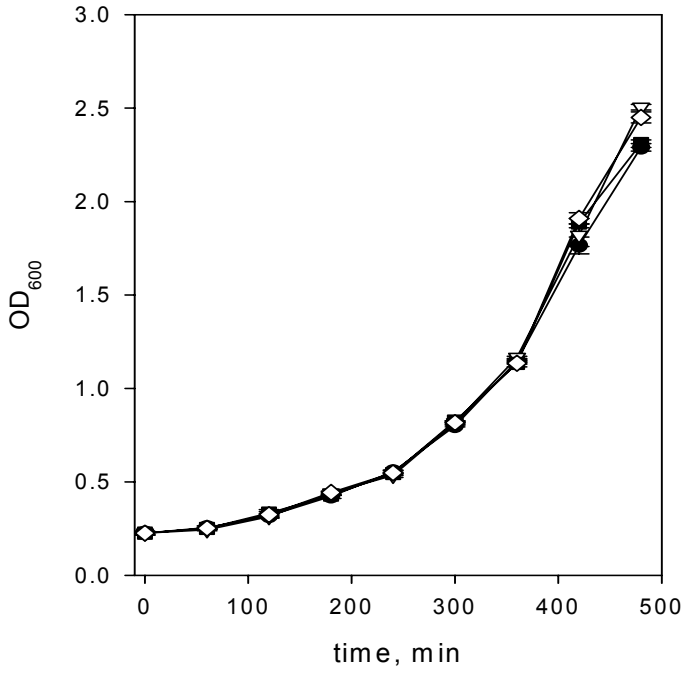

Fig. 3.13: Effect on bacterial growth in presence of 10µM single or combined elicitor treatment with flg22 and elf18

No effects towards bacterial growth were observed in the time range (8h) of this experiment. Furthermore no obvious difference between single or combined elicitor applications was detected. Our findings suggest that the effect on enhanced disease resistance (Chapter 1, Chapter 2) is not caused by a direct inhibiting effect from the peptides stimuli used to activate the plant defense system.

4 General Discussion

In this study the model plant *Arabidopsis thaliana* was used to analyze the microbial-plant interaction towards activation of early plant defense mechanisms. Arabidopsis was shown to respond to stimuli of diverse chemical nature and from a variety of different plant pathogens. The components that are able to activate immune reactions includes structures which are characteristic for bacteria such as flagellin (Felix, G. et al. 1999), lipopolysachharides (Zeidler, D. et al. 2004), harpins (Li, C. M. et al. 2005) and EF-Tu (this study). Sensing fungi and oomycetes components was demonstrated in case of PaNie, NPP1, Ergosterol, Chitin and Xylanase (Amborabe, B. E. et al. 2003, Fellbrich, G. et al. 2002, Ramonell, K. et al. 2005, Veit, S. et al. 2001, Zhang, B. et al. 2002)

A crucial step of a successful active defense in all living organisms is the early recognition of potential microbial invaders. In eukaryotes pattern recognition receptors (PRRs) are responsible for sensing microbial signals - the pathogen associated molecular patterns (PAMPs) (Janeway, C. A., Jr. and Medzhitov, R. 2002). Contradictory to the name, PAMPs are not only restricted to pathogens and are also present in non-pathogenic organisms. In plants the characterization of elicitors revealed a multitude of viral, bacterial, or fungal components, which trigger initiation of plant pathogen defense (Boller, T. 2005, Jones, D. A. and Takemoto, D. 2004, Nürnberger, T. et al. 2004). In addition plants signal perception is not limited to exogenous pathogen-derived signals. They are also able to detect endogenous plant-derived structures (D'Ovidio, R. et al. 2004) as result of a limited degradation of the plant cell wall, indicating a potential invasion. The structural diversity of elicitors suggests that plants must have evolved an enormous arsenal of perception systems for microbe-associated molecules.

PAMPs and defense

A common feature of all PAMPs is their highly conserved structure, an essential function for the pathogen, an exposed localization, absence in the (potential) host and they are characteristic for whole classes of microorganisms (Medzhitov, R. and Janeway, C. A. Jr. 2002). This PAMP-definition is based on observations from the animal field and our findings towards activation of plant defense mechanisms by the elicitor's flagellin and Elongation factor Tu raise a few doubts for a stringent definition like this.

Flagellin is a characteristic molecule in Gram-negative bacteria and highly conserved in the N-terminal elicitor-active motif (flg22) (Felix, G. et al. 1999). Furthermore no sequence homology exists in plants and this molecule is of functional importance for the bacteria. Plants are sessile and therefore require pathogen mobility, whereas mammals are mobile and able to spread infections by contact (Jones, D. A. and Takemoto, D. 2004). The flagellum is necessary for the initial colonization of the host tissue and acts as a virulence factor (Ramos, H. C. et al. 2004) in non-host plants. For example, non-motile mutants of *Pseudomonas syringae* pv. *tabaci* that are not able to produce flagellin, did not induce HR in tomato suspension cultured cells, whereas a mutant deficient in flagellum-assembling (release large amount of flagellin-monomers) induced a strong HR. This findings suggest that flagellin monomers of *Pseudomonas syringae* pv. *tabaci* acts as a strong elicitor to induce HR-associated cell death in non-host tomato cells (Shimizu, R. et al. 2003). The conserved domains, including the epitope that is recognized by the plants (flg22), are embedded at the inner part of the flagellum structure and only exposed in flagellin monomers (Smith, K. D. et al. 2003, Yonekura, K. et al. 2002, Yonekura, K. et al. 2003). Here the question arise how a plant receptor is able to sense this hidden motif? It is tempting to speculate if a pathogen however managed to overcome constitutive plant barriers (e.g. wax, cell walls) the flagellum is not of use anymore and probably released or enzymatically digested. In addition the process of assembling the flagellum is

related with high energetic throughput in biosynthesis (Blocker2003) and this resources might be needed elsewhere, e.g. for the infection process. This hypothesis is consistent with the finding that during early infection period, the flagellum production is down-regulated when the formation of the T-pilus (basis for secretion of effectors with the Type III secretion system, (TTSS)) is initiated (Lai, E. M. et al. 2000). A growing amount of evidence reported from plant and animal research, suggests that flagellin production is regulated by changes in the environment and like discussed before, might be deactivated after the infection process has started (Wang, Q. et al. 2005, Wolfgang, M. C. et al. 2004).

EF-Tu is a highly conserved (Lathe, W. C. and Bork, P. 2001) and essential protein, involved in protein translation (Jeppesen, M. G. et al. 2005, Rodnina, M. V. et al. 2005). Therefore it was considered to be a restrictive cytoplasmatic protein, but increasing amounts of publications showed that this is not the exclusive location for EF-Tu. In addition to the major cytoplasmic biosynthetic and metabolic role this protein might have several additional functions (e.g. EF-Tu in plants is able to protect chloroplast proteins from thermal aggregation and inactivation (Rao, D. et al. 2004) and might be involved in regulation of plant adaptation towards environmental stress (Singh, B. N. et al. 2004)).

Studies on bacterial EF-Tu are important in pharmaceutical research as a target for antibiotics (Hogg, T. et al. 2002) inhibiting bacterial biosynthesis and therefore stopping bacterial development in infected tissues. The interaction between pathogenic bacteria and components of the host's extracellular matrix strongly influences the initiation and establishment of a successful infection (Prithiviraj, B. et al. 2005b, Prithiviraj, B. et al. 2005a). EF-Tu was found to mediate attachment of *Lactobacillus johnsonii* to human intestinal cells and mucins (Granato, D. et al. 2004a) and therefore determined at the bacterial surface. Another similar adhesion mediation function was reported in case of *Mycoplasma pneumonia,* where EF-Tu was found to be surface translocated and contributes to the infection of potential hosts cells. Despite from our work no bacterial EF-Tu was

reported to implicate in immune responses in plants, but concerning the PAMP definition (see above) a number of questions can not answered easily. Even when human-pharmaceutical reports revealed the surface location of this protein, the recognized epitope in plants (elf18) is not fully exposed concerning the 3-dimensional structure (Introduction). Only the first 8 amino acids can be easily targeted by the putitative receptor, the other part is embedded in Domain 1. However, similar like the regulation of flagellin biosynthesis towards changes in the extracellular environment, *E.coli* EF-Tu was reported to be released upon osmotic downshock to the periplasm by an unknown mechanism (Berrier, C. et al. 2000) consistent with our observation of EF-Tu-based activity in the supernatant of bacteria cultures (Chapter 1). This finding allow to speculate that the change of conditions after a bacterium has entered the apoplast-region in host plants led to release of this protein, underlined by the detection of released EF-Tu from *Xanthomonas campestris* pv. c*ampestris* in secretome-analysis (Watt, S. A. et al. 2005). These experimental results can still not answer the question how an EF-Tu receptor (Appendix 4) can target internal protein sequences of this protein and has to be further evaluated (e.g. search for plant hydrolytic enzymes). In addition the experimental evidence that direct interaction between both PAMPs and their potential receptors (FLS2, EFR1) takes place (Bauer, Z. et al. 2001, Chinchilla, D. et al. 2005), Appendix 4) are not targeted to address the exact binding domains. The LRR-domain, present in both receptors may act as a protein-protein interaction domain (Dievart, A. and Clark, S. E. 2004), like recently demonstrated for brassinosteroid binding between LRR-repeats of BRI1 receptor kinase (Kinoshita, T. et al. 2005).

Furthermore homologues of EF-Tu protein exists in all living organisms and Arabidopsis contain at least three EF-Tu proteins (Chapter1). It is tempting to speculate that the differentiation between self and non-self EF-Tu is regulated though sequence variations (Appendix 1) and posttranslational modifications (Kunze, G. et al. 2004), like reported for the participation of EF-Tu in virulence of

Listeria monocytogenes (Archambaud, C. et al. 2005) and the flagellin recognition in human TLR5 (Lee, S. K. et al. 2003).

PAMP perception in innate immune system of plants and animals is based on redundancy. Arabidopsis can sense flagellin, EF-Tu and LPS, all characteristics from Gram-negative bacteria. Therefore this higher efficiency of recognition is of great advantage for the plant. In case of one failing perception system, still other mechanisms are present to activate defense mechanisms (Pfund, C. et al. 2004) (Chapter2). Considering the huge repertoire of PRRs in the plants, pathogens must have evolved ways to hide or to manipulate PAMP perception for a successful colonization. PAMPs are highly conserved, but small differences in sequences without loss of functionality exist. For example flagellin sequences (flg22) of plant-associated bacteria like *Sinorrhizobium meliloti, Agrobacterium tumefaciens* and *Ralstonia solanacearum* have no capability to induce defense reactions (Felix, G. et al. 1999, Pfund, C. et al. 2004), but elf18 from this bacteria is highly elicitor-active (Chapter 1) (EC_{50} < 0.5 nM). In direct comparison the elf18 peptides derived from sequences of the phytopathogenic bacteria *Pseudomonas syringae* pv. *tomato* DC3000 and *Xylella fastidiosa* show reduced activity (EC_{50}: 35-190 nM). Contrastive to the very potent *Xanthomonas axonopodis* pv. *citri* (Xac) elf-motif (unpublished results, EC_{50} < 0.5 nM), *Xanthomonas campestris* (Xcc) EF-Tu is 100-fold weaker active. Interestingly the relation found for flg22 sequences is identical, suggesting that these bacteria have managed to modify their EF-Tu and Flagellin-sequences evolutionary without loosing functionality and virulence – again corroborating by very recently results found in flagellin TLR5 perception (ndersen-Nissen, E. et al. 2005) in human.

In addition of sequences variations, pathogens are able to actively interfere in the plants basal- or Avr-mediated host defense (Alfano, J. R. and Collmer, A. 2004a). Invasive pathogenic bacteria are trying to overcome the defense mechanisms of their animal/plant host and to proliferate in its tissues. One of these systems

involves the delivery of bacterial proteins with different functions inside target cells by extracellularly located bacteria in close contact with the host cell surface (Espinosa, A. and Alfano, J. R. 2004). In this context the fact that bacterial EF-Tu might be released and was found to be involved in binding to potential host cells (see before), offers the possibility that EF-Tu might act as a required virulence factor for initial infection processes. Pathogens might have evolved the effector proteins and their secretion into the host cell (with Type III secretion system, TTSS), as a consequence of the high amount of potential detection mechanisms in the plant. A functional TTSS is of major importance for the virulence of many bacteria. TTSSs were found in various pathogens, including the animal pathogens *Yersinia* spp., *Salmonella typhimurium*, *Shigella flexneri, Escherichia coli* and *Pseudomonas aeruginosa* and the plant pathogens *Pseudomonas syringae, Erwinia amylovora*, *Xanthomonas campestris* and *Ralstonia solanacearum* (Espinosa, A. and Alfano, J. R. 2004). For example YopE (Yops, *Yersinia* outer protein), a Yersinia secreted protein, can disrupt the actin microfilament structure of host cells leading to cell-rounding which is thought to contribute to yersinia resist against phagocytosis (Cornelis, G. R. and Van Gijsegem, F. 2000). Another secreted protein YopJ/P *(Yersinia* cysteine protease) inhibits defense-related MAPK kinase activity and therefore suppresses PAMP triggered innate immunity (Meijer, L. K. et al. 2000).

The major functions for TTSS secreted effectors in plants (named Hrp-system, hypersensitive response and pathogenicity (Collmer, A. et al. 2002) have been postulated to be acquiring of nutrients and suppressing the host defense mechanisms (Chang, J. H. et al. 2004). Interestingly a recent report analyzed the possibility that secreted (or otherwise delivered) bacterial proteins, enable epiphytic living bacteria to have excess to nutrients below the cuticle, without damaging of cuticular membranes. 46 bacterial proteins, including Flagellin and Elongation Factor Tu, were found below the plant cuticle membrane, suggesting an important role for Flagellin and EF-Tu in this specific interaction (Singh, P. et

al. 2004) and again demonstrate the existence of an unknown release mechanisms for these proteins.

More details are known for the second suppression function of effector proteins. For example a protein tyrosine phosphatase (HopPtoD2) is secreted by *Pseudomonas syringae* and inhibits defense-related MAPK kinase activity (Chapter 2) (Asai, T. et al. 2002, Cardinale, F. et al. 2000) in plants. The only known proteins phosphorylated at threonine and tyrosines are the MAPKs (Zhang, S. and Klessig, D. F. 2000), even when recent studies suggest a similar regulation process existing for SYMRK (Yoshida, Satoko and Parniske, Martin 2005) (Introduction). Thus, type III effectors can suppress signal transduction pathways activated by PRR surveillance systems. Furthermore the effector AvrPtoB is able to suppress programmed cell death (PCD) and immunity in the in tomato (Abramovitch, Robert B. and Martin, Gregory B. 2005). Interestingly and underlining the problem with the definition of PAMPs, Type III effectors are not only limited to pathogens. A type III effector from the symbiontic bacterium *Rhizobium*, NopL, was found to actively suppress induction of PR genes in tobacco, suggesting that symbionts may also benefit from suppressing plant innate immunity (Bartsev, A. V. et al. 2004) to a certain extent.

The intracellular plant R proteins perceive bacterial Avr proteins (Introduction) and mediate resistance to the particular pathogens, whereas extracellular PAMP perception is mostly based on receptor like kinases e.g. FLS2 (Zipfel, C. et al. 2004). Similar in the animal system: Toll-like receptors (TLRs) sense PAMPs extracellularly, whereas the NOD-proteins act as an intracellular perception system (Girardin, S. E. and Philpott, D. J. 2004b). The plant R proteins contain different structures. CC-NBS-LRR R proteins consist of an N-terminal coiled-coil (CC) domain, a central nucleotide binding site (NBS) and a C-terminal LRR domain (Jones, D. A. and Takemoto, D. 2004), whereas the TIR-NBS-LRR possess a TIR (TIR IL-1 domain) instead of a CC domain. The first and largest group is mainly responsible for detecting fungal and viral Avr proteins (Nimchuk,

Z. et al. 2003, Peart, J. R. et al. 2005), whereas the second family in addition plays also a role in sensing bacterial derived signals (Gassmann, W. et al. 1999, van der Biezen, E. A. et al. 2002). R proteins containing WRKY domains (Chapter 3) (Deslandes, L. et al. 2002) were also identified. The Pto R protein, a serine/threonine kinase involved in the detection of Avr proteins from *Pseudomonas syringae* (see before), is interestingly not a NBS-LRR. However, the Pto mediated recognition is based on Prf, a CC-NBS-LRR type R protein (Pedley, K. F. and Martin, G. B. 2004), indicating that receptor complexes in plants are used for signal perception. Association with different proteins was shown for other receptor-ligand systems in plants. CLV1, involved in maintaining the apical meristem in Arabidopsis (Trotochaud, A. E. et al. 2000), is a two component complex (Torii, K. U. 2004) probably consists of a CLV1-CLV2 heterodimer (Jeong, S. et al. 1999). In addition CLV3 and other proteins (e.g. kinase-associated protein phosphatase (KAPP)), are required to activate this receptor complex (Cock, J. M. et al. 2002). Similar during establishing of symbiosis two receptors, NFR1 and NFR5, are required for sensing bacterial Nod-factor (Parniske, M. and Downie, J. A. 2003b), whereas in this case no direct interaction was demonstrated. Furthermore the perception of pathogenic signals in animals is also not a direct interaction and involves many different proteins and individual steps. This was demonstrated for sensing LPS, which requires in addition to the transmembrane receptor TLR4 the proteins sCD14 and MD-2 (Miyake, K. 2004). This indicates that perception systems across kingdom borders are conceptual similar.

Plants have no direct homologue for TLR receptors, but a huge amount of RLKs (Shin-Han, S. and Bleecker, A. B. 2003) and receptor-like proteins (RLPs) (Greenberg, J. T. and Yao, N. 2004a), transmembrane proteins that also possess an extracellular LRR structure, but lacking an intracellular kinase domain (Shiu, S. H. et al. 2004). The receptor-like proteins are also part of the PAMP perception network as demonstrated e.g. for fungal Xylanase (Ron, M. and Avni, A. 2004) and resistance towards *Peronospora parasitica* (Tor, M. et al. 2004).

A common feature of perception components involved in diverse regulatory cases are the LRR-domains. Not only in plants also in animals this structure seemed to be of importance for sensing various defense-related (Ballvora, A. et al. 2002) and developmental (Matsubayashi, Y. et al. 2002) signals. The fact that in animals (TLR5, (ndersen-Nissen, E. et al. 2005, Tallant, T. et al. 2004)) and plants Flagellin (FLS2, (Gómez-Gómez, L. and Boller, T. 2002) can be detected from receptors both containing LRRs and without sharing sequence similarity, underlines that they might have evolved in a convergent evolutionary way in this organisms.

Despite of the high number of receptor-like kinases in the genome of *Arabidopsis thaliana* (Dievart, A. and Clark, S. E. 2004, Shin-Han, S. and Bleecker, A. B. 2003) only very little is known about their implications in biological processes and their functional mechanisms. Bacterial extracts (with or without flagellin) induce similar basal defenses (Chapter1, Chapter2, (Zipfel, C. et al. 2004)). FLS2 and EFR1 are the so far only known PRRs in plants, involved in Arabidopsis innate immunity. With distinct highly specific receptors belonging to the same family (LRR-XII), Arabidopsis can sense flagellin (Felix, G. et al. 1999) and EF-Tu (Kunze, G. et al. 2004). The gene expression profile (Chapter2) after elicitor treatment showed a large extent of induced RLKs from many families, including regulated FLS2 and EFR1, suggesting that many more RLKs might act as PRRs as known so far. Our postulated hypothesis, based on the ATH1 analysis, that sensing one PAMP is a regulating perception mechanism for other elicitor signals, could be demonstrated (Chapter2) and give indications for the existence of a interacting signaling network.

The identification of a novel elicitor purified from an elicitor-active bacterial preparation is one possibility to determine other structures to further investigate their roles in plant defense. In combination with approaches in our laboratory and

other recent findings by studying in particular RLKs from the LRR-class, these results contribute for our understanding of PAMP-Ligand-receptor interaction and signaling and demonstrate the importance of this group towards the activation of plant defense mechanisms. Nevertheless considering the 600 RLKs of Arabidopsis a huge amount of ligands (elicitors, regulatory signals) and their corresponding receptors has still to be identified. Furthermore, based on experimental results discussed before, it would be interesting to evaluate the role of bacterial EF-Tu with regard to animal immunity in more details.

Appendix

Appendix 1: Alignment of EF-Tu (elf18) sequences:

			ALIGNMENTS			
	Query sequence: Elf18		1_14925	1	SKEKFERTKPHVNVGTIG	18
gi|31076641|sp|Q877T5|EFTU_VIBPA Elongation factor Tu (EF-Tu)	46	3e-06	31076641	2	.....	19
gi|24211680|sp|Q8ZAN8|EFT2_YERPE Elongation factor Tu-B (EF-Tu-B)	46	3e-06	24211680	2	.....	19
gi|24211692|sp|Q9KUZ6|EFT2_VIBCH Elongation factor Tu-B (EF-Tu-B)	46	3e-06	24211692	2	.....	19
gi|24211693|sp|Q9KV37|EFT1_VIBCH Elongation factor Tu-A (EF-Tu-A)	46	3e-06	24211693	2	.....	19
gi|24211681|sp|Q8ZJB2|EFT1_YERPE Elongation factor Tu-A (EF-Tu-A)	46	4e-06	24211681	2	.....	19
gi|1169492|sp|P43926|EFTU_HAEIN Elongation factor Tu (EF-Tu)	46	4e-06	1169492	2	.....	19
gi|119201|sp|P02990|EFTU_ECOLI Elongation factor Tu (EF-Tu) (P-43)	46	5e-06	119201	2	.....	19
gi|33301069|sp|Q83JC4|EFTU_SHIFL Elongation factor Tu (EF-Tu)	46	5e-06	33301069	2	.....	19
gi|119212|sp|P21694|EFTU_SALTY Elongation factor Tu (EF-Tu)	46	5e-06	119212	2	.....	19
gi|13431463|sp|P57966|EFT2_PASMU Elongation factor Tu-B (EF-Tu-B)	45	5e-06	13431463	2	.....	19
gi|13431462|sp|P57939|EFT1_PASMU Elongation factor Tu-A (EF-Tu-A)	45	6e-06	13431462	2	.....	19
gi|24211676|sp|Q8XGZ0|EFTU_RALSO Elongation factor Tu (EF-Tu)	45	1e-05	24211676	2	A.....	19
gi|31076650|sp|Q8DCQ7|EFTU_VIBVU Elongation factor Tu (EF-Tu)	44	1e-05	31076650	2	.....V.....	19
gi|416937|sp|P33165|EFTU_BACFR Elongation factor Tu (EF-Tu)	44	1e-05	416937	2	A.....I....	19
gi|24211685|sp|Q97EH5|EFTU_CLOAB Elongation factor Tu (EF-Tu)	43	2e-05	24211685	2	A.....I....	19
gi|24211691|sp|Q9JRI5|EFTU_NEIMA Elongation factor Tu (EF-Tu)	43	3e-05	24211691	2	A.....S.....	19
gi|1352356|sp|P48864|EFTU_NEIGO Elongation factor Tu (EF-Tu)	43	3e-05	1352356	2	A.....S.....	19
gi|24211673|sp|Q8R7V2|EFT1_THETN Elongation factor Tu-A (EF-Tu-A)	43	3e-05	24211673	2	A.Q.....	19
gi|24211672|sp|Q8R7T8|EFT2_THETN Elongation factor Tu-B (EF-Tu-B)	43	3e-05	24211672	2	A.Q.....	19
gi|12230896|sp|P09591|EFTU_PSEAE Elongation factor Tu (EF-Tu)	42	4e-05	12230896	2	A.....N.....	19
gi|24211667|sp|Q8NL22|EFTU_XANAC Elongation factor Tu (EF-Tu)	42	4e-05	24211667	2	A.A.....	19
gi|1169498|sp|P42481|EFTU_THICU Elongation factor Tu (EF-Tu)	42	6e-05	1169498	2	A.S.....	19
gi|29611729|sp|P59506|EFTU_BUCBP Elongation factor Tu (EF-Tu)	41	8e-05	29611729	2	.....K.S...I.....	19
gi|2506377|sp|P42479|EFTU_STIAU Elongation factor Tu (EF-Tu)	41	8e-05	2506377	2	A.....N.....I....	19
gi|3122064|sp|O33594|EFTU_STRAU Elongation factor Tu (EF-Tu)	41	8e-05	3122064	2	A.A.....I....	19
gi|1169488|sp|P42439|EFTU_CORGL Elongation factor Tu (EF-Tu)	41	8e-05	1169488	2	A.A.....I....	19
gi|22654233|sp|O31298|EFTU_BUCAP Elongation factor Tu (EF-Tu)	41	8e-05	22654233	2	.....Q.V...I.....	19
gi|24211678|sp|Q8YHQ4|EFTU_BRUME Elongation factor Tu (EF-Tu)	41	9e-05	24211678	2	A.S.....I....	19
gi|232043|sp|P29542|EFT1_STRRA Elongation factor Tu-1 (EF-Tu-1)	41	1e-04	232043	2	A.A.....I....	19
gi|24211688|sp|Q99QM0|EFTU_CAUCR Elongation factor Tu (EF-Tu)	41	1e-04	24211688	2	A.....C.I....	19
gi|416939|sp|P33167|EFTU_BURCE Elongation factor Tu (EF-Tu)	41	1e-04	416939	2	A.G.....	19
gi|24211675|sp|Q8XFP8|EFTU_CLOPE Elongation factor Tu (EF-Tu)	41	1e-04	24211675	2	..A....S.....I....	19
gi|2494258|sp|Q53871|EFT1_STRCU Elongation factor Tu-1 (EF-Tu-1)	41	1e-04	2494258	2	A.A.....I....	19
gi|6226607|sp|P13537|EFTU_THEMA Elongation factor Tu (EF-Tu)	41	1e-04	6226607	2	A....V.....	19
gi|3122093|sp|P95724|EFTU_STRCJ Elongation factor Tu (EF-Tu)	41	1e-04	3122093	2	A.A.....I....	19
gi|729407|sp|P40174|EFT1_STRCO Elongation factor Tu-1 (EF-Tu-1)	41	1e-04	729407	2	A.A.....I....	19

gi|1706620|sp|P50068|EFTU_UREPA Elongation factor Tu (EF-Tu)	41	1e-04	1706620	2	A.A.....I....	19
gi|24211677|sp|Q8Y422|EFTU_LISMO Elongation factor Tu (EF-Tu)	41	1e-04	24211677	2	A....D.S.....I....	19
gi|11182421|sp|O31297|EFTU_BUCAI Elongation factor Tu (EF-Tu)	41	1e-04	11182421	2	.....Q.L...I.....	19
gi|119203|sp|P26184|EFTU_FLESI Elongation factor Tu (EF-Tu)	41	1e-04	119203	2	..Q.Y..K.........	19
gi|24211683|sp|Q927I6|EFTU_LISIN Elongation factor Tu (EF-Tu)	41	1e-04	24211683	2	A....D.S.....I....	19
gi|34222600|sp|Q8KTA3|EFTU_RICRH Elongation factor Tu (EF-Tu)	41	2e-04	34222600	2	A.A.....I....	19
gi|416941|sp|P30768|EFTU_MYCLE Elongation factor Tu (EF-Tu)	41	2e-04	416941	2	A.A.....I....	19
gi|6015080|sp|O50306|EFTU_BACST Elongation factor Tu (EF-Tu)	41	2e-04	6015080	2	A.A.....I....	19
gi|24211684|sp|Q92GW4|EFTU_RICCN Elongation factor Tu (EF-Tu)	41	2e-04	24211684	2	A.A.....I....	19
gi|34222595|sp|Q8KI92|EFTU_RICRI Elongation factor Tu (EF-Tu)	41	2e-04	34222595	2	A.A.....I....	19
gi|34222597|sp|Q8KT97|EFTU_RICFE Elongation factor Tu (EF-Tu)	41	2e-04	34222597	2	A.A.....I....	19
gi|34222599|sp|Q8KTA1|EFTU_RICMO Elongation factor Tu (EF-Tu)	41	2e-04	34222599	2	A.A.....I....	19
gi|34222601|sp|Q8KTA6|EFTU_RICPA Elongation factor Tu (EF-Tu)	40	2e-04	34222601	2	A.A.....I....	19
gi|31340063|sp|Q8D240|EFTU_WIGBR Elongation factor Tu (EF-Tu)	40	2e-04	31340063	2	.....Q.I...I.....	19
gi|3913574|sp|O50293|EFTU_AQUPY Elongation factor Tu (EF-Tu)	40	2e-04	3913574	2	A.....E........	19
gi|3913576|sp|O66429|EFTU_AQUAE Elongation factor Tu (EF-Tu)	40	2e-04	3913576	2	A.....E........	19
gi|34222596|sp|Q8KT95|EFTU_RICTY Elongation factor Tu (EF-Tu)	40	2e-04	34222596	2	A.A.....I....	19
gi|34222598|sp|Q8KT99|EFTU_RICHE Elongation factor Tu (EF-Tu)	40	2e-04	34222598	2	A.A.....I....	19
gi|6226606|sp|P48865|EFTU_RICPR Elongation factor Tu (EF-Tu)	40	2e-04	6226606	2	A.A.....I....	19
gi|2494256|sp|P56003|EFTU_HELPY Elongation factor Tu (EF-Tu)	40	2e-04	2494256	2	A....N......I....	19
gi|18202638|sp|Q981F7|EFTU_RHILO Elongation factor Tu (EF-Tu)	40	2e-04	18202638	2	A.G.....I....	19
gi|24211671|sp|Q8R603|EFTU_FUSNN Elongation factor Tu (EF-Tu)	40	3e-04	24211671	2	A...Y..S.....I....	19
gi|38372189|sp|Q81VT2|EFTU_BACAN Elongation factor Tu (EF-Tu) >g...	40	3e-04	38372189	2	A.A....S.....I....	19
gi|24211679|sp|Q8YP63|EFTU_ANASP Elongation factor Tu (EF-Tu)	39	3e-04	24211679	2	ARA.....I....	19
gi|416942|sp|P33169|EFTU_SHEPU Elongation factor Tu (EF-Tu)	39	4e-04	416942	2	A.A....I.....	19
gi|2506376|sp|P23568|EFTU_MYCPN Elongation factor Tu (EF-Tu)	39	4e-04	2506376	2	AR...D.S.....	19
gi|232044|sp|P29543|EFT2_STRRA Elongation factor Tu-2 (EF-Tu-2)	39	4e-04	232044	2	A.A..Q......I....	19
gi|1169493|sp|P42477|EFTU_HERAU Elongation factor Tu (EF-Tu)	39	4e-04	1169493	2	A.Q....N...I.I....	19
gi|119208|sp|P13927|EFTU_MYCGE Elongation factor Tu (EF-Tu)	39	4e-04	119208	2	AR...D.S.....	19
gi|24211674|sp|Q8UE16|EFTU_AGRT5 Elongation factor Tu (EF-Tu)	39	4e-04	24211674	2	A.S....N.....I....	19
gi|38257505|sp|Q88VE0|EFTU_LACPL Elongation factor Tu (EF-Tu)	39	4e-04	38257505	2	A..HY.......I....	19
gi|2494255|sp|P75022|EFTU_AGRTU Elongation factor Tu (EF-Tu)	39	5e-04	2494255	2	A.S....N.....I....	19
gi|26006962|sp|P72483|EFTU_STRMU Elongation factor Tu (EF-Tu)	39	5e-04	26006962	2	A...YD.S.....I....	19
gi|24211682|sp|Q925Y6|EFTU_RHIME Elongation factor Tu (EF-Tu)	39	5e-04	24211682	2	A.S....N.....I....	19
gi|6015081|sp|O69303|EFTU_CAMJE Elongation factor Tu (EF-Tu)	39	5e-04	6015081	2	A....S.N.....I....	19
gi|399422|sp|P31501|EFTU_MYCTU Elongation factor Tu (EF-Tu)	39	5e-04	399422	2	A.A..Q......I....	19
gi|119207|sp|P18906|EFTU_MYCGA Elongation factor Tu (EF-Tu)	39	5e-04	119207	2	A..R.D.S.....I....	19
gi|14194714|sp|P82559|EFTU_STRPY Elongation factor Tu (EF-Tu)	38	6e-04	14194714	2	A...YD.S.....I....	19
gi|25090248|sp|Q8K872|EFTU_STRP3 Elongation factor Tu (EF-Tu)	38	6e-04	25090248	2	A...YD.S.....I....	19
gi|37999654|sp|Q88QN7|EFT2_PSEPK Elongation factor Tu-B (EF-Tu-B)	38	6e-04	37999654	2	A....D.SL.....	19
gi|1169497|sp|P42480|EFTU_TAXOC Elongation factor Tu (EF-Tu)	38	6e-04	1169497	2	A..T.D.S.....I....	19
gi|37999655|sp|Q88QP8|EFT1_PSEPK Elongation factor Tu-A (EF-Tu-A)	38	6e-04	37999655	2	A....D.SL.....	19
gi|1169485|sp|P42471|EFTU_BRELN Elongation factor Tu (EF-Tu)	38	6e-04	1169485	2	A.AS.....I....	19
gi|24211668|sp|Q8P1W4|EFTU_STRP8 Elongation factor Tu (EF-Tu)	38	7e-04	24211668	2	A...YD.S.....I....	19

gi|24211686|sp|Q97PV3|EFTU_STRPN Elongation factor Tu (EF-Tu)	38	7e-04	24211686	2	A..YD.S.....I....	19
gi|416940|sp|P33168|EFTU_DEISP Elongation factor Tu (EF-Tu)	38	7e-04	416940	2	A.GT..........	19
gi|416943|sp|P33170|EFTU_STROR Elongation factor Tu (EF-Tu)	38	7e-04	416943	2	A..YD.S.....I....	19
gi|416944|sp|P33171|EFTU_SYNP7 Elongation factor Tu (EF-Tu)	38	8e-04	416944	2	ARA.........A.I....	19
gi|119193|sp|P18668|EFTU_SYNP6 Elongation factor Tu (EF-Tu)	38	8e-04	119193	2	ARA.........A.I....	19
gi|7404358|sp|P26622|EFTU_CHLTR Elongation factor Tu (EF-Tu)	38	0.001	7404358	2	...T.Q.N...I.I....	19
gi|13626414|sp|Q9PK73|EFTU_CHLMU Elongation factor Tu (EF-Tu)	38	0.001	13626414	2	...T.Q.N...I.I....	19
gi|1706598|sp|P50062|EFTU_BORBU Elongation factor Tu (EF-Tu)	38	0.001	1706598	2	A..V.Q.....M.....	19
gi|7674029|sp|Q9ZK19|EFTU_HELPJ Elongation factor Tu (EF-Tu)	38	0.001	7674029	2	A....N..N....I....	19
gi|9789747|sp|Q9R342|EFTU_DEIRA Elongation factor Tu (EF-Tu)	38	0.001	9789747	2	A.GT.........I....	19
gi|33301059|sp|Q822I4|EFTU_CHLCV Elongation factor Tu (EF-Tu)	38	0.001	33301059	2	...T.Q.N...I.I....	19
gi|119206|sp|P09953|EFTU_MICLU Elongation factor Tu (EF-Tu)	38	0.001	119206	2	A.A.......A...I....	19
gi|119213|sp|P13552|EFTU_SPIPL Elongation factor Tu (EF-Tu)	38	0.001	119213	2	ARA....N.....I....	19
gi|6831536|sp|Q9Z9A7|EFTU_CHLPN Elongation factor Tu (EF-Tu)	37	0.001	6831536	2	...T.Q.N...I.I....	19
gi|38258922|sp|P50064|EFTU_GLOVI Elongation factor Tu (EF-Tu)	37	0.001	38258922	2	ARA....N.....I....	19
gi|1169489|sp|P42474|EFTU_CYTLY Elongation factor Tu (EF-Tu)	36	0.002	1169489	2	A..T.D.S...L.I....	19
gi|2494257|sp|P72231|EFTU_PLARO Elongation factor Tu (EF-Tu)	36	0.003	2494257	2	A.A.L.......M.I....	19
gi|7676153|sp|O83217|EFTU_TREPA Elongation factor Tu (EF-Tu)	36	0.003	7676153	2	A....A...V.M......	19
gi|37999596|sp|Q889X3|EFTU_PSESM Elongation factor Tu (EF-Tu)	36	0.004	37999596	2	A....D.SL..C......	19
gi|37538296|sp|P42482|EFTU_WOLSU Elongation factor Tu (EF-Tu)	36	0.004	37538296	2	A....VKN.....I....	19
gi|38372234|sp|Q8ETY4|EFTU_OCEIH Elongation factor Tu (EF-Tu)	36	0.004	38372234	2	A....D.S.S......L.	19
gi|24211690|sp|Q9CEI0|EFTU_LACLA Elongation factor Tu (EF-Tu)	35	0.006	24211690	2	A..VYD.S.....I....	19
gi|1169491|sp|P42476|EFTU_FLAFE Elongation factor Tu (EF-Tu)	35	0.006	1169491	2	A..T.K.E.....I....	19
gi|42560199|sp|P60338|EFT1_THETH Elongation factor Tu-A (EF-Tu-A)	35	0.006	42560199	2	A.GE.V...........	19
gi|2494260|sp|P74227|EFTU_SYNY3 Elongation factor Tu (EF-Tu)	35	0.006	2494260	2	ARA.....D...I....	19
gi|38257610|sp|Q8EX18|EFTU_MYCPE Elongation factor Tu (EF-Tu)	35	0.008	38257610	2	A.Q..D.S.A...I....	19
gi|38257578|sp|Q8CQ81|EFTU_STAEP Elongation factor Tu (EF-Tu)	35	0.008	38257578	2	A....D.S.E.A.I....	19
gi|25452937|sp|Q8KAH0|EFTU_CHLTE Elongation factor Tu (EF-Tu)	35	0.008	25452937	2	A..SYK.D.....I....	19
gi|42560544|sp|P60339|EFT2_THETH Elongation factor Tu-B (EF-Tu-B)	35	0.009	42560544	2	A.GE.I...........	19
gi|24211689|sp|Q99W61|EFTU_STAAM Elongation factor Tu (EF-Tu)	35	0.009	24211689	2	A....D.S.E.A.I....	19
gi|399423|sp|Q01698|EFTU_THEAQ Elongation factor Tu (EF-Tu)	35	0.009	399423	2	A.GE.I...........	19
gi|1169487|sp|P42473|EFTU_CHLVI Elongation factor Tu (EF-Tu)	35	0.010	1169487	2	A..SYK.D.....I....	19
gi|7674027|sp|Q9Z9L6|EFTU_BACHD Elongation factor Tu (EF-Tu)	35	0.010	7674027	2	A....D.S.T.A.I....	19
gi|32129506|sp|Q877P8|EFTU_XYLFT Elongation factor Tu (EF-Tu)	35	0.010	32129506	2	AQD..K...L........	19
gi|20138044|sp|Q9P9Q9|EFTU_XYLFA Elongation factor Tu (EF-Tu)	34	0.011	20138044	2	AQD..K...L........	19
gi|416938|sp|P33166|EFTU_BACSU Elongation factor Tu (EF-Tu) (P-40)	34	0.015	119209	2	A.LD.D.S.....I....	19
gi|6015082|sp|O50340|EFTU_FERIS Elongation factor Tu (EF-Tu)	33	0.020	1169495	2	A.QN.V.S...I...A..	19
gi|19859281|sp|P40175|EFT3_STRCO Elongation factor Tu-3 (EF-Tu-3)	33	0.034	19859281	2	..TAYV.....L.I..M.	19
gi|232045|sp|P29544|EFT3_STRRA Elongation factor Tu-3 (EF-Tu-3)	32	0.037	232045	2	..TAYV.....L.I..M.	19
gi|544234|sp|P35644|EFTU_EIKCO Elongation factor Tu (EF-Tu)	32	0.043	544234	2	A.G....S..........	19
gi|24211670|sp|Q8PC59|EFT1_XANCP Elongation factor Tu-A (EF-Tu-A)	30	0.23	24211670	2	ARA..L.E.L........	19
gi|24211669|sp|Q8PC51|EFT2_XANCP Elongation factor Tu-B (EF-Tu-B)	30	0.23	24211669	2	ARA..L.E.L........	19

Appendix 2: T-DNA sequence of pAC102

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   1 TCGAGGGGGG GCCCGGTACC GTGAACGTCG GCTCGATTGT ACCTGCGTTC
  51 AAATACTTTG CGATCGTGTT GCGCGCCTGC CCGGTGCGTC GGCTGATCTC
 101 ACGGATCGAC TGCTTCTCTC GCAACGCCAT CCGACGGATG ATGTTTAAAA
 151 GTCCCATGTG GATCACTCCG TTGCCCCGTC GCTCACCGTG TTGGGGGGAA
 201 GGTGCACATG GCTCAGTTCT CAATGGAAAT TATCTGCCTA ACCGGCTCAG
 251 TTCTGCGTAG AAACCAACAT GCAAGCTCCA CCGGGTGCAA AGCGGCAGCG
 301 GCGGCAGGAT ATATTCAATT GTAAATGGCT TCATGTCCGG GAAATCTACA
 351 TGGATCAGCA ATGAGTATGA TGGTCAATAT GGAGAAAAAG AAAGAGTAAT
 401 TACCAATTTT TTTTCAATTC AAAAATGTAG ATGTCCGCAG CGTTATTATA
 451 AAATGAAAGT ACATTTTGAT AAAACGACAA ATTACGATCC GTCGTATTTA
 501 TAGGCGAAAG CAATAAACAA ATTATTCTAA TTCGGAAATC TTTATTTCGA
 551 CGTGTCTACA TTCACGTCCA AATGGGGGCT TAGATGAGAA ACTTCACGAT
 601 CGATGCCTTG ATTTCGCCAT TCCCAGATAC CCATTTCATC TTCAGATTGG
 651 TCTGAGATTA TGCGAAAATA TACACTCATA TACATAAATA CTGACAGTTT
 701 GAGCTACCAA TTCAGTGTAG CCCATTACCT CACATAATTC ACTCAAATGC
 751 TAGGCAGTCT GTCAACTCGG CGTCAATTTG TCGGCCACTA TACGATAGTT
 801 GCGCAAATTT TCAAAGTCCT GGCCTAACAT CACACCTCTG TCGGCGGCGG
 851 GTCCCATTTG TGATAAATCC ACCATCACAA TAGATAGTCT AATGGACGAA
 901 AAAGGCGAAT ATTTCGATGC TGAGATTCGA CGCAATTAAT TCGAGAAAAA
 951 TCCCGTGATT GATGCTGTTG AGTTACCAAT AATATGGGCA GCGAAGGCCA
1001 TTTAATTATA AGATCTGATC CCCGGGTACC GAGCTCGGTA CCCACTGGAT
1051 TTTGGTTTTA GGAATTAGAA ATTTTATTGA TAGAAGTATT TTACAAATAC
1101 AAATACATAC TAAGGGTTTC TTATATGCTC AACACATGAG CGAAACCCTA
1151 TAAGAACCCT AATTCCCTTA TCTGGGAACT ACTCACACAT TATTATAGAG
1201 AGAGATAGAT TTGTAGAGAG AGACTGGTGA TTTCAGCGGC ATGCCTGCAG
1251 GTCGACCTGC AGCCAAGCTA GGCATGATCT AACCCTCGGT CTCTGGCGTC
1301 GCGACTGCGA AATTTCGCGA GGGTTTCCGA GATGGTGATT GCGCTTCGCA
1351 GATCTCCAGG CGCGTGGGTG CGGACGTAGT CAGCGCCATT GCCGATCGCG
1401 TGAAGTTCCG CCGCAAGGCT CGCTGGACCC AGATCCTTTA CAGGAAGGCC
1451 AACGGTGGCG CCCAAGAAGG ATTTCCGCGA CACCGAGACC AATAGCGGAA
1501 GCCCCAACGC CGACTTCAGC TTTTGAAGGT TCGACAGCAC GTGCAGCGAT
1551 GTTTCCGGTG CGGGGCTCAA GAAAAATCCC ATCCCCGGAT CGAGGATGAG
1601 CCGGTCGGCA GCGACCCCGC TCCGTCGCAA GGCGGAAACC CGCGCCTCGA
1651 AGAACCGCAC AATCTCGTCG AGCGCGTCTT CGGGTCGAAG GTGACCGGTG
1701 CGGGTGGCGA TGCCATCCCG CTGCGCTGAG TGCATAACCA CCAGCCTGCA
1751 GTCCGCCTCA GCAATATCGG GATAGAGCGC AGGGTCAGGA AATCCTTGGA
1801 TATCGTTCAG GTAGCCCACG CCGCGCTTGA GCGCATAGCG CTGGGTTTCC
1851 GGTTGGAAGC TGTCGATTGA AACACGGTGC ATCTGATCGG ACAGGGCGTC
1901 TAAGAGCGGC GCAATACGTC TGATCTCATC GGCCGGCGAT ACAGGCCTCG
1951 CGTCCGGATG GCTGGCGGCC GGTCCGACAT CCACGACGTC TGATCCGACT
2001 CGCAGCATTT CGATCGCCGC GGTGACAGCG CCGGCGGGGT CTAGCCGCCG
2051 GCTCTCATCG AAGAAGGAGT CCTCGGTGAG ATTCAGAATG CCGAACACCG
2101 TCACCATGCA CTTTACTCTT CCACCATTGC TTGTAATGGA AGTAATGTCA
2151 GTGTTGACCT TCTTCACTGG GAATCCAGTC ATGGATTTGA GGCCGCCGAA
2201 TGGAGCCACT GCGGCGGATT GCCCCCTAGA GGCACGGCTG ACTGTTGTCA
2251 CAGCGGAAGA GGATATCATA GAAGCCATTT TTTCTGACTT TCTTAGTTCT
2301 TGTGGTTGAA TTGCAAAGCT GGGTACCCTG TCCTCTCCAA ATGAAATGAA
2351 CTTCCTTATA TAGAGGAAGG GTCTTGCGAA GGATAGTGGG ATTGTGCGTC
2401 ATCCCTTACG TCAGTGGAGA TATCACATCA ATCCACTTGC TTTGAAGACG
2451 TGGTTGGAAC GTCTTCTTTT TCCACGATGC TCCTCGTGGG TGGGGGTCCA
2501 TCTTTGGGAC CACTGTCGGC AGAGGCATCT TCAACGATGG CCTTTCCTTT
2551 ATCGCAATGA TGGCATTTGT AGGAGCCACC TTCCTTTTCC ACTATCTTCA
2601 CAATAAAGTG ACAGATAGCT GGGCAATGGA ATCCGAGGAG GTTTCCGGAT
2651 ATTACCCTTT GTTGAAAAGT CTCAATTGCC CTTTGGTCTT CTGAGACTGT
2701 ATCTTTGATA TTTTTGGAGT AGACAAGCGT GTCGTGCTCC ACCATGTTGA
2751 CGAAGATTTT CTTCTTGTCA TTGAGTCGTA AGAGACTCTG TATGAACTGT
2801 TCGCCAGTCT TTACGGCGAG TTCTGTTAGG TCCTCTATTT GAATCTTTGA
2851 CTCCATGGGA ATTCGAGCTC GGTACCCGGG GATCCTCTAG AGTCGACCTG
2901 CAGGCATGCA AGCTTGCATG CCTGCAGGTC AACATGGTGG AGCACGACAC
2951 TCTCGTCTAC TCCAAGAATA TCAAAGATAC AGTCTCAGAA GACCAGAGGG
3001 CTATTGAGAC TTTTCAACAA AGGGTAATAT CGGGAAACCT CCTCGGATTC
3051 CATTGCCCAG CTATCTGTCA CTTCATCGAA AGGACAGTAG AAAAGGAAGA
3101 TGGCTTCTAC AAATGCCATC ATTGCGATAA AGGAAAGGCT ATCGTTCAAG
3151 AATGCCTCTA CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGAGGAA
3201 CATCGTGGAA AAAGAAGACG TTCCAACCAC GTCTTCAAAG CAAGTGGATT
3251 GATGTGATAT CTCCACTGAC GTAAGGGATG ACGCACAATC CCACTATCCT
3301 TCGCAAGACC CTTCCTCTAT ATAAGGAAGT TCATTTCATT TGGAGAGGAC
3351 CTCGAGTGGC CACCATGGXX XGATCCATGA GCCCAGAACG ACGCCCGGCC
3401 GACATCCGCC GTGCCACCGA GGCGGACATG CCGGCGGTCT GCACCATCGT
3451 CAACCACTAC ATCGAGACAA GCACGGTCAA CTTCCGTACC GAGCCGCAGG
3501 AACCGCAGGA GTGGACGGAC GACCTCGTCC GTCTGCGGGA GCGCTATCCC
3551 TGGCTCGTCG CCGAGGTGGA CGGCGAGGTC GCCGGCATCG CCTACGCGGG
3601 CCCCTGGAAG GCACGCAACG CCTACGACTG GACGGCCGAG TCGACCGTGT
3651 ACGTCTCCCC CCGCCACCAG CGGACGGGAC TGGGCTCCAC GCTCTACACC
3701 CACCTGCTGA AGTCCCTGGA GGCACAGGGC TTCAAGAGCG TGGTCGCTGT
3751 CATCGGGCTG CCCAACGACC CGAGCGTGCG CATGCACGAG GCGCTCGGAT
3801 ATGCCCCCCG CGGCATGCTG CGGGCGGCCG GCTTCAAGCA CGGGAACTGG
3851 CATGACGTGG GTTTCTGGCA GCTGGACTTC AGCCTGCCGG TACCGCCCCG
3901 TCCGGTCCTG CCCGTCACCG AGATCTGATC TCACGCGTCT AGGATCCTCT
3951 AGAGTCCGCA AATCACCAGT CTCTCTCTAC AAATCTATCT CTCTCTATTT
4001 TCTCCAGAAT AATGTGTGAG TAGTTCCCAG ATAAGGGAAT TAGGGTTCTT
4051 ATAGGGTTTC GCTCATGTGT TGAGCATATA AGAAACCCTT AGTATGTATT
4101 TGTATTTGTA AAATACTTCT ATCAATAAAA TTTCTAATTC CTAAAACCAA
4151 AATCCAGTGA CCTGCAGGCA TGCAAGCTTG GCGTAATCAT GGTCATAGCT
4201 GTTTCCTGTG TGAAATTGTT ATCCGCTCAC AATTCCACAC AACATACGAG
4251 CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT GAGCTAACTC
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4301 ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC
4351 GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC
4401 GTATTGGGCG CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC
4451 GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT
4501 ATCCACAGAA TCAGGGGATA ACGCAGGAAA GAACATGTGA GCAAAAGGCC
4551 AGCAAAAGGC CAGGAACCGT AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT
4601 AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG
4651 GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA
4701 GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG
4751 TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCAAT GCTCACGCTG
4801 TAGGTATCTC AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC
4851 ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT
4901 CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC
4951 TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT
5001 TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC
5051 TGCGCTCTGC TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG
5101 ATCCGGCAAA CAAACCACCG CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC
5151 AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC TTTGATCTTT
5201 TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT
5251 GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA
5301 AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC
5351 AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT
5401 TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC
5451 GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA
5501 CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC
5551 CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA
5601 ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC
5651 AACGTTGTTG CCATTGCTAC AGGCATCGTG GTGTCACGCT CGTCGTTTGG
5701 TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT
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5801 GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT
5851 GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG
5901 GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT
5951 TGCTCTTGCC CGGCGTCAAT ACGGGATAAT ACCGCGCCAC ATAGCAGAAC
6001 TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA
6051 GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC
6101 AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA
6151 AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT
6201 GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG
6251 GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA
6301 ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA CCTGACGTCT
6351 AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG
6401 AGGCCCTTTC GTCTCGCGCG TTTCGGTGAT GACGGTGAAA ACCTCTGACA
6451 CATGCAGCTC CCGGAGACGG TCACAGCTTG TCTGTAAGCG GATGCCGGGA
6501 GCAGACAAGC CCGTCAGGGC GCGTCAGCGG GTGTTGGCGG GTGTCGGGGC
6551 TGGCTTAACT ATGCGGCATC AGAGCAGATT GTACTGAGAG TGCACCATAT
6601 GCGGTGTGAA ATACCGCACA GATGCGTAAG GAGAAAATAC CGCATCAGGC
6651 GCCATTCGCC ATTCAGGCTG CGCAACTGTT GGGAAGGGCG ATCGGTGCGG
6701 GCCTCTTCGC TATTACGCCA GCTGGCGAAA GGGGGATGTG CTGCAAGGCG
6751 ATTAAGTTGG GTAACGCCAG GGTTTTCCCA GTCACGACGT TGTAAAACGA
6801 CGGCCAGTGA ATTGATCCCC AATTCCCATG GAGTCAAAGA TTCAAATAGA
6851 GGACCTAACA GAACTCGCCG TAAAGACTGG CGAACAGTTC ATACAGAGTC
6901 TCTTACGACT CAATGACAAG AAGAAAATCT TCGTCAACAT GGTGGAGCAC
6951 GACACGCTTG TCTACTCCAA AAATATCAAA GATACAGTCT CAGAAGACCA
7001 AAGGGCAATT GAGACTTTTC AACAAAGGGT AATATCCGGA AACCTCCTCG
7051 GATTCCATTG CCCAGCTATC TGTCACTTTA TTGTGAAGAT AGTGGAAAAG
7101 GAAGGTGGCT CCTACAAATG CCATCATTGC GATAAAGGAA AGGCCATCGT
7151 TGAAGATGCC TCTGCCGACA GTGGTCCCAA AGATGGACCC CCACCCACGA
7201 GGAGCATCGT GGAAAAAGAA GACGTTCCAA CCACGTCTTC AAAGCAAGTG
7251 GATTGATGTG ATATCTCCAC TGACGTAAGG GATGACGCAC AATCCCACTA
7301 TCCTTCGCAA GACCCTTCCT CTATATAAGG AAGTTCATTT CATTTGGAGA
7351 GGACAGGGTA CCCGGGGATC AGATTGTCGT TTCCCGCCTT CAGTTTAAAC
7401 TATCAGTGTT TGACAGGATA TATTGGCGGG TAAACCTAAG AGAAAAGAGC
7451 GTTTATTAGA ATAACGGATA TTTAAAAGGG CGTGAAAAGG TTTATCCGTT
7501 CGTCCATTTG TATGTGCATG CCAACCACAG GGTTCCCCTC GGGAGTGCTG
7551 GCATTCC
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Appendix 3: Nucleotide sequence of EFR1, including marked primer sequences. Direction is indicated through direction of the arrows

2221 aaagttttga acctcctaaa gcatggagcg acgaaaagct ttatggcgga atgtgaaacc

2281 ttcaagggta tacgacatcg taaccttgta aaactgataa cggtttgttc aagccttgat

M544334rev

2341 tccgagggaa atgatttcag agctctggtc tatgagttca tgccaaaagg aagtctggat

2401 atgtggctgc agctagaaga tctggaaagg gtaaacgatc actcgagatc tttaacaccc

Chip1_seq.8

2461 gcagagaaac tcaacatagc aatagatgtg gcttcagctt tggagtatct gcacgttcat

2521 tgtcatgacc ctgtagctca ctgtgatatt aagccaagca acattcttct agacgatgat

2581 ctgactgctc atgttagtga ctttggtttg gctcagctcc tctataaata cgatcgagaa

2641 tcctttctaa accagtttag ttctgctggt gtcagaggca ccattggcta tgccgcgcca

2701 gagtatggaa tgggaggcca accatcaata caaggagatg tgtacagctt cggaattcta

2761 cttttggaga tgtttagtgg aaaggaacca acagatgaat catttgcagg cgattataac

Chim1.seq.9

2821 ctccacagct acacaaagtc tatattatcg ggttgcacga gcagtggagg cagcaacgcc

2881 attgatgagg ggttgagact ggttttgcag gtggggataa agtgttctga agaatatccg

2941 agggatagga tgagaacgga tgaagcagta cgagaattaa tctcaataag atctaagttc

3001 ttcagttcca agacgactat tacagagagt cctcgagatg ctccgcaaag ttctcctcag

3061 gaatggatgt taaatacgga catgcatact atgtag

Appendix 4: Manuscript in preparation

Recognition of Bacterial EF-Tu by the Arabidopsis LRR Receptor Kinase EFR

Cyril Zipfel, Gernot Kunze, Delphine Chinchilla, Anne Caniard, Georg Felix, Thomas Boller

Abstract

A conserved aspect of innate immune response is the ability to sense microbial invaders through the perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Although many PRRs have been identified over the last few years in mammals and insects, plants PRRs remain largely unknown. Here, we describe a new gene, *EF-Tu Response* (*EFR*), required for perception and response to the bacterial elongation factor EF-Tu. *EFR* encodes a receptor kinase protein with a predicted extracellular domain containing leucine-rich repeats and an intracellular serine-threonine kinase domain (LRR-RLK). Plants mutated in *EFR* are insensitive to EF-Tu treatment, correlating with the absence of binding and cross-linking to the elicitor-active peptide elf18. Furthermore, heterologous transient expression of *EFR* in the non-responsive plant *Nicotiana benthamiana* results in responsiveness to elf18, but not to the inactive peptide elf12. Therefore, our data demonstrate that EFR is the EF-Tu receptor and is involved in bacterial recognition in Arabidopsis.

Comparable to the innate immune response in mammals and insects, plants possess highly specific and sensitive recognition systems for pathogen-associated molecular patterns (PAMPs) (Nurnberger and Brunner, 2002; Nurnberger et al., 2004; Medzhitov and Janeway, 2002). In human and mice, Toll-like receptors (TLRs) sense various bacterial PAMPs such as flagellin, lipopolysaccharides (LPS), peptidoglycan, lipoproteins and nucleic acids (Akira and Takeda, 2004; O'Neill, 2004). The NOD-1 and -2 proteins are involved in intracellular recognition of bacterial peptidoglycan (Philpott and Girardin, 2004). In Drosophila, members of the peptidoglycan-recognition protein (PGRP) family and the Gram-negative binding protein GNBP1 have been recently shown to be involved in bacterial sensing through peptidoglycan perception (Royet et al., 2005). PAMPs signaling the presence of bacteria in Arabidopsis comprise flagellin, the main building block of the flagellum, and LPS, a cell wall component of Gram-negative bacteria (Gerber et al., 2004; Felix et al., 1999). In addition, we recently identified the elongation factor EF-Tu as a novel bacterial PAMP that is highly active in Arabidopsis, and other Brassicaceae. The corresponding active epitope could be determined as the N-acetylated first 18-amino-acid residues, elf18 (Kunze et al., 2004). The flagellin receptor FLS2, a leucine-rich repeat receptor kinase (LRR-RLK), represents so far the only known PRR in Arabidopsis (Gòmez-Gòmez and Boller, 2000; Chinchilla D. et al., 2005). The perception systems for flagellin and EF-Tu involve different receptors since EF-Tu is also active in plants mutated in the flagellin receptor (Kunze et al., 2004).

In order to identify the EF-Tu receptor in Arabidopsis, we attempted a reverse-genetic approach. In a previous genome-wide expression study, we identified about 1000 genes whose expression was induced 30 minutes after flg22 treatment (Zipfel et al., 2004). Strikingly, among these induced genes there were 106 *RLK* out of the 610 *RLK* genes present in the Arabidopsis genome (Shiu and Bleecker, 2001). Notably, a similar survey of transcriptional changes following elf18 treatment revealed an identical set of induced genes (Kunze et al., in preparation). This suggested that the perception of a single PAMP, either flagellin or EF-Tu), enhances the synthesis of many receptors which might lead to increased sensitivity of the plant to microbial stimuli signaling the presence of invading microorganisms. In particular, since flg22 as well as elf18 treatment increased FLS2 transcript level one might speculate that some of the induced RLKs could be involved in the recognition of other PAMPs, notably the perception of EF-Tu. In Drosophila, a positive feedback regulation on the transcriptional level was reported for several PGRPs and GNBPs that are involved in innate immune recognition of peptidoglycans (Irving et al., 2001; De Gregorio et al., 2001).
The flagellin receptor FLS2 possesses extracellular LRR repeats and directly interacts with flg22 (Chinchilla D. et al., 2005). LRR domains are found in diverse eukaryotic proteins and typically participate in protein-protein interactions (Kobe and Kajava, 2001). Elf18, like flg22, is a peptidic PAMP, opening the hypothesis that the receptor for EF-Tu might be one of the 28 LRR-RLKs induced by flg22 and efl18. Thus, we set out to obtained homozygous mutant lines for most of these genes starting from lines provided by the Salk Institute Genomic Analysis Laboratory collection (Alonso et al., 2003). To test the functionality of the corresponding proteins in EF-Tu response, these mutants were tested for their ability to respond to elf18 treatment. Similarly to flg22, elf18 treatment leads to a strong inhibition of

seedling growth (Kunze et al., in preparation; Fig. 1A and B,). This readout was already successfully used in a screen for flg22-insensitive plants, and led to the identification of the FLS2 receptor (Gòmez-Gòmez and Boller, 2000) One of the lines in the LRR-RLK collection, *SALK_044334*, proved clearly insensitive to elf18 application and was named *efs-1*, for *EF-Tu sensing-1* (Fig. 1A and B). The growth inhibition triggered by flg22 treatment was identical in wild-type and in *efs-1* seedlings, suggesting that *efs-1* plants were specifically affected in EF-Tu responses (Fig. 1B).

To further characterize the *efs-1* phenotype, we analyzed its response to EF-Tu in different bioassays. Similarly to flg22, elf18 treatment induces numerous defense-related responses such as an increase in the production of the stress hormone ethylene, and a rapid production of reactive oxygen species in an oxidative burst (Kunze et al., 2004). Elf18 treatment did not induce any ethylene production, nor an oxidative burst in *efs-1*, but in wild-type (Fig. 1C and D). This was likely not due to a general defect in the ability to generate these responses, as *efr-1* leaf pieces were still responsive to flg22 treatment (Fig. 1C and 1D). Pre-treatment of Arabidopsis leaves with elf18 peptide, but not with the inactive peptide elf12, restricts the growth of the virulent bacterium *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) (Kunze et al., 2004). This effect was completely abolished in *efs-1* mutant plants (Fig. 1E). The *efs-1* mutation did neither trigger any developmental, nor growth defect through the plant life cycle, and this also other several generations (data not shown).

A second line mutated in the same gene, *efs-2* (*SALK_068675*), was isolated and proved as insensitive towards treatment with elf18 as *efs-1* (data not shown). This strongly indicates that non-responsiveness to EF-Tu was due to the insertions at this locus rather than to unrelated changes at second sites. The fact that the *efs-1 and efs-2* mutants are impaired in all responses triggered by elf18, but not by flg22, show that EFS is specifically required for EF-Tu responses. Thus we provisionally termed this gene *EFR* for *EF-Tu response* or *EF-Tu receptor*.

The *EFR* gene (At5g20480) codes for a LRR-RLK of the subfamily XII, which comprises also *FLS2* and 8 additional members (Shiu and Bleecker, 2001). No biological function has been previously assigned to this gene which, on two exons, encodes encodes a predicted protein of 1031 amino-acids residues with an estimated molecular mass of 113 kD (Fig. 2A and C). The deduced protein has all characteristic of a typical LRR-RLK (Fig. 2C). The N-terminus contains a hydrophobic sequence predicted to act as a signal peptide for secretion, followed by the LRR domain with 21 tandem copies of a 24-residue LRR (residues 96 to 606). Each unit of the LRR domain has the consensus LxxLxxLxLxxNxLxGxIPxxLGx. The LRR domain is flanked by pairs of cysteines with spacing observed in several LRR-RLKs (Dievart and Clark, 2003). A single trans-membrane domain (amino-acids 650 to 673) is predicted to separate the extracellular domain from the intracellular domain which shows all the signatures of a serine-threonine protein kinase (amino-acids 712 to 1000) (Hanks and Quinn, 1991) (Fig. S1). The presence of 21 potential N-glycosylation sites (N-X-S/T)

indicates that EFR might be a glycosylated protein, as recently demonstrated for FLS2 (Chinchilla D. et al., 2005). In contrary to FLS2 (Gòmez-Gòmez and Boller, 2000), EFR protein sequence does not contain any Leucine Zipper, or PEST motif, but a potential endocytosis motif (YXXØ), where Ø is an amino-acid with a hydrophobic side chain. This motif was recently shown to be essential for the function of the tomato receptor LeEIX2 that perceive fungal xylanase (Ron and Avni, 2004).

EFR mRNA was not detectable in *efr-1* (Fig. 2B), establishing that *efr-1* is a null allele.

Evidence for the existence of high-affinity, saturable and irreversible EF-Tu binding has been provided by binding assays with radiolabeled elf peptides in Arabidopsis cells (Kunze et al., in preparation). The radiolabeled derivative of elf18, ^{125}I-elf18, bound specifically to wild-type plant extracts but not with extracts from *efr-1* plants (Fig. 3A), suggesting that EFR is essential for elf18 perception. In addition, chemical cross-linking analysis with ^{125}I-elf18 has shown that the putative receptor for EF-Tu in Arabidopsis is a protein with an apparent molecular weight of ~150 kD (Kunze et al., in preparation).

To test the hypothesis that EFR is directly binding to elf18, we performed chemical crosslinking experiments on wild-type and *efr-1* mutant plants. Unexpectedly, cross-linking with wild-type plant extracts with ^{125}I-elf18 labeled specifically two polypeptides of high molecular weight (~150 and 100 kD), which could be competed in presence of an excess of cold elf26. In *efr-1* plant extracts, none of these bands are present suggesting that both may be products of the *EFR* gene. These bands are in good agreement with the predicted molecular mass of EFR, which is without signal peptide ~111 kD. Higher band may correspond to the EFR protein modified by e.g. glycosylation as it was demonstrated for FLS2 (Chinchilla et al., 2005).

These experiments showed that EFR is necessary for elf18 binding, and that it might directly interacts with elf18.

Nicotiana benthamiana plants, as all plants outside the family of *Brassicaceae* tested so far are non-responsive to EF-Tu (Kunze et al., 2004). To test whether this is due to lack of functional EFR, we transiently expressed *EFR* under the control of its native promoter in *Nicotiana benthamiana* leaves by agroinfiltration (Van der Hoorn et al., 2000). Leaves were injected with Agrobacteria carrying either the *EFR* gene or the *FLS2* gene as a control. When tested for responsiveness four days later, leaves transformed with the *EFR* gene, but not leaves transformed with the control *FLS2* construct, showed a clear induction of an oxidative burst (Fig. 4A) and enhanced ethylene biosynthesis (Fig. 4B) when treated with elf18. No induction of both responses was observed after treatment with the truncated, inactive derivative elf12 (Fig. 4 A and B).

In summary, our results demonstrate that *EFR* encodes a functional binding site for EF-Tu that is also capable to activate signaling and induce physiological responses. Based on these results we conclude that EFR is the EF-Tu receptor.

We recently showed that flagellin perception participates in the basal resistance against virulent bacterium *Pst* DC3000 (Zipfel et al., 2004). To test if EF-Tu perception also contributes to this defense, we tested *efr-1* mutant plants for their susceptibility to *Pst* DC3000 infection. However, under the conditions tested, *efr-1* plants were as susceptible as wild-type plants to *Pst* DC3000 (Fig. 5). Several non-exclusive hypotheses could explain this observation. EF-Tu of *Pst* DC3000 has a N-terminal amino-acid sequence that exhibits reduced elicitor activity (Kunze et al., 2004). Although correlative, this peculiar alteration of EF-Tu in this plant pathogen might hint at an evolutionary pressure on this pathogen to modify this part of their EF-Tu protein and to avoid recognition by the defense system of the plants. This is reminiscent of the sequence variations observed for the elicitor-active domain in flagellins of bacterial plant pathogens. Several of these bacteria carry sequence variations that renders them undetectable for the flagellin detection system of the plant (Felix et al., 1999). Thus, *Pst* DC3000 might not be the strain of choice to test the involvement of EF-Tu perception for activation of defense. In future work, we test susceptibility of *efr* plants to bacterial strains with EF-Tu that exhibits normal elicitor-activity in Arabidopsis.
A second hypothesis is that the effect of the *efr* mutation on bacterial detection might be masked by the presence of a functional perception system for other PAMPs like flagellin. To test this hypothesis, we generated an *fls2 efr* double mutant. As expected, the resulting *fls2 efr* double mutant was insensitive to both flg22 and elf18 peptides (Fig. 6). We will now be able to test the susceptibility of wild-type, *efr*, *fls2* and *fls2 efr* plants to diverse virulent and avirulent bacterial strains. However, as hypothesized for the single *efr* mutant, the discovery of an enhanced disease susceptibility phenotype might be again rendered difficult by redundant perception systems for bacterial PAMPs. Indeed, we found that extracts from bacteria were still able to induce plant defense responses in the *fls2 efr* double mutant. For example, clear induction of ethylene production was found with extracts from *Agrobacterium tumefaciens*, *Ralstonia solanacearum* and *Xanthomonas campestris* pv *campestris* (Fig. 7). This result suggests that Arabidopsis plants possess, in addition to flg22/FLS2 and elf18/EFR, other detection systems for bacterial factors. LPS and HrpZ are primary candidate of further PAMP that might get recognized by Arabidopsis (Zeidler et al., 2004; Dong et al., 1999), but treatment with 100 □g/ml LPS or 200 nM HrpZ from *Pst* DC3000 did not lead to significant stimulation of ethylene biosynthesis (Fig. 7). Thus, at present, we have no clue on the nature of this/these additional elicitor active pattern(s) present in the bacterial extracts tested.

In summary, in this manuscript we report the identification by reverse-genetic of the Arabidopsis EF-Tu receptor, EFR. Together with FLS2, this constitutes the only examples of known PRR in Arabidopsis. Interestingly, EFR and FLS2 are similar and belong to the same subfamily (LRR-XII) of Arabidopsis receptor kinases (Shiu and Bleecker, 2001). This is consistent with our previous observations that EF-Tu and flagellin perceptions by plant cells exhibit similar characteristics. In both cases, elicitor-activity could be attributed to a highly conserved epitope comprising a single stretch of 18 to 22 amino-acid residues of the respective protein (Felix et al., 1999; Kunze et al., 2004). Synthetic peptides representing the

genuine amino-acid sequences of these domains display activity at subnanomolar concentrations. Truncating peptides at their C-termini leads to elicitor–inactive forms that specifically antagonize elicitor-activity of flagellin (Meindl et al., 2000; Bauer et al., 2001) and EF-Tu (Kunze et al., 2004). Functionally, these elicitors can be divided in a part responsible for binding and a part required for activation of the receptor. As postulated for flagellin perception (Meindl et al., 2000), perception of EF-Tu appears to involve two consecutive steps according to the address-message concept, a concept originally put forward to explain functioning of peptide hormones in animals (Schwyzer, 1987).
In addition to EFR and FLS2, the LRR-XII subfamily comprises 8 additional members. It would be interesting to test in the future if they are also involved in PAMP perception. Interestingly, we found that at least one other member is also induced by flg22 and elf18 (data not shown).

Despite the large number of PRRs involved in innate immune responses in mammals and Drosophila, whether all of them are actually receptors is still a matter of debate because, for most of them, direct binding of microbial ligands has yet to be demonstrated. Whereas TLR5 directly binds flagellin (Smith et al., 2003; Mizel et al., 2003), TLR4-mediated LPS perception requires two additional proteins, CD14 and MD2 (Miyake, 2004). In addition many TLRs, for example, are still orphan receptors, in the sense that their potential ligands are still unknown. Interestingly, EF-Tu was observed to act as a stimulator of a proinflammatory response in the presence of soluble CD14 (sCD14) (Granato et al., 2004). This opens the possibility that EF-Tu, similar to flagellin, might act as a PAMP for the innate immune system in both animals and plants, and that EF-Tu perception in mammals might involve TLR4. However, as already observed with flagellin (Smith et al., 2003; Donnelly and Steiner, 2002; Felix et al., 1999), plants and animals probably evolved independently to recognize different epitopes, and animals might not respond to the N terminus of EF-Tu, but rather to another part of this bacterial hallmark protein. A convergent evolution is also suggested by the fact that plants belonging to the *Brassicaceae* family and mammals, but not other plants, respond to EF-Tu. In plants, the appearance of EFR and the capacity to recognize EF-Tu could be easily explained by gene duplication/diversification events that occurred early in the *Brassicaceae* lineage. This mechanism has been indeed proposed to explain the expansion of the RLK gene family in Arabidopsis (Shiu and Bleecker, 2003; Shiu et al., 2004). Since *EFR* expressed in *Nicotiana benthamiana* forms a functional binding site and also induces physiological responses it seems to properly interact with the downstream signaling components of this plant. This indicates conservation of these signaling elements in both species.

Although, elf18/EFR and flg22/FLS2 perceptions systems might have an overlapping function for the detection of many bacteria strains, EF-Tu perception might be necessary in certain cases, such as for defense against strains that evolved to avoid flagellin recognition, or non-flagellated bacteria. In fact, the importance of EF-Tu perception in disease resistance against bacteria is suggested by the apparent inactivity of elf18 peptides derived from some plant pathogenic bacterial strains. This suggests that these bacteria evolved to avoid EF-Tu recognition by mutating some residues in the elf18 peptide, although EF-Tu is considered as

one of the slowest evolving protein. This is, for example, the case for *Pst* DC3000, Xylella fastidiosa (Kunze et al., 2004), or *X. campestris* pv *campestris* (data not shown).
Future studies should help us to decipher how perception of different bacterial PAMPs by Arabidopsis contribute to efficient defense against bacteria, and how, in certain cases, individual PAMP perception system are already sufficient to limit bacteria evasion, as recently demonstrated for flagellin perception.

Experimental procedures

Materials

The peptides and bacterial extracts used in this study were described elsewhere (Kunze et al., 2004; Zipfel et al., 2004; Felix et al., 1999).

Plant growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and *Nicotiana benthamiana* were grown in single pots at 20-21 °C with 65% humidity under ~100 □mol m^{-2} s^{-1} light in an 8h-light/16h-dark cycle in controlled-environment chambers, or on plates containing 1x MS medium (Duchefa), 1% sucrose and 0,8% agar under continuous light (60 µE m^{-2} sec^{-1}, Biolux lamps) at 22°C. Seeds were surface-sterilized prior sowing on Petri plates. All seeds were treated at 4°C for 2 days before moving them to the growth environment.

Isolation of T-DNA insertion mutants

The *EFR* T-DNA insertion lines SALK_044334 (*efr-1*) and SALK_068675 (*efr-2*) were generated by SIGnAL (Alonso et al., 2003) and obtained from the NASC (Nottingham, UK). To select plants homozygous for the T-DNA insertion, gene-specific primers (forward and reverse) 5'-GCTGCAGCCACATATCCAGAC-3' and 5'-GGAAGGGTGCC
AACAACAGGAG-3', 5'-GGATTGCTTGGCCCTGAG-3' and 5'-ACTAGTAGTCTCTCC-3', were used for *efr-1* and *efr-2*, respectively. Plants yielding no PCR product with the gene-specific primers were subsequently tested for the presence of the T-DNA insertion, using the gene-specific forward primer in combination with the T-DNA left border specific primer LBb1 5'- GCGTGGACCGCTTGCTGCAACT -3'.

Bioassays

Growth inhibition, ethylene production, oxidative burst, and induced-resistance experiments were performed as previously described (Gòmez-Gòmez et al., 1999; Felix et al., 1999; Kunze et al., 2004; Zipfel et al., 2004). For growth inhibition assay, seedlings were treated with peptides immediately after their transfer into liquid medium, or directly treated on solid MS plates, 5 days post-germination. The oxidative burst measurements were here performed in 96-well plate over a 35-minute time period using a MicroLumat LB96P luminometer (EG&G Berthold).

Bioinformatic analysis

Nucleotidic and proteic sequences were retrieved from the MIPS Arabidopsis database (http://mips.gsf.de/proj/thal/db/index.html) or the TIGR Arabidopsis database (http://www.tigr.org/tdb/e2k1/ath1/index.shtml). Protein domains, localization and properties were predicted using a combination of programs available on the Expasy website (http://www.expasy.org/) (Gasteiger et al., 2003).

Multiple sequence alignment of EFR (At5g20480), FLS2 (At5g46330), Xa21 (LoC-Os02g12420), BRI1 (At4g39400), BAK1 (At4g33430), CLV1 (At1g75820) and ERECTA (At2g26330) kinase domains was generated by the Tcoffee software (http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) (Poirot et al., 2004) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

RT-PCR analysis

Total RNAs were extracted from Col-0 and *efr-1* seedlings using the RNeasy Plant Mini kit (Qiagen). Five micrograms of DNase-treated RNA were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) accordingly to the manufacturer's instructions. One microliter of the reverse transcription reaction was used as template in a 50-□l PCR reaction (30 cycles) using primers specific for EFR (5'-CGGGTTGCACGAGCAGTG-3' and 5'-ACTAGTAGTCTCTCC-3') and for *RPL4* (*At1g07320*) (5'-GTGATAGGTCAGGTCAGGGAACAAC-3' and 5'CCACCACCACGAA CTTCACCGCGAGTC-3') used as constitutive control.

Binding assays

One hundred milligrams of liquid nitrogen-ground leaves were resuspended in 500 µl of binding buffer (25 mM MES pH 6, 50 mM NaCl, 2 mM KCl, 5 mM KI, 1 mM DTT, 10 mM $MgCl_2$) and centrifuged at 14.000 rpm for 25 min at 4°C. Supernatant was discarded and pellet (P1)resuspended in 500 µl binding buffer and used for the binding experiment. Aliquots of P1 were incubated in binding buffer in a total volume of 100 µl with ^{125}I-Tyr-elf26 (30 fmol in standard assays; >2000 Ci/mmol) for 25 min either alone (total binding) or with an excess (10 µM) of competing peptides (non-specific binding). Extracts were collected by vacuum filtration on chromatography paper (Whatman 3 mm CHr, pre-incubated with 1% bovine serum albumin, 1% bactotrypton, and 1% bactopepton in binding buffer) and washed for 10 s with 15 ml of binding buffer. Radioactivity retained on the filters was determined by gamma-counting.

Chemical cross-linking

Aliquots of P1 supplied with 30 fmol ^{125}I-Tyr-elf26 and the unlabeled elf26 peptide used as competitor were incubated for 30 min at 4°C. Crosslinking was initiated by addition of 10 □l 25 mM EGS (ethylene glycol bis(succinimidylsuccinate) (Pierce) in dimethylsulfoxide directly to the incubation mixture. After further incubation for 30 min at room temperature the reaction was stopped by addition of 2.5 □l 1 M Tris-HCl (pH 7.5). Samples were solubilized in Laemmli buffer (5 min, 95 °C). Proteins were separated by SDS-PAGE on gels containing 7 % (w/v) acrylamide. Gels were fixed, dried and analyzed using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA).

Plasmid construction

A fragment of 7.1kb including a region of 1080 bp upstream the ATG of the EFR gene as well as the coding sequence of *EFR* was amplified from Col-0 genomic DNA using the Expand High Fidelity System (Roche) and primers specific for EFR. These primers were designed to delete the stop codon of EFR (forward primer: 5'-TTAACCCGGGGGTGGAACCTGCATCATGTAAAC-3') and add a *KpnI* restriction site in 3' (reverse primer: 5'-TAATGGTACCGCCATAGTATGCATGTCCGTATTTAAC-3'). The resulting fragment was subcloned in the pGEM®-T Easy plasmid (Promega) to produce the construct termed *pGEM-EFRp::EFR*. The *GFP* coding sequence was amplified with specific primers containing KpnI restriction sites (forward primer: 5'-ATTAGGTACCAATGGTGAGCAAGGGCGAGGAGCTG-3' and reverse primer: 5'-TTAAGGTACCTTACTTGTACAGCTCGTCCATGGCG-3') and cloned in the KnpI site of *pGEM-*

EFRp::EFR in frame with the EFR coding sequence. After digestion with NotI, a *EFRp::EFR-GFP* fragment was cloned into the binary vector *pGREENII/T-0229* (Hellens et al., 2000). The final construct called *pGREENII-EFRp::EFR-GFP* was verified by sequencing and electroporated into *Agrobacterium tumefaciens* EHA101 containing the helper plasmid *pSOUP* (Tetracyclin resistant).

Transient expression analysis

Agrobacterium strains harbouring the *EFRp::EFR-GFP* construct in *pGREENII/T-0229* or the *FLS2p::FLS2-cmyc* construct in *pCAMBIA2300* (Zipfel et al., 2004) constructs were grown in YEB medium overnight, diluted into an induction medium (10 mM MES, pH 5.6, 0.1% (w/v) glucose, 0.1% (w/v) fructose, 0.4% (v/v) glycerol, 60 mM K_2HPO_4, 33 mM KH_2PO_4, 8 mM $(NH_4)_2SO_4$, 2 mM sodium citrate, 1 mM $MgSO_4$, and 50 □M acetosyringone) and grown for additional 4h until OD_{600} reached 0.4 to 0.5. The Agrobacterium cultures were diluted to OD_{600}=0.2 in infiltration medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 □M acetosyringone), and the suspensions were injected with a needleless syringe into leaves of 4- to 5-week-old *N. benthamiana* plants. Infiltrated leaves were analyzed 4 days after injection.

Bacterial infections

Bacterial infection experiments were performed as previously described (Zipfel et al., 2004).

Generation of efr fls2 double-mutant

The *EFR fls2* double-mutant was generated by crossing *fls2* (SAIL_691C4) (Zipfel et al., 2004) with *efr-1* (SALK_044334). The F1 and F2 were allowed to self-fertilize, and F3 plants were initially screened for their insensitivity to both elf18 and flg22 peptides using the oxidative burst and ethylene bioassays. Insensitive plants were finally genotyped by PCR to checked the presence of T-DNA in the *EFR* and *FLS2* genes.

Figures

Figure 1

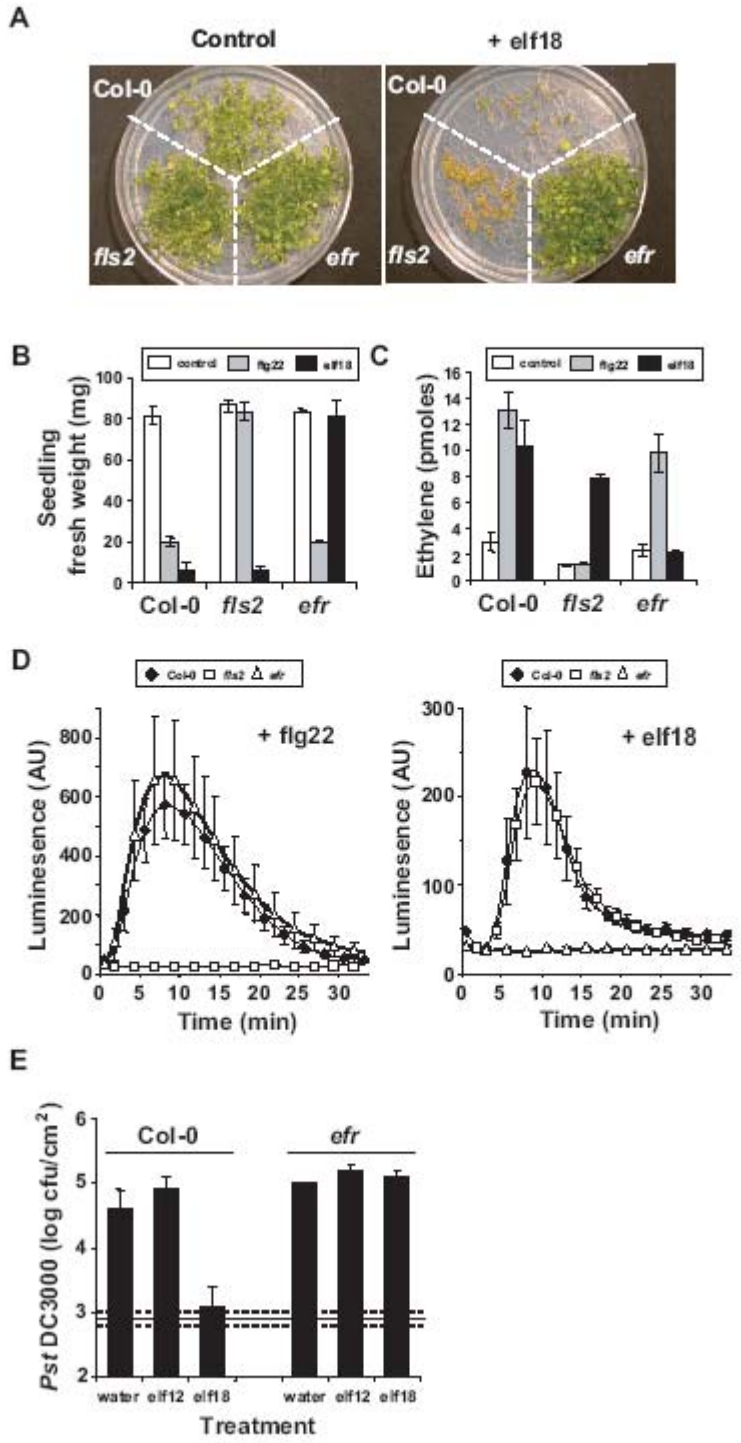
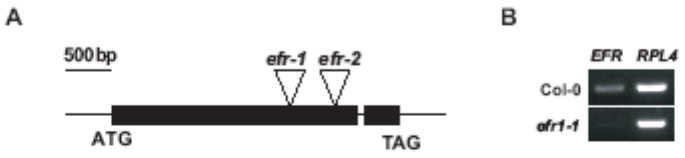


Figure 2



C

a. MKLSFSLVFNALTLLLQVCIFAQA

b. RFSNETDMQALLEFKSQVSENNKRE
VLASWNHSSPFCNWIGVTCGRRRER
VISLNLGGFKLTGVISPSIGN

c.

LxxLxxLxLxxNxLxGxIPxxLGx

d. RNATAVSVFGNTNICSGVREMQLKPC
IVQASPRKRKPLSVRKK

e. VVSGICIGIASLLLIIIVASLCW

f FMKRKKK

g. NNASDGNPSDSTTLGMFHEKVSYEELHSATSR

h. FSSTNLIGSNFGNVFKGLLGPENKLVAVK
VLNLLKHGATKSFMAECETFKGIRHRNLV
KLITVCSSLDEGNDFRALVYEFMPKGSLD
MWLQLEDLERVNDHSRSLTPAEKLNIAID
VASALEYLHVHHDPVAHCDIKPSNILLDD
DLTAHVSDFGLAQLLYKYDRESFLNQFSS
AGVRGTIGYAAPYGMGGQPSIQGDVYSFG
ILLLEMFSGKEPTDESFAGDYNLHSYTKS
ILSGCTSSGGSNAIDEGLRLVLQVGIKCS
EEYPRDRMRTDEAVRELISIRSKFF

i. SSKTTITESPRDAPQSSPQEWMLNTDMHTM

Figure 3

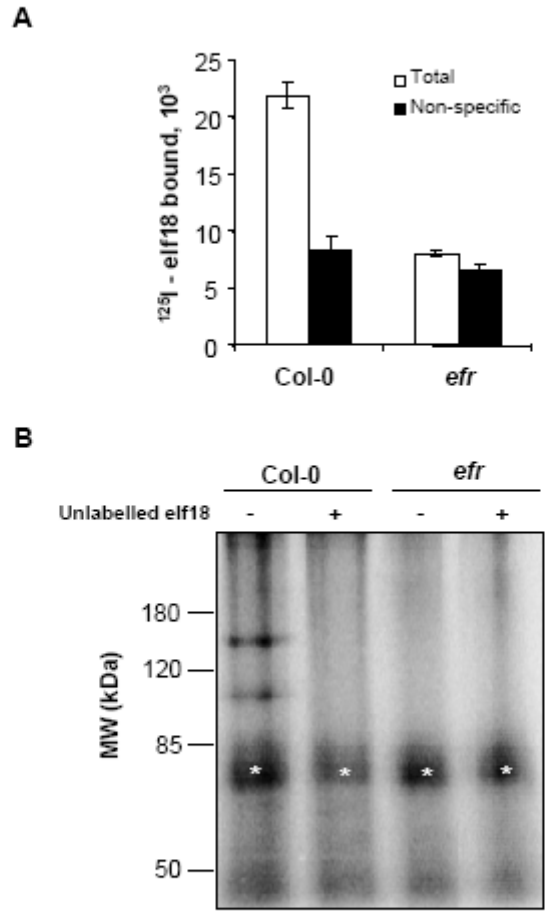
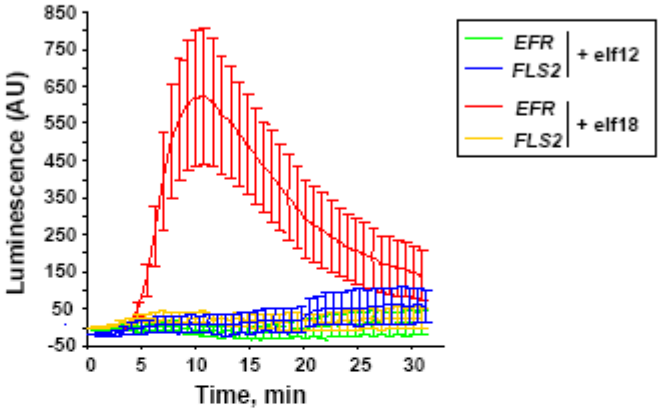

A
25
20
15
10
5
0
Total
Non-specific
125I - elf18 bound, 10^3
Col-0
efr
B
Col-0
efr
Unlabelled elf18
-
+
-
+
180
120
85
50
MW (kDa)
*
*
*
*

Figure 4

A


Luminescence (AU)
850
750
650
550
450
350
250
150
50
-50
0
5
10
15
20
25
30
Time, min
EFR
FLS2
+ elf12
EFR
FLS2
+ elf18

B

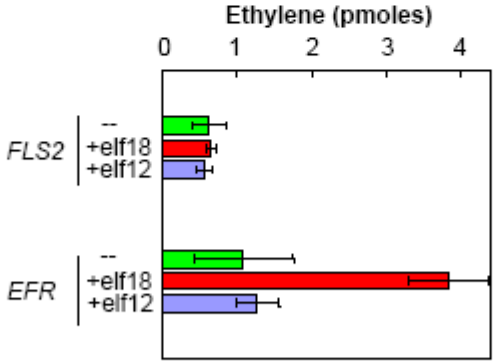

Ethylene (pmoles)
0
1
2
3
4
FLS2
--
+elf18
+elf12
EFR
--
+elf18
+elf12

Figure 5

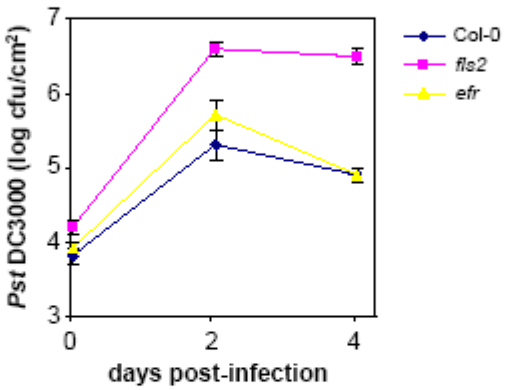
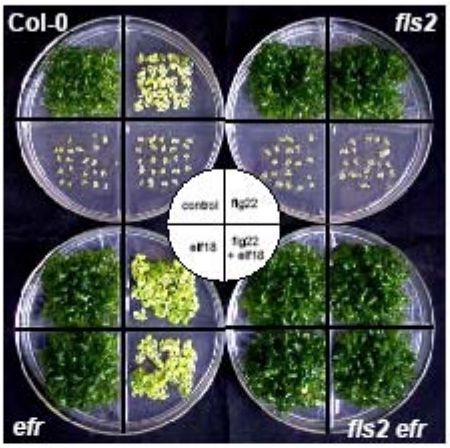

7
6
5
4
3
0
2
4
Pst DC3000 (log cfu/cm²)
days post-infection
Col-0
fls2
efr

Figure 6


Col-0
fls2
control
flg22
elf18
flg22 + elf18
efr
fls2 efr

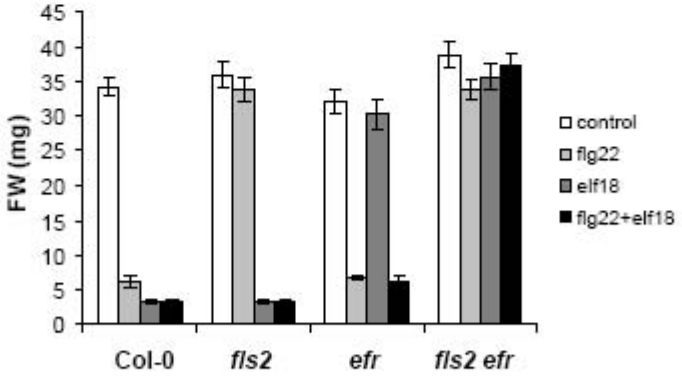

FW (mg)
45
40
35
30
25
20
15
10
5
0
Col-0
fls2
efr
fls2 efr
control
flg22
elf18
flg22+elf18

Figure S1

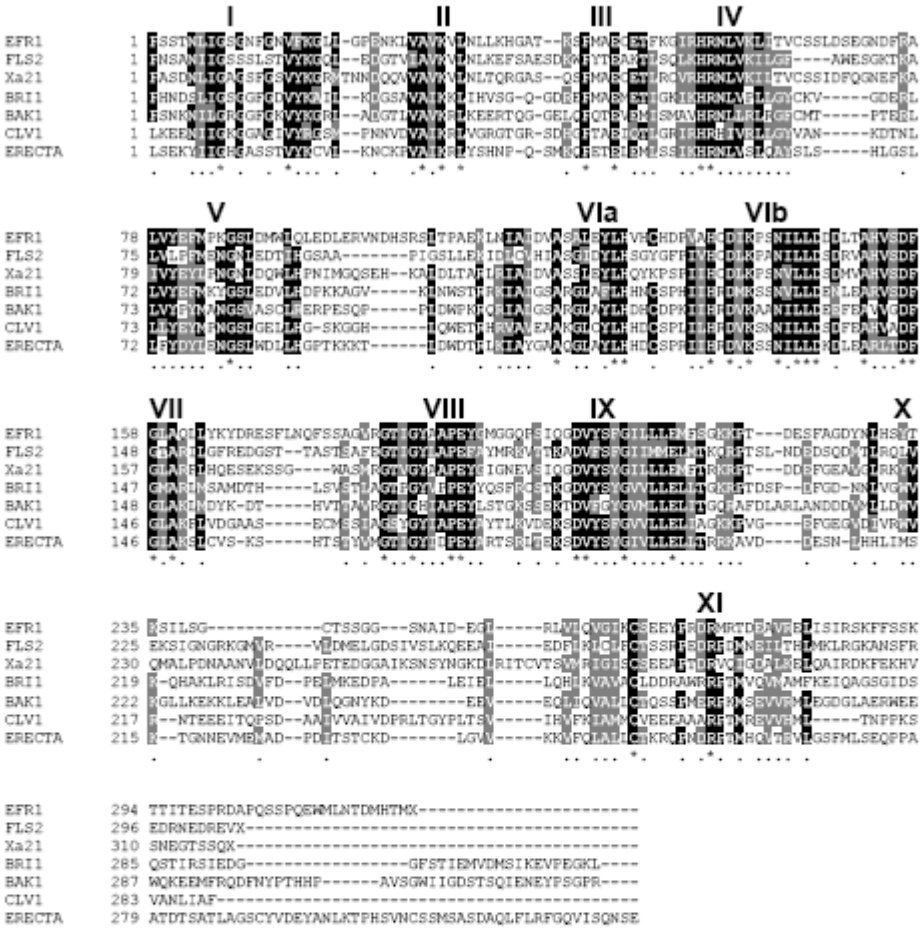

I
II
III
IV
V
VIa
VIb
VII
VIII
IX
X
XI
EFR1
FLS2
Xa21
BRI1
BAK1
CLV1
ERECTA

Figure legends

Figure 1 The *efr* mutant is insensitive to elf18.
(A) Qualitative measurement of seedlings growth inhibition. Five-day-old wild-type Col-0, *efr* and *fls2* seedlings grown on agar plates were treated with liquid MS medium alone (left panel), or supplemented with 1 □M elf18 peptide (right panel). Pictures were taken one week after treatment.
(B) Quantitative measurement of seedlings growth inhibition. Five-day-old wild-type Col-0, *efr* and *fls2* seedlings were transferred from solid agar plates to liquid medium alone, or supplemented with 1 µM elf18 or 1 µM flg22. Seedling fresh weight was measured one week after treatment. Results are averages ± standard errors (n=6).
(C) Induction of ethylene biosynthesis in leaf of wild-type Col-0, *efr* and *fls2* plants. Leaf pieces were mock treated (control) or treated with 1 µM flg22 or 1 □M elf18, and ethylene was measured after 3 h of incubation. Results are averages ± standard errors (n=6).
(D) Oxidative burst in leaf tissues of wild-type Col-0, *efr* and *fls2* plants. Luminescence of leaf slices in a solution with peroxidase and luminol was measured over the time after addition of 1 µM flg22 (left panel) or 1 µM elf18 (right panel). Results are averages ± standard errors (n=8).
(E) Elf18-induced resistance in wild-type Col-0 and *efr* plants. Plants were pretreated for 24 h by leaf infiltration with water, 1 µM elf12, or 1 □M elf18. Elf12 is an inactive analogue of elf18. Subsequently, leaves were infected with 10^5 cfu/ml *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000), and bacterial growth was assessed 2 days post-infection (dpi). The solid and dashed lines indicate respectively average and standard error of cfu extractable from leaves at 0 dpi. Results are averages ± standard errors (n=8).

Figure 2 *EFR* encodes a LRR receptor kinase.
(A) Schematic representation of the *EFR* gene. Exons are represented are black boxes. The start ant stop codons are indicated. The sites of insertion of T-DNA in the mutants *efr-1* and *efr-2* are shown by open triangles.
(B) *efr-1* is a null mutant. RT-PCR was performed using cDNA from seedlings to analyze EFR expression in Col-0 and *efr-1*. RPL4 was used as constitutive control.
(C) Primary structure of the EFR protein. The amino-acid sequence predicted from the DNA sequence of EFR is shown divided in nine domains (a-i) indicated as follows: a, potential signal peptide; b, unknown domain containing paired cysteines (underlined); c, LRR domain (conserved residue with the consensus sequence are highlighted in black); d, extracellular juxta-membrane domain containing paired cysteines (underlined); e, transmembrane domain (hydrophobic residues are highlighted); f, charged intracellular juxta-membrane domain; g, intracellular juxta-membrane domain containing the putative endocytosis motif YXXØ (underlined); h. Serine/Threonine kinase domain; I, C-terminal tail.

Figure 3 EFR is required for specific elf18 perception.
(A) Specific ^{125}I-elf18 binding is impaired in *efr* plants. Binding activity of wild-type Col-0 and *efr* plant extracts was tested by adding ^{125}I-elf18 alone (total binding) or with 10 □M unlabeled elf18 as competitor (non-specific binding). Results are averages ± standard deviations (n=2).
(B) Chemical crosslinking in presence of ^{125}I-elf18 reveals specific bands in wild-type Col-0, but not in *efr* plants. Aliquots of plant extracts were incubated with ^{125}I-elf18 alone or together with an excess of 10 µM of unlabeled elf18. After incubation on ice for 30 min, crosslinking was initiated by the addition of 2.5 mM EGS. Radiolabeled proteins in plant extracts were analyzed after separation by SDS-PAGE with a Phosphoimager.

Figure 4 Transient expression of *EFR* in the non-responsive *N. benthamiana* plant restores elf18 responsiveness.
(A) Oxidative burst in leaf tissues of *N.benthaniana* plants expressing *EFR* or *FLS2*. Luminescence of leaf slices in a solution with peroxidase and luminol was measured over the time after addition of 100 nM el18, or the inactive analogue elf12. Results are averages ± standard deviations (n=3).
(B) Induction of ethylene biosynthesis in leaf of *N.benthaniana* plants expressing EFR or FLS2. Leaf pieces were mock treated (control) or treated with 10 □M elf18, or the inactive analogue elf12, and ethylene was measured after 3 h. Results are averages ± standard deviations (n=3).

Figure 5 EFR is not required for basal resistance against *Pst* DC3000.
Wild-type Col-0, *fls2* and *efr* plants were spray-infected with 10^8 cfu/ml *Pst* DC3000, and bacterial growth was measured over the time. Results are averages ± standard errors (n=8).

Figure 6 The *efr fls2* double-mutant is insensitive to both elf18 and flg22.
For the qualitative measurement of seedlings growth inhibition (upper panel), five-day-old wild-type Col-0, *efr*, *fls2* and *efr fls2* seedlings on agar plates were treated with liquid medium alone (control), or supplemented with 1 µM elf18 alone, flg22 alone, or both together. Pictures were taken one week after treatment. For the quantitative measurement of seedlings growth inhibition (lower panel), five-day-old wild-type Col-0, *efr*, *fls2* and *efr fls2* seedlings were transferred from solid agar plates to liquid medium alone, or supplemented with 1 µM elf18 alone, flg22 alone, or both together. Seedling fresh weight was measured one week after treatment. Results are averages ± standard errors (n=6).

Figure 7 The *efr fls2* double-mutant still responds to bacterial extracts.
Induction of ethylene biosynthesis in leaf of *efr fls2* double-mutant. Leaf pieces were mock treated (control) or treated as follows: *Agrobacterium tumefaciens* C58 (*At*, the asterisk indicates that the extract was boiled, 10 □l), *Xanthomonas campestris* pv *campestris* (*Xcc*, 10 □l), *Ralstonia solanacearum* GMI1000 (*Rs*, the asterisk indicates that the extract was boiled, 10 □l), *Pseudomonas syringae* pv *syringae* (*Pss*, 200 □g/ml), LPS from Pst DC3000 (100 □g/ml), or HrpZ (200 nM). Ethylene was then measured after 3 h. Results are averages ± standard deviations (n=4).

Figure S1 Amino-acid sequence of the EFR kinase domain.
The EFR kinase domain was aligned with kinase domains from other plant LRR-RLKs. EFR contains all 12 conserved kinase subdomains (shown in Roman numerals), the ATP-binding site motif in subdomain I, the predicted catylytic Lysine residue in subdomain II, and the APE kinase catalytic domain indicator in domain VIII. Identical and similar amino-acids are highlighted by black and grey boxes, respectively.

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Chinchilla,D., Bauer,Z., Regenass,M., Boller,T., and Felix,G. (2005). The Arabidopsis receptor kinase FLS2 interacts directly with flg22 and determines specificity of flagellin perception. submitted.

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Acknowledgements

I would like to thank Prof. Thomas Boller for the opportunity to continue the EF-Tu diploma work during my thesis in his laboratory. Furthermore I would like to thank him for the possibility and his encouragement to present my work and also to be representative of our laboratory on congresses and meetings. I appreciate this and would like to state out that something like this is not always a common practice of a supervisor. In addition I want to thank Prof. Karsten Niehaus for his responsibility that I was able to do this work in Switzerland and his helpful comments and his support of my work.

I warmly thank Dr. Jürg Felix for his patience and continuous practical help, his ideas and his support for writing publications by telling secrets from his experience. I always received support to continue my work from Thomas and Jürg and all other lab members. Important parts of my work would not have been successful without the advices from Dr. Silke Robatzek, Dr. Delphine Chinchilla, Dr. Barbara Thürig and Dr. Cyril Zipfel towards experimental setup, handling and afterwards analysis of the revealed results. Many thanks to all other not namely mentioned people at Hebelstrasse for their support.

Finally, I would like to thank my wife Monika for her continuous support and patience when I was coming home later as promised. And furthermore I am grateful for the pressure she put on me and motivates me when my enthusiasm starts to decrease.

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