Receptor-like kinases and pathogen-associated molecular patterns perception in Arabidopsis

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Summary

Comparable to the innate immune response in mammals and insects, plants possess highly specific and sensitive recognition systems for pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin. In Arabidopsis, perception of flagellin occurs via recognition of the most conserved domain in its N-terminus, represented by the peptide flg22. Perception of this elicitor-active domain depends on the LRR receptor kinase FLS2.

Here, using transcriptome analysis in Arabidopsis, we first present evidences that treatment with flg22, bacteria or avirulence proteins are similar, and that virulent bacteria are likely to suppress flg22-induced responses (Chapter 1).

Second, we demonstrated that flagellin perception contributes to the basal resistance against bacteria, as *fls2* mutants are more susceptible to bacterial infection, proving for the first time that perception of a single PAMP is sufficient to restrict bacterial growth (Chapter 2).

This study also showed that PAMPs other than flagellin trigger resistance against virulent bacteria. Wild-type and *fls2* mutants both display enhanced resistance when treated with crude bacterial extracts, even devoid of elicitor-active flagellin, indicating the existence of functional perception systems for PAMPs other than flagellin. One of these novel PAMPs was just identified as the elongation factor EF-Tu, and the corresponding active peptidic epitope identified (Appendix 1). FLS2 constituted so far the only PAMP receptor identified in Arabidopsis. Using ATH1 Affymetrix arrays, we identified about 1000 genes whose expression was induced 30 minutes after flg22 treatment (Chapter 2). Strikingly, among these induced genes there were 106 *RLK* out of the 610 *RLK* genes present in Arabidopsis. We hypothesize that the perception of a single PAMP (e.g. flg22) could enhance the sensitivity of the plant to microbial stimuli sensing the presence of invading microorganisms. In particular, some of the induced RLKs might be involved in the recognition of other PAMPs. We generated a collection of mutants for the induced LRR-RLKs to test the implication of the corresponding proteins in innate immune responses (Chapter 3.1). This approach led to the discovery of the EF-Tu receptor (Chapter 3.2) and suggested a new role for the previously described *BAK1* gene in flg22- and probably elf18-signaling (Chapter 3.3).

Introduction

All organisms are constantly confronted to a vast array of microbes, such as bacteria, fungi, oomycetes or viruses. However, disease is not the rule, and most organisms are resistant to most microbes. A key aspect of active defense mechanism is the early detection of potential microbial invaders. In higher eukaryotes, this is achieved by the perception of microbial patterns by germ-line encoded receptors, named pattern recognition receptors (PRRs) (Janeway, Jr. and Medzhitov, 2002; Medzhitov and Janeway, Jr., 1997). The microbial patterns recognized by PRRs are structural motifs that are conserved in a wide range of microbes, and play pivotal functions for the microorganism. Therefore these structures represent prime targets for the detection of infectious agents (Janeway, Jr. and Medzhitov, 2002; Medzhitov and Janeway, Jr., 1997). Although termed pathogen-associated molecular patterns (PAMPs), these motifs are not restricted to pathogens, but are rather characteristic of a whole class of microorganisms in general. They should rather be called microbe-associated molecular patterns (MAMPs), but the term PAMPs will be maintained here for historical reason, as it has been essential to unify the work that has accumulated on the innate immune responses in a large variety of species. In addition to this PAMP-based “non-self” recognition, PRRs also sense “danger” signals, endogenous molecules that are normally not available for recognition, but are released upon microbial contact (Matzinger, 2002). After the detection event, PRRs activate several, complex signalling cascades, which ultimately regulate the transcription of target genes that encode effectors and regulators of the immune response. The detection of PAMPs and danger signals constitutes the basis of innate immunity, and is necessary for the activation of the adaptive immunity in mammals (Janeway, Jr. and Medzhitov, 2002). With the exception of vertebrates, all other organisms (invertebrates, plants and fungi) rely exclusively on innate immunity.

1. Innate immune recognition in plants

Plant surfaces (phyllosphere and rhizosphere) are always in close contact with vast number of microorganisms that could be beneficial, or pathogenic for the plant. If a microbe wants to become pathogenic on a host plant, it has to be able to overcome an arsenal of plant defenses. Most potential pathogens fail to surmount these barriers and are never able to colonize a potential host plant. The resistance shown by an entire plant species to all members of a specific pathogen species is the most common form of disease resistance and is named non-host resistance or general resistance (Thordal-Christensen, 2003; Heath, 2000; Mysore and Ryu, 2004). This type of resistance is determined by several intermingled layers of defense, which include both constitutive barriers and inducible reactions (Thordal-Christensen, 2003; Heath, 2000; Mysore and Ryu, 2004).

Preformed barriers constitutively present on the plant surface (e.g. wax layer, rigid cell walls, anti-microbial enzymes, or secondary metabolites) prevent ingress of the pathogen, subsequent activation of inducible defense responses, or disease symptom development. An effective role of pre-formed chemical compounds during a natural infection process has been for example described for saponins that protect cereals against pathogenic fungi (Bouarab et al., 2002; Papadopoulou et al., 1999).

Should a pathogen, however, manage to overcome constitutive defensive layers, for example by entering plant tissues via wounds or natural openings (e.g. stomata), it may become subject to recognition by plant cells, and induce plant defense reactions.
Plants have a broad, basal perception system for patterns characteristic for entire groups or classes of microorganisms, so-called “general elicitors” (Table 1) (Boller, 1995; Ebel and Cosio, 1994). This recognition occurs in response to attack by host and non-host pathogens and is independent of the genotype of the individual pathogen. The responses to these general elicitors include production of reactive oxygen species (ROS) (referred to an oxidative burst) and ethylene, ion fluxes, and the induction of pathogenesis-related (PR) proteins, but rarely the hypersensitive response (HR) (Nurnberger and Scheel, 2001). It is now clear that general elicitors are conceptually equivalent to the PAMPs activating innate immune responses in animal systems

(Nurnberger et al., 2004; Nurnberger and Brunner, 2002). During their interaction with microorganisms *in vivo*, higher organisms encounter a variety of signals, and recognition of several PAMPs is likely to determine the efficiency of inducible innate defense mechanisms.

All the steps mentioned above constitute the basal level of resistance of plant species (basal resistance), and probably contribute to non-host resistance to most potential pathogens (Thordal-Christensen, 2003). Should one of these events failed; disease development is initiated by the pathogen. The discovery of plant mutants with enhanced-disease susceptibility showed that even when they are susceptible to a pathogen, plants defend themselves to slow down it, but this basal level of defense is not effective enough to completely stop the pathogen (Glazebrook, 2001). Most of the genes affected in these mutants encode signalling elements (Hammond-Kosack and Parker, 2003), and do not allow ruling out an implication of specific perception of a single or several PAMPs in the observed basal defense.

In addition to basal resistance, plant disease resistance also occurs at the level of individual cultivars. It is assumed that, during evolution, basal resistance was overcame by individual phytopathogenic races or strains of a given pathogen species through the acquisition of virulence factors, which enabled them to either evade or suppress plant defense mechanisms (Dangl and Jones, 2001; Jones and Takemoto, 2004). In such cases, plants that became host to such microbes were rendered susceptible to microbial colonization and disease ensued. However, as a result of co-evolution to microbial pathogenicity factors, individual cultivars of an otherwise susceptible plant species have evolved resistance (*R*) genes products that specifically recognize pathogen strain or pathogen race-specific factor and allow the plant to resist infection by this particular pathogen strain/race. This so-called race-specific resistance conforms to the gene-for-gene resistance hypothesis (Flor, 1971) and is genetically determined by complementary pairs of pathogen-encoded avirulence (*Avr*) genes and plant *R* genes. Lack or non-functional products of either gene result in disease. Most Avr proteins are considered as virulence factors required for the colonization of host plants,

which, upon recognition by resistant host plant cultivars, act as “specific elicitors” of plant defense and thereby trigger the plant’s surveillance system.

Common to non-host and host pathogens are the presence of general elicitors, or PAMPs. In addition, the spectrum of reactions induced by general elicitor treatment or by R-gene mediated recognition is strikingly similar (Thordal-Christensen, 2003; Scheel, 1998; Dangl and Jones, 2001). It is tempting to speculate that the plant basal resistance is a consequence of PAMP recognition by pattern recognition receptors. However, in plants a causal link between PAMP-induced defense responses and disease resistance has to be established. As of today, such a relationship is based upon correlative data rather than causal (genetic) evidence. For example, the crucial question as to whether PAMPs or general elicitors also exhibit their proven defense-eliciting activity in natural encounters between plants and would-be pathogens has yet to be answered (Nurnberger and Brunner, 2002; Nurnberger et al., 2004; Gòmez-Gòmez and Boller, 2002).

2. PAMPs perceived by plants

2.1 A case study: flagellin perception

Flagellin is the major structural protein of eubacterial flagella. Consistently with a PAMP definition, flagellin is essential for bacterial motility, and is a proven virulence factor required for bacterial pathogenicity (Ramos et al., 2004). Flagellin from various bacteria have highly conserved N- and C-termini but hypervariable central regions. The N- and C-terminal regions, necessary for filament architecture and motility functions, are embedded in the flagellum inner core, whereas the hypervariable region is exposed at the surface of the flagellum (Yonekura et al., 2003; Ramos et al., 2004). Although most of the secreted flagellin is usually assembled in the flagellum, flagellin can also accumulate in the bacterial environment as a result of leaks and spillover during the construction of flagella, or after its degradation due to environmental conditions (Komoriya et al., 1999). The hypervariable central region of flagellin could allow a strain-specific recognition by the adaptive immunity, whereas the conserved N- and C-terminal regions that are exposed in flagellin monomers may constitute prime targets for recognition by innate receptors.

Our laboratory identified flagellin as a potent elicitor of defenses at subnanomolar concentrations (Table 1) (Felix et al., 1999; Gòmez-Gòmez and Boller, 2002). A synthetic peptide corresponding to the most highly conserved region in the N-terminal domain of flagellin, flg22 was found to be the elicitor-active epitope (Felix *et al.*, 1999). The use of synthetic flg22 peptide enables to study flg22 effects on plant cells without the risk of any other bacterial contaminations. The minimal active epitope can however differ from one plant species to the other, as exemplified in tomato, where the flg15 peptide is fully active, whereas it acts as an antagonist in Arabidopsis (Felix et al., 1999; Meindl et al., 2000; Chinchilla et al., submitted). Flg22 induced strong defense responses in all plants tested, except for rice (Felix *et al.*, 1999). Apparently, rice (as well as tobacco) developed a bacterial strain-specific recognition system based on flagellin sequences (different from flg22) and/or post-translational modifications (e.g. glycosylation) (Che et al., 2000; Fujiwara et al., 2004; Tanaka et al., 2003; Shimizu et al., 2003; Taguchi et al., 2003; Takeuchi et al., 2003).

A genetic screen for flg22-insensitive Arabidopsis mutants mapped to a single locus *FLS2* (*Flagellin Sensing 2*), encoding a putative transmembrane receptor-like kinase with an extracellular leucine-rich repeat (LRR) domain (LRR-RLK) (Gòmez-Gòmez and Boller, 2000). In addition, the Arabidopsis ecotype Wassilewskaya (Ws-0) presents an flg22-insensitive phenotype, formerly attributed to a mutation in a hypothetical locus, named *FLS1* (Gòmez-Gòmez et al., 1999), but recently shown to be a natural *fls2* mutant carrying a point mutation that resulted in a stop codon in the kinase domain of FLS2 (Gòmez-Gòmez and Boller, 2002). A close correlation between the flagellin sensitivity and the presence of flg22-binding sites in different Arabidopsis ecotypes and *fls2* mutants strongly supported that FLS2 is part of the flagellin perception complex (Bauer et al., 2001). Indeed, recent biochemical studies using cross-linking with radio-labelled flg22 and immuno-precipitation showed that flg22 interacts directly with FLS2, demonstrating that FLS2 is the *bona-fide* flg22 receptor in Arabidopsis (Chinchilla et al., submitted). However, the exact region of FLS2 that is responsible for flg22 binding is still unknown.

Perception of flg22 by FLS2 activates in the cytoplasm a downstream MAP kinase pathway composed of MEKK1, MKK4/5 and MPK3/6 and is likely to involve WRKY transcription factors in Arabidopsis (Asai et al., 2002; Nühse et al., 2000). Flg22 treatment induced several general defense responses, such as production of ethylene and ROS, ion fluxes, callose deposition and PR-gene expression (Felix et al., 1999; Gòmez-Gòmez et al., 1999; Asai et al., 2002).

2.2 Other examples of bacterial PAMPs

In animal systems, classical PAMPs signalling the presence of bacteria comprised, in addition to flagellin, structural molecules as lipopolysaccharides (LPS), a component of the cell wall of Gram-negative bacteria, and peptidoglycans (PGN) common to all bacteria (Akira and Takeda, 2004; Janeway, Jr. and Medzhitov, 2002).

LPS is a glycolipid component of the outer membrane of Gram-negative bacteria that exhibits the most potent immunostimulating activity of the PAMPs known in mammals (Miyake, 2004). Trace amounts of LPS are able to activate the innate immune system, leading to the production of an array of proinflammatory mediators. LPS perception has been thoroughly studied in mammals and is considered as a prototypic model PAMP. In contrast, much remains to be elucidated about the effect of LPS on plants (Erbs and Newman, 2003; Zeidler et al., 2004). However, a growing amount of evidences suggest that LPS also acts as a PAMP in plant cells (Table 1). LPS purified from different bacterial strains induces typical defense responses such as oxidative burst, production of nitric oxide (NO), defense-gene expression, ions fluxes, and protein phosphorylation (Zeidler et al., 2004; Gerber et al., 2004; Erbs and Newman, 2003; Gerber and Dubery, 2004). It participates in the induction of systemic resistance by nonpathogenic plant growth-promoting rhizobacteria, and potentiates plant defenses in response to bacteria (Zeidler et al., 2004; Gerber et al., 2004; Erbs and Newman, 2003). LPS is composed of three distinct regions: lipid A, the oligosaccharide core, and commonly a long-chain polysaccharide O antigen. In Arabidopsis cells, lipid A, the most conserved part of LPS, was as effective on NO production as most LPS preparations used (Zeidler et al., 2004). Thus, lipid A may serve as the active part of LPS in plants, as reported in mammals (Miyake, 2004). However, LPS operates at pg/ml or ng/ml range in

mammals, whereas in most plants, defense responses require higher amounts of LPS (μg/ml). This suggests that the plant putative LPS receptor may be of a low-affinity type (Zeidler et al., 2004), or that LPS responses in plants could result from a minor but highly active inducer present in LPS preparations used. In view of the later hypothesis, it should be noted that LPS seems unable to activate the immune response in *Drosophila*, and that previously reported effect of LPS in flies resulted from PGN contamination (Leulier et al., 2003).

It appeared in fact that PGN is the major PAMP recognized by Drosophila innate immune system (Royet et al., 2005). PGN is the major component of the cell wall of Gram-positive bacteria, whereas in Gram-negative bacteria it resides as a thin layer in the periplasmic space. PGN are composed of a common core of β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid strands, with little variation between bacteria, but they are cross-linked by short peptides that are highly variable. Drosophila and mammals detect PGNs that are specific for Gram-positive or Gram-negative bacteria (Royet et al., 2005; Philpott and Girardin, 2004). For Drosophila at least, this discriminative detection system would be the basis for the differential activation of innate immune responses by Gram-negative and Gram-positive bacteria (Leulier et al., 2003).

In plants, a single report suggests that PGN preparations of *Staphylococcus. aureus* at the μg/ml range triggered elicitor responses in tobacco cells (Felix and Boller, 2003). This activity was not characterized in details, and we cannot exclude that it was due to the presence of a contaminant in the PGN preparation.

In the same study, bacterial cold-shock protein (CSP) was identified as a new PAMP that acts as a highly active elicitor of defense responses in tobacco and other *Solanaceae* (Table 1) (Felix and Boller, 2003). Arabidopsis was not responsive to CSP-derived elicitors. The minimal active epitope could be defined as a 15 amino-acids peptide (csp15) representing the most conserved part of the CSP protein.

Recently, we showed that elongation factor Tu (EF-Tu), the most abundant bacterial protein, acts as a PAMP in Arabidopsis and other *Brassicaceae*, but not in other plant families (Table 1) (Kunze et al., 2004). EF-Tu is highly conserved in all bacteria and is

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, elf18, is fully active as inducer of defense responses (Kunze et al., 2004).

2.3 Oomycetes and fungal PAMPs

Oomycetes and fungi constitute major classes of plant pathogens and have been therefore extensively studied.

Oligosaccharide elicitors from the β-glucans of pathogenic oomycetes, such as *Phytophtora sojae*, have been well characterized (Table 1). A classic elicitor is the branched β-1,3,β-1,6-heptaglucoside (HG), isolated as the smallest elicitor-active compound from cell walls of the oomycete *Phytophtora megasperma* f.sp *glycinea* (Shibuya and Minami, 2001).

Plants can recognize a number of other cell-wall or secreted oomycetes proteins (Table 1) (Nurnberger *et al.*, 2004). A well-studied example is the 42 kDa *P. megasperma glycineae* glycoprotein that elicits defense response in parsley and potato. The minimal active-epitope has been identified as a surface-exposed 13 amino-acid peptide, Pep13 (Nürnberger et al., 1994), which is present within a transglutaminase enzymatic domain (Brunner et al., 2002). The notion of PAMP (i.e. pivotal function for the microorganism) was there nicely demonstrated as mutational analysis within the Pep13 sequence identified amino-acid residues indispensable for both transglutaminase activity and the activation of plant defense responses (Brunner *et al.*, 2002).

Additional oomycetes-derived elicitors recognized by plants include the necrosis-inducing *Phytophtora* protein (NPP1) (Fellbrich et al., 2002) and the *Pythium aphanidermatum*-derived protein PaNie (Veit et al., 2001) that both induce defense responses in a variety of plants, including Arabidopsis.

Chitin, a β-1,4-linked linear polymer of N-acetylglucosamine, is a major constituent of the cell walls of most higher fungi and its fragments, N-acetylchitooligosaccharides, have been shown to act as potent elicitor signals in several plant species (Table 1) (Shibuya and Minami, 2001). The requirement for the size and structure of active chitin fragments are different depending on the experimental systems

Table 1. Selected pathogen-associated molecular patterns (PAMPs) recognized by plants

PAMP	Active epitope	Responsive plants	Receptor	Refs
Bacteria				
Lipopolysaccharides (LPS)	Lipid A?	Arabidopsis, tobacco, and pepper	Unknown	1-4
Flagellin	flg22 (most conserved domain in N-terminus)	Most plants (except rice)	AtFLS2 (LRR-RLK)	5-7
Harpin	Undefined	Various plants	Unknown	8-10
Cold-shock protein	csp15 (RNP-1 motif)	Solanaceae	Unknown	11
Elongation factor Tu (EF-Tu)	elf18 (acetylated N-terminus)	Brassicaceae	Unknown	12
Oomycetes				
Necrosis-inducing proteins	Undefined	Many dicot plants	Unknown	13-15
Transglutaminase	Pep-13 (surface-exposed epitope of the enzyme)	Parsley and potato	Unknown	16, 17
Lipid-transfer proteins (elicitins)	Undefined	Tobacco	Unknown	18, 19
β-glucans	Branched hepta-β-glucoside	Legumes	GnGBP (soluble protein with 1,3-β-glucanase activity)	20, 21
	Linear oligo-β-glucosides	Tobacco	Unknown	22
Fungi				
Xylanase	TKLGE pentapeptide (surface exposed epitope of the xylanase)	Tobacco and tomato	LeEIX2 (LRR-RLP)	23-26
Invertase	N-mannosylated peptide (fungal-type N-glycosylation)	Tomato	Unknown	27,28
β-glucans	Tetraglucosyl glucitol-branched hepta-β-glucoside	Rice	Unknown	29
Chitin	Chitin oligosaccharides (degree of polymerization > 3)	Tomato, Arabidopsis, rice, and wheat	Unknown	30-34
Ergosterol	Undefined	Tomato	Unknown	35

1. Newman et al., 2002; **2.** Meyer et al., 2001; **3.** Zeidler et al., 2004; **4.** Dow et al., 2000; **5.** Felix et al., 1999; **6.** Gòmez-Gòmez and Boller, 2000; **7.** Chinchilla et al., submitted; **8.** He et al., 1993; **9.** Lee et al., 2001; **10.** Wei et al., 1992; **11.** Felix and Boller, 2003; **12.** Kunze et al., 2004; **13.** Veit et al., 2001; **14.** Qutob et al., 2002; **15.** Fellbrich et al., 2002; **16.** Nürnberger et al., 1994; **17.** Brunner et al., 2002; **18.** Ricci et al., 1989; **19.** Osman et al., 2001; **20.** Umemoto et al., 1997; **21.** Fliegmann et al., 2004; **22.** Klarzynski et al., 2000; **23.** Hanania and Avni, 1997; **24.** Enkerli et al., 1999; **25.** Rotblat et al., 2002; **26.** Ron and Avni, 2004; **27.** Basse et al., 1992; **28.** Basse et al., 1993; **29.** Yamaguchi et al., 2000; **30.** Baureithel et al., 1994; **31.** Felix et al., 1993; **32.** Ito et al., 1997; **33.** Barber et al., 1989; **34.** Zhang et al., 2002; **35.** Granado et al., 1995.

(Shibuya and Minami, 2001). Recently, chitin has also been used as an elicitor in Arabidopsis, where it triggers rapid changes on defense-related gene expression (Ramonell et al., 2002; Zhang et al., 2002; Salinas-Mondragon et al., 1999) and the activation of a MAP kinase pathway similar to the one reported in flagellin studies (Wan J. et al., 2004).

Similarly to oomycetes, fungi secrete a large variety of proteins that are recognized as general elicitors, such yeast glycopeptides and xylanase (Table 1) (Boller, 1995). The 22 kDa fungal ethylene-inducing xylanase (EIX) acts as a potent elicitor in diverse plant species, such as tobacco and tomato (Boller, 1995). Analysis of EIX mutant lacking enzymatic activity (β-1-4-endoxylanase) but retaining elicitor activity showed that the EIX protein itself functions as elicitor, and not the xylan fragments that could be released from the plant cell walls due to the xylanase activity (Enkerli et al., 1999; Furman-Matarasso et al., 1999). The TKLGE pentapeptide, a surface-exposed epitope of EIX, was shown to be essential for its elicitor activity (Rotblat et al., 2002).

Interestingly, fungi can also indirectly induce plant defenses. Fungal pectolytic enzymes that are required during the infection to digest plant cell walls have been early found to induce plant defense responses (Cervone et al., 1997). However, it became clear that the elicitor active principle was not the enzymes themselves, but oligosaccharides released from the plant cell wall by these enzymes (Shibuya and Minami, 2001). The most intensively studied oligosaccharides are the oligo-α-galacturonides (OGAs), which are produced from degraded homogalacturonans by fungal endo-polygalacturonases (Shibuya and Minami, 2001; Vorwerk et al., 2004). Because they are plant-derived, OGAs are endogenous elicitors. Their perception by plants nicely illustrates the concept of “danger signal” perception by the innate immune system (Matzinger, 2002).

Several viral compounds act as PAMPs in mammals, such as single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) and viral DNA (Wagner, 2004). It is still unknown if any of these molecules also trigger defense responses in plants.

3. Pattern recognition receptors

3.1 Current status in animal systems

3.1.1 In mammals

The hunt for PRRs culminated with the discovery of the role of Toll-like receptor (TLR) proteins in mammalian innate immunity (O'Neill, 2004). They are transmembrane proteins with extracellular LRRs and an intracellular Toll-IL-1 receptor homology (TIR) domain. Thirteen mammalian TLR paralogues have now been identified (10 in humans and 12 in mice). Humans express ten TLRs, enumerated 1 through 10. Mice do not express TLR10, but do express TLRs 1 through 9, and have two additional paralogs (11 and 12) that are not represented in humans (Beutler, 2004).

Table 2. Mammalian TLRs and NODs [a]

	Agonists	Microorganisms	Co-receptors	Cellular localization
TLR1	Triacyl lipopeptides	Bacteria	TLR2	Plasma membrane
TLR2	Lipoteichoic acid GPI-linked proteins Atypical LPS Lipoproteins Zymosan	Gram-positive bacteria Trypanosomes Gram-negative bacteria Mycobacteria Fungi	TLR1, TLR6	Plasma membrane
TLR3	dsRNA	Viruses		Endosomes
TLR4	LPS F protein Hsp60? FN fragments?	Gram-negative bacteria Respiratory Syncytial Virus Host Host	CD14, MD-2	Plasma membrane
TLR5	Flagellin	Bacteria		Plasma membrane
TLR6	Diacyl lipopeptides Zymosan	Mycobacteria Fungi	TLR2	Plasma membrane
TLR7	ssRNA	Viruses		Endosomes
TLR8	ssRNA	Viruses		Endosomes
TLR9	CpG motifs	Viruses and bacteria		Endosomes
TLR10	?			?
TLR11	?	Uropathogenic bacteria		?
TLR12	?			?
NOD1	DAP-peptidoglycan	Gram-negative bacteria		Cytoplasm
NOD2	Peptidoglycan	Bacteria		Cytoplasm

[a]Abbreviations: DAP, meso-diominopimelic acid; ds, double-stranded; FN, fibronectin; GPI, glycosylphosphatidylinositol; LPS, lipopolysaccharides; NOD, nucleotide oligomerisation domain; ss, single-stranded; TLR, Toll-like receptor. Adapted from O'Neill (2004) and Akira and Takeda (2004).

These TLRs are activated by a wide range of microbial stimuli allowing them to sense organisms ranging from protozoa to bacteria to fungi to viruses (Table 2) (Akira and Takeda, 2004). Some TLRs are still orphan receptors in the sense that their agonist is unknown, whereas others can sense several microbial inducers. In addition, the

number of recognized PAMPs can be significantly enhanced through cooperation between different TLRs (Akira and Takeda, 2004). Whether all TLRs are actually receptors is still a matter of debate because, for most of them, direct binding of microbial ligands has yet to be demonstrated (O'Neill, 2004). Two TLRs, TLR4 and TLR5, have been characterized in details.

TLR4 is essential for the response to LPS (Poltorak *et al.*, 1998). However, recognition of LPS requires other molecules in addition to TLR4. LPS, which sticks in the outer membrane of Gram-negative bacteria, has to be first processed by the soluble LPS-binding protein (LBP) and CD14, an LRR-containing protein. LBP removes LPS from the bacterial membrane and forms complexes consisting of LPS, LBP and soluble CD14 (sCD14). These complexes deliver LPS to membrane glycosylphosphatidylinositol (GPI)-anchored CD14 for LPS signalling. LPS bound to CD14 is then transferred to TLR4-MD-2 complex, which undergoes oligomerization and triggers signalling (Miyake, 2004). It is however not fully understood how LPS interacts with TLR4-MD-2.

In addition to LPS, TLR4 appears to mediate inflammatory responses to many different self and non-self ligands, such as the fusion protein (F protein) of respiratory syncytial virus, the endogenous heat-shock protein 60 (Hsp60) and host-derived fibronectin fragments (Takeda et al., 2003). However, it is still unclear whether these additional ligands also bind the CD14-TLR4-MD-2 complex directly.

In mammals, flagellin triggers innate immune responses via direct interaction with the Toll-like receptor TLR5 (Hayashi et al., 2001; Smith et al., 2003; Mizel et al., 2003). Other than sharing the common feature of an extracellular LRR domain, there is no notable amino acid similarity between FLS2 and TLR5. In addition, TLR5 detects a specific conformation of flagellin domain D1, which is different from the flg22 epitope, suggesting that both detection systems evolved independently by convergent evolution (Smith et al., 2003; Donnelly and Steiner, 2002; Jacchieri et al., 2003). Further investigations are needed to decipher the molecular interactions of TLR5 with flagellin, as available results are not conclusive (Jacchieri et al., 2003; Mizel et al., 2003). The significance of flagellin/TLR5 interaction in disease resistance was highlighted with the

findings that a natural TLR5 polymorphism is associated with enhanced susceptibility to *Legionella pneumophila* in human (Hawn et al., 2003), and that bacteria evolved strategies to escape flagellin recognition (Ramos et al., 2004).

Despite the major importance of TLRs, mammals possess additional PRRs such as mannose binding lectins, dectin and, of increasing interest, the intracellular nucleotide oligomerisation domain (NOD) family (Table 2) (Herre et al., 2004; Philpott and Girardin, 2004).

3.1.2 In Drosophila

In Drosophila, two major pathways control the expression of antimicrobial genes during microbial infection (Figure 1) (Hoffmann, 2003). Gram-positive bacteria and fungi predominantly induce the Toll signalling pathway, whereas Gram-negative bacteria activate the Imd pathway. Toll is a transmembrane protein with extracellular LRRs and an intracellular TIR domain, whereas Imd is a cytoplasmic protein. Toll, the namesake of the TLR family, is involved in immunity but most Toll receptor orthologues seem to exert developmental rather than immune functions (Lemaitre, 2004; Bilak et al., 2003). Toll receptor itself does not act as a PRR but rather as a cytokine receptor, with Spaetzle as an endogenous ligand (Figure 1) (Lemaitre, 2004). Recent research indeed demonstrated a critical role for peptidoglycan recognition proteins (PGRPs) and Gram-negative bacteria binding proteins (GNBPs) as recognition receptors of microbial infections in Drosophila (Figure 1) (Royet et al., 2005). The Drosophila genome encodes 13 PGRPs and 3 GNBPs; all of them are not likely to play a role in perception (Royet et al., 2005).

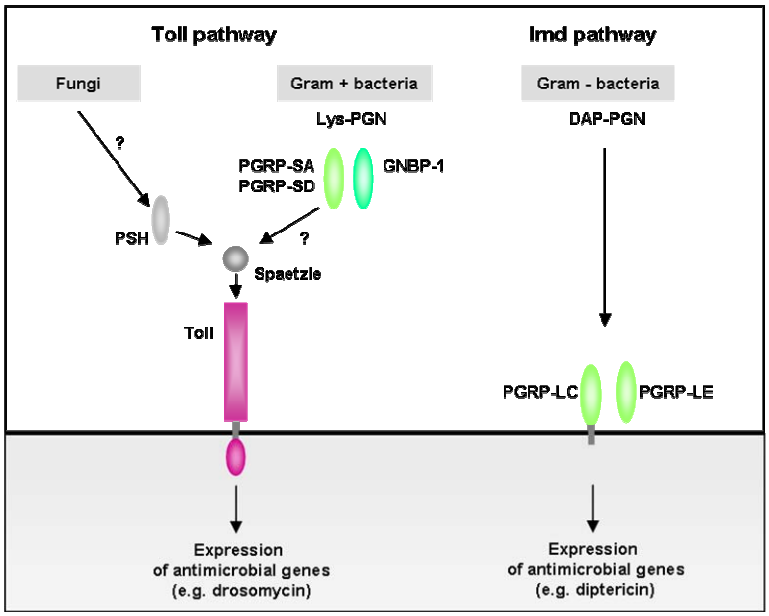

Figure 1 Drosophila innate immune recognition.
In Drosophila, the Toll pathway is essentially triggered during infection by fungi and Gram-positive bacteria. Toll activation is not mediated by direct interaction with microbial ligands, but by binding to a cleaved form of the cytokine Spaetzle. This cytokine is cleaved in the blood by a circulating protease, which has not been formally identified. Upstream activation of this cascade during Gram-positive infection requires the presence of peptidoglycan-recognition protein PGRP-SA (and frequently PGRP-SD), concomitantly with the Gram-negative bacteria binding protein GNBP-1. Activation of a proteolytic cascade during fungal infection can involve the circulating serine protease Persephone (PSH). During Gram-negative bacterial infection, microbial sensing occurs at the plasma membrane of immune-responsive cells by the transmembrane receptor PGRP-LC in synergy with PGRP-LE leading to activation of the Imd pathway by an unknown mechanism (adapted from Royet et al., 2005).

3.2 Plant pattern recognition receptors

In plants, despite the isolation and characterization of numerous potential PAMPs, and the development of binding studies with labelled PAMPs, there is still a great deal to learn about the PRRs (Montesano et al., 2003; Nurnberger et al., 2004). Only three PRR/ligand pairs have been until now identified in all plants, each in different species and involving different classes of PRR protein.

3.2.1 Receptor-like kinases (RLKs)

Receptor-like kinases form one of the largest gene families in plants with at least 610 members in Arabidopsis (Shiu and Bleecker, 2001b), and about 1131 members in rice (Shiu et al., 2004). In general, RLKs are transmembrane proteins with versatile N-terminal extracellular domains and a C-terminal intracellular kinase domain that is related to the Drosophila Pelle kinase (Shiu and Bleecker, 2001b).They can be classified according to the structural characteristics of their extracellular domains (Shiu and Bleecker, 2001a). Some RLKs do not have any signal peptide and/or transmembrane region, and are named receptor-like cytoplasmic kinases (RLCKs). The diversity and large number of plant RLKs suggest that they may be involved in the perception of a wide range of stimuli. Only a few RLKs have been functionally characterised and they seem to play roles in development, growth, plant defense and symbiosis (Tables 3 and 4).

The LRR-receptor kinase FLS2 protein represents so far the only known PRR in Arabidopsis, and the only receptor-like kinase involved in PAMP perception in plants. It is however tempting to speculate that some other might play a similar function.
Structural features of some RLKs suggest a PRR function. The largest subfamily of RLK possesses LRRs as extracellular domain. The LRR motif often participates in protein-protein interaction (Kobe and Deisenhofer, 1995). LRR could therefore directly bind proteinaceous ligands, or ligands in complex with proteins. In animals, LRRs are found in various membrane proteins involved in pathogen recognition such as the Drosophila Toll and mammalian TLRs (Bell et al., 2003). In addition, the predominant structural motif found in plant R proteins is LRR (Nimchuk et al., 2003).

Table 3 Plant RLKs with known functions in growth or development

Name	Plant	Class	Function	Putative ligands	References
BRI1	*Arabidopsis thaliana*	LRR	Brassinosteroid perception	BL	Kinoshita et al., 2005; Li and Chory, 1997
BRL1	*Arabidopsis thaliana*	LRR	Brassinosteroid perception	BL	Cano-Delgado et al., 2004; Zhou et al., 2004
BRL3	*Arabidopsis thaliana*	LRR	Brassinosteroid perception	BL	Cano-Delgado et al., 2004
BAK1/SERK3	*Arabidopsis thaliana*	LRR	Brassinosteroid response	?	Li et al., 2002; Nam and Li, 2002
CLV1	*Arabidopsis thaliana*	LRR	Meristem maintenance	?	Clark et al., 1997; Trotochaud et al., 2000; Nishihama et al., 2003
ERECTA	*Arabidopsis thaliana*	LRR	Organ shape	?	Torii et al., 1996
HAESA/RLK5	*Arabidopsis thaliana*	LRR	Floral organ abscission	?	Jinn et al., 2000
EMS1/EXS	*Arabidopsis thaliana*	LRR	Microspore development	?	Canales et al., 2002; Zhao et al., 2002
VH1/BRL2	*Arabidopsis thaliana*	LRR	Leaf patterning	?	Clay and Nelson, 2002; Cano-Delgado et al., 2004
ACR4	*Arabidopsis thaliana*	LRR	Cell layer organization	?	Gifford et al., 2003; Watanabe et al., 2004
SCM	*Arabidopsis thaliana*	LRR	Positional development in root	?	Kwak et al., 2005
CDG	*Arabidopsis thaliana*	RLCK	Organ growth and elongation		Muto et al., 2004
SERK1	*Daucus carota/Arabidopsis thaliana*	LRR	Ovule development and early embryogenesis?	?	Hecht et al., 2001
PSKR	*Daucus carota*	LRR	Phytosulfokine mediated growth response	Phytosulfokine	Matsubayashi et al., 2002
CRINKLY4	*Zea mays*	CR4L	Epidermal cell development	?	Becraft et al., 1996
PRK1	*Petunia inflata*	LRR	Post meiotic development of microspores	?	Lee et al., 1996
SRK	*Brassica oleracea*	S domain	Self incompatibility	SP11/SCR	Takayama et al., 2001; Kachroo et al., 2001
MLPK	*Brassica rapa*	RLCK	Self incompatibility		Murase et al., 2004
FON1	*Oryza sativa*	LRR	Floral meristem size	?	Suzaki et al., 2004
MSP1	*Oryza sativa*	LRR	Early sporogenic development	?	Nonomura et al., 2003
SR160/CURL3	*Lycopersicon esculentum*	LRR	Systemin/Brassinosteroid perception	BL ?	Scheer and Ryan, Jr., 2002; Montoya et al., 2002
Le PRK2	*Lycopersicon esculentum*	LRR	Pollination	LAT52?	Muschietti et al., 1998; Tang et al., 2002

Table 4 Plant RLKs with known functions in plant-microbe interaction

Name	Plant	Class	Function	Putative ligands	References
FLS2	*Arabidopsis thaliana*	LRR	Flagellin perception	flg22	Gòmez-Gòmez and Boller, 2000; Chinchilla et al., submitted
PBS1	*Arabidopsis thaliana*	RLCK	Specific resistance to *Pseudomonas syringae* pv *phaseolicola*		Swiderski and Innes, 2001
ERECTA	*Arabidopsis thaliana*	LRR	Resistance to *Ralstonia solanacearum*	?	Godiard et al., 2003
Pto	*Lycopersicon esculentum*	RLCK	Specific resistance to *Pseudomonas syringae* pv *tomato*	AvrPto	Martin et al., 1993; Tang et al., 1996; Scofield et al., 1996
Pti	*Lycopersicon esculentum*	RLCK	Specific resistance to *Pseudomonas syringae* pv *tomato*	?	Zhou et al., 1995
SR160/CURL3	*Lycopersicon esculentum*	LRR	Systemin perception? Brassinosteroid perception?	Systemin? BL?	Scheer and Ryan, Jr., 2002; Montoya et al., 2002
Xa21	*Oryza sativa*	LRR	Specific resistance to *Xanthomonas oryzae* pv *oryzae*	?	Song et al., 1995
Xa26	*Oryza sativa*	LRR	Specific resistance to *Xanthomonas oryzae* pv *oryzae*		Sun et al., 2004
LRK10	*Triticum aestivum*	LRK10L	Specific resistance to wheat rust fungi	?	Feuillet et al., 1997
HAR1/NARK	*Lotus japonicus/Glycine max*	LRR	Nodule development during nitrogen fixation symbiosis	?	Searle et al., 2003; Krusell et al., 2002; Nishimura et al., 2002
NORK/SYMRK	*Medicago sativa/L. japonicus*	LRR	Root nodule and mycorrhiza formation	?	Stracke et al., 2002; Endre et al., 2002
NFR1/LYK3	*L. japonicus//M. truncatula*	LysM	Early events during nitrogen fixation symbiosis	Nod factors?	Limpens et al., 2003; Radutoiu et al., 2003
LYK4	*M. truncatula*	LysM	Early events during nitrogen fixation symbiosis	Nod factors?	Limpens et al., 2003
NFR5/SYM10	*L. japonicus/Pisum sativum*	LysM	Early events during nitrogen fixation symbiosis	Nod factors?	Madsen et al., 2003

The second largest class of extracellular motifs found in RLKs are various sugar-binding motifs or lectins. In the case of lectin receptor protein kinases (LecRKs) (Herve et al., 1996), their extracellular domains contain lectin motifs originally found in the seeds of leguminous plants. Legume lectins can bind various disaccharides and complex sugars (Loris et al., 1998). Another type of lectin found in plant RLKs is the C-type lectin. This motif is found in proteins that mediate innate immune responses in mammals, and bind to a diverse range of sugar moieties on the surface of non-self biological entities and cells (Cambi and Figdor, 2003).

Other types of sugar-binding motifs than lectins are also present in RLKs. The first one is the lysin motif (LysM) originally identified in bacteria and thought to function as a general peptidoglycan-binding motif (Bateman and Bycroft, 2000).

Another class of RLK has been proposed to act as a putative chitin PRR. The chitinase receptor kinase CHRK1, an RLK with a chitinase extracellular domain, exhibiting autophosphorylation activity but no chitinase activity, was identified in tobacco plasma membranes (Kim et al., 2000). However recent data suggest that CHRK1 is rather involved in plant development and cytokinin homeostasis (Lee et al., 2004; Lee et al., 2003).

Another motif potentially implicated in the perception of fungal cell wall component is the thaumatin domain, which is found in the extracellular region of Arabidopsis PR5K (Wang et al., 1996). Thaumatin domains have antifungal activity and in vitro chitinase activity (Fritig et al., 1998).

In Drosophila, a positive feedback transcriptional regulation was also reported for several PGRPs and GNBPs that are involved in innate immune recognition (Irving et al., 2001; De Gregorio et al., 2001). Differential expression has been observed for some RLK genes in response to general elicitors, pathogens or signal molecules related to defense responses such salicylic acid (SA), and suggests the implication of additional RLKs in PAMP recognition.

Four Arabidopsis RLKs with unknown DUF26 extracellular domain called *RLK-3, -4, -5, -6* are induced by pathogenic bacteria, oxidative stress and SA treatment (Czernic et al., 1999; Du and Chen, 2000). These genes were later found to be members of the family of cysteine-rich RLKs (CRKs) and were renamed *CRK11, -10, -6* and *-5*,

respectively (Chen, 2001). Overexpression of *CRK5*/*RLK6* correlated with increased resistance to the bacterial pathogen *Pseudomonas syringae* in Arabidopsis (Chen et al., 2003). SA-inducible expression has also been observed for the Arabidopsis RLK *RKC1* (DUF26 domain), *RKL1* (LRR domain) and the S-domain RLKs *RKS1* and *RKS2* (Ohtake et al., 2000). Other S-domain RLKs from *Brassica oleracea* and from Arabidopsis are induced by wounding, bacterial infection and SA treatment (Pastuglia et al., 1997; Pastuglia et al., 2002; Rocher et al., 2005). An Arabidopsis lectin RLK, *lecRK-a1*, is induced during senescence, wounding and in response to OGAs (Riou et al., 2002). The Arabidopsis LRR-RLK *SIRK/FRK1* (*Senescence-induced receptor kinase/Flagellin-induced receptor kinase 1*) is induced during leaf senescence, but also by flagellin treatment (Asai et al., 2002; Robatzek and Somssich, 2002).
Interestingly, the expression of several of these RLKs seems to be regulated by WRKY transcription factors (Du and Chen, 2000; Robatzek and Somssich, 2002; Rocher et al., 2005). These transcription factors are involved in plant defenses (Eulgem et al., 2000), and some members (WRKY6, -29 and -22) are activated by flagellin treatment (Asai et al., 2002; Robatzek and Somssich, 2001).

3.2.2 Receptor-like proteins (RLPs)

Receptor-like proteins (RLPs) are transmembrane proteins with extracellular LRRs, a short cytoplasmic tail, but lacking any intracellular signalling domain, like a kinase domain. There are 57 and 90 RLPs in Arabidopsis (Tor et al., 2004) and rice (Fritz-Laylin et al., 2005), respectively. RLPs seem to be involved in growth, development as well as in plant defense (Shiu and Bleecker, 2003). The majority of known RLPs are in fact *R* genes, as exemplified by the *Cf* (resistance to *Cladosporium fulvum*) genes in tomato (Rivas and Thomas, 2002) and *RPP27* (Resistance to *Peronospora parasitica* 27) in Arabidopsis (Tor et al., 2004). As RLPs do not possess any known signaling, or interaction domain in their short intracellular tail, it has been therefore suggested that RLPs might work together with RLKs (Shiu and Bleecker, 2003). This is indeed illustrated in developmental process, where the RLP CLV2 may function with the LRR-RLK CLV1 in meristem maintenance (Jeong et al., 1999).

The first example of a RLP-type PRR involved in PAMP perception has been recently provided by the identification of the tomato receptor for the fungal elicitor ethylene-inducing xylanase (EIX) (Ron and Avni, 2004). In this study, the authors characterized the locus that confers EIX response in tomato (*LeEIX*). The *LeEIX1* and *LeEIX2* genes both encoding RLPs, were shown to be capable of binding EIX independently. However, only LeEIX2 can transmit the signal to activate HR, when expressed transiently in tobacco. Interestingly, a putative endocytosis motif, YXXØ, present in the LeEIX2 sequence is essential for EIX-induced HR, suggesting a role of LeEIX2-endocytosis for EIX signalling.

3.2.3 Others

The third example of plant PRR is provided by the heptaglugoside receptor of soybean. Based on the initial findings of a high-affinity binding site for *Phytophtora* glucans in soybean plasma membranes, the 75 kDa glucan-binding protein (GBP) could be identified (Umemoto et al., 1997; Mithofer et al., 1996). GBP is a soluble protein with a 1,3-β-glucanase activity (Fliegmann et al., 2004). It is therefore proposed that, during initial contact with *Phytophtora*, the intrinsic endo-1,3-β-glucanase activity of the GBP could release oligoglucoside fragments enriched in motifs that constitute ligands for the high affinity binding site present in GBP. A similar mechanism is involved in LPS recognition in mammals, where initial contact with and release of LPS is assumed by the LPS-binding protein (LBP), allowing later interaction with CD14 and MD-2, and finally with TLR4 (Miyake, 2004). Absence of recognizable functional domains for transmembrane signalling within GBP, detection of multiple labelled proteins in photoaffinity experiments, and the presence of GBP in non-responsive plants (e.g. Arabidopsis) suggest that GBP may form part of a multicomponent recognition complex, for example with a yet unknown transmembrane receptor (Fliegmann et al., 2004; Mithofer et al., 2000).

Interestingly, a few proteins that exhibit characteristics of receptors and confer broad resistance against pathogens have been identified in plants. The NHL3 gene, a member of the Arabidopsis nonrace-specific disease resistance (*NDR1*)/and the tobacco harpin-induced (*HIN1*) gene family, encodes a plasma membrane protein and

its overexpression correlates with increased resistance to pathogenic *Pseudomonas syringae* (Varet et al., 2003). The Arabidopsis RPW8, which carries a putative anchor signal to the plasma membrane at its N-terminus and possesses a single coiled-coil (CC) domain, confers resistance to all Arabidopsis-infecting powdery mildew isolated tested (Xiao et al., 2003; Xiao et al., 2001). RPG1, which controls stem rust in barley, has tandem protein kinase domains at its C-terminus and appears not to be membrane bound (Brueggeman et al., 2002; Horvath et al., 2003). It will be interesting to see whether NHL3, RPW8 and/or RPG1 act as receptors for PAMPs or would function further downstream in the defense response. If RPW8 and RPG1 were proven PRR, they would constitute prime examples of cytoplasmic PAMP recognition in plants.

4. Pathogen recognition in host cultivar-specific resistance

Many R proteins have been identified in diverse plant species. The most prevalent R proteins include the cytoplasmic nucleotide-binding site (NBS)-LRR proteins, and to a lesser extent, transmembrane LRR-RLKs and membrane-anchored RLPs. The amino-terminal of the NBS-LRR proteins can be a TIR homology or a CC effector domain (Nimchuk et al., 2003). It is interesting to see that proteins involved in R-gene mediated resistance and PAMP perception are similar. This suggests that R proteins probably evolved from PAMP receptors. However, the major class of R proteins is the cytoplasmic NBS-LRR family; to date, no NBS-LRRs were found to be involved in PAMP recognition. In addition, the three known examples of plant PRRs (AtFLS2, LeEIX2 and GmGBP1) directly bind with their corresponding ligands (Chinchilla et al., submitted; Ron and Avni, 2004; Umemoto et al., 1997). This is likely not the rule for R-Avr interactions (Nimchuk et al., 2003), although direct interaction between Avr and R proteins has been demonstrated in a few cases (Jia et al., 2000; Scofield et al., 1996; Tang et al., 1996; Deslandes et al., 2003). Indeed, several studies have provided evidence that LRR-type R proteins constitute components of larger signal perception complexes, and do not necessarily bind directly to their matching Avr proteins (Mackey et al., 2003; Mackey et al., 2002; Axtell and Staskawicz, 2003; Luderer et al., 2001). These findings confirm the “guard hypothesis”, which predicts that Avr proteins act as virulence factors that contact their cognate pathogenicity targets in host plants or even non-host plants, but function as elicitors of cultivar-

specific plant resistance only when the complementary R protein is recruited into a functional signal perception complex (Dangl and Jones, 2001; Van der Hoorn et al., 2002). Thus, the role of R protein is to monitor (i.e. “guard” against) the Avr mediated perturbation of cellular functions.

5. Aim of the work

Similarly to Drosophila and mammals, plants have perception systems for PAMPs. In animal system, most of the advances in the understanding of PAMP perception have been possible thanks to genetic and genomic tools available in the model species *Drosophila melanogaster* and *Mus musculus*. As of today, many potential PRRs have been identified in these systems, but the corresponding ligands are rarely known.

In contrast, in plants, the ease of bioassays, and particularly the use of cell cultures to study elicitor effects enabled the identification of a wide range of PAMPs acting in diverse plant species, but little is known about the proteins involved in their recognition. It is clear that although not all plant species may recognize and respond to all of these signals, plant cells have recognition systems for multiple signals derived from individual microbial species. For example, Arabidopsis can recognize invading bacteria through at least flagellin, LPS and EF-Tu perception.

The goal of this work was to use the plant model *Arabidopsis thaliana* to understand how different bacterial PAMPs are perceived by a single plant species, and how their perceptions could contribute to bacterial basal disease resistance, using the paradigmatic flg22/FLS2 perception system as a starting point.

Chapter 1

The Transcriptional Innate Immune Response to flg22. Interplay and Overlap with Avr Gene-Dependent Defense Responses and Bacterial Pathogenesis

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The Transcriptional Innate Immune Response to flg22. Interplay and Overlap with Avr Gene-Dependent Defense Responses and Bacterial Pathogenesis[1[w]]

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Animals and plants carry recognition systems to sense bacterial flagellin. Flagellin perception in Arabidopsis involves FLS2, a Leu-rich-repeat receptor kinase. We surveyed the early transcriptional response of Arabidopsis cell cultures and seedlings within 60 min of treatment with flg22, a peptide corresponding to the most conserved domain of flagellin. Using Affymetrix microarrays, approximately 3.0% of 8,200 genes displayed transcript level changes in flg22 elicited suspension cultures and seedlings. *FLARE* (Flagellin Rapidly Elicited) genes mostly encode signaling components, such as transcription factors, protein kinases/phosphatases, and proteins that regulate protein turnover. Approximately 80% of flg22-induced genes were also up-regulated in Arabidopsis seedlings treated with cycloheximide. This suggests that many *FLARE* genes are negatively regulated by rapidly turned-over repressor proteins. Twenty-one tobacco Avr9/Cf-9 rapidly elicited (*ACRE*) cDNA full-length sequences were used to search for their Arabidopsis orthologs (*AtACRE*). We identified either single or multiple putative orthologs for 17 *ACRE* genes. For 13 of these *ACRE* genes, at least one Arabidopsis ortholog was induced in flg22-elicited Arabidopsis suspension cells and seedlings. This result revealed a substantial overlap between the Arabidopsis flg22 response and the tobacco Avr9 race-specific defense response. We also compared *FLARE* gene sets and genes induced in basal or gene-for-gene interactions upon different *Pseudomonas syringae* treatments, and infer that *Pseudomonas syringae* pv *tomato* represses the flagellin-initiated defense response.

Plants and animals mount defense responses upon recognition of numerous pathogen-derived molecules. These pathogen-associated molecular patterns (PAMPs) include bacterial cell wall components such as lipopolysaccharide (Ulevitch and Tobias, 1999). PAMPs are (1) highly conserved (2) present in different organisms and (3) usually play a pivotal role for the life of the microorganism (Janeway and Medzhitov, 1998). In mammals, the perception of PAMPs occurs through Toll-like receptors (TLRs). For instance, in mice, the innate immune response is activated through perception of the Salmonella flagellin by the TLR5 receptor (Hayashi et al., 2001). Several plant species, including Arabidopsis, have a specific recognition system for a conserved, 22-amino acid motif (flg22) of the bacterial flagellin (Felix et al., 1999). The Arabidopsis innate immune response to flg22 involves a host recognition protein complex that contains the FLS2 Leu rich repeat (LRR) receptor kinase (Gómez-Gómez et al., 2001). The flg22-FLS2 interaction leads to production of reactive oxygen species (ROS), medium alkalinization, activation of mitogen-activated protein (MAP) kinases, and induction of pathogen-responsive genes (Felix et al., 1999; Gómez-Gómez et al., 1999; Nühse et al., 2000; Asai et al., 2002).

In gene-for-gene relationships, plants carrying a resistance (*R*) gene resist pathogen races with the corresponding avirulence (*Avr*) gene (Flor, 1971; Keen, 1990). This specific recognition leads to activation of defense responses and local cell death referred to as the hypersensitive response (HR). A well-characterized example of HR elicitation through gene-for-gene interaction is provided by the tomato (*Lycopersicon esculentum*) *Cf-9* gene, which confers resistance to races of the fungus *Cladosporium fulvum* expressing the *Avr9* gene (Van den Ackerveken et al., 1992). The product of Avr9 is secreted and subsequently processed by fungal and plant proteases to produce a peptide of 28 amino acids (Joosten et al., 1994). Treatment of leaves of Cf9 tomato or transgenic Cf9 tobacco (*Nicotiana tabacum*) with the Avr9 peptide induces HR within 24 h (Hammond-Kosack et al., 1998). In addition, Avr9-treated Cf9 tobacco cell cultures show rapid production of ROS and activation of MAP kinases and calcium-dependent protein kinases (CDPKs;

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Romeis et al., 1999, 2000). Gene expression profiling of Avr9-treated Cf9 tobacco cells revealed a set of *Avr9/Cf-9* rapidly elicited (*ACRE*) genes induced within 15 to 30 min after elicitation (Durrant et al., 2000).

Bacterial plant pathogens can also be recognized in a gene-for-gene manner. Bacterial Avr proteins are translocated into the host cells through a type III protein secretion system (Galan and Collmer, 1999) which, in the case of *Pseudomonas syringae* DC3000, is thought to deliver more than 30 effector proteins (Boch et al., 2002; Collmer et al., 2002; Fouts et al., 2002; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002). AvrRPM1 and AvrRpt2 from *P. syringae* provide examples of such type III effector proteins that are recognized by the products of the *RPM1* and *RPS2* resistance genes, respectively (Dangl et al., 1992; Innes et al., 1993). This recognition initiates the plant HR response through modification or loss of the host RIN4 protein (Mackey et al., 2002; Mackey et al., 2003; Axtell and Staskawicz, 2003). Although the mechanisms of bacterial Avr defense activation is becoming clearer, very little is known about the potential connection between race-specific and PAMP-mediated innate immune responses to bacterial pathogens.

Most plants are resistant to most pathogens through a basal defense mechanism referred to as nonhost resistance, which is based on both constitutive and inducible defense responses. For instance, the nonhost bacterium *P. syringae* pv *tabaci* induces accumulation of defense transcripts in *Phaseolus vulgaris,* leading to antimicrobial phytoalexin production (Jakobek et al., 1993). Interestingly, type III secretion system mutants of the same bacterial strain trigger the same set of genes in *Phaseolus vulgaris* (Jakobek et al., 1993), suggesting that general elicitors such as PAMPs (e.g. flg22) are likely to play a crucial, albeit yet uncharacterized, role in elicitation of nonhost resistance.

The goal of this study was to investigate the possible connections between innate immunity, race-specific, and nonhost types of resistance responses. Using a high-density oligonucleotide microarray (Affymetrix, La Jolla, CA), we studied the rapid changes in gene expression that occur in Arabidopsis cell cultures and seedlings treated with the flg22 peptide. We found that these flagellin rapidly elicited (*FLARE*) genes mostly encode signaling components. The flg22-rapidly elicited genes in cell cultures were called c*FLARE* genes and in seedlings s*FLARE* genes. The majority of these genes were also up-regulated upon treatments with the protein synthesis inhibitor cycloheximide (CHX), suggesting that *FLARE* genes are negatively regulated by rapidly turned-over repressor proteins. Analysis of a set of Arabidopsis *ACRE* orthologs revealed a substantial overlap between the Avr9 race-specific response in tobacco and the flg22-elicited innate immune response in Arabidopsis, suggesting that at least some polymorphic race-specific resistance mechanisms have evolved from mechanisms that recognize PAMPs. Finally, a comparison of genes that were up-regulated upon treatments with either virulent, avirulent, or nonhost *P. syringae* strains revealed that (1) genes induced in nonhost interactions might be regulated through PAMP perception, (2) some type III effector proteins could suppress PAMP-induced genes, and (3) Avr proteins, if recognized through an *R* gene, might positively regulate the PAMP-mediated innate immune response.

RESULTS

Validation of Cell Culture and Seedling Systems for flg22 Inducibility

To monitor gene expression changes in response to flg22, cell suspension cultures of Arabidopsis ecotype Landsberg *erecta* (L*er*) were exposed in two independent experiments to 100 nM flg22. RNA was prepared from cells 30 and 60 min after elicitation. Control samples were taken from cultures treated with dimethyl sulfoxide, and from untreated cell cultures. Elicitors, such as flg22, induce medium alkalinization and ethylene production (Felix et al., 1999; Gómez-Gómez et al., 1999). The pH in the extracellular medium of the cell cultures was monitored upon flg22 addition and a very reproducible response was observed (Fig. 1A; Felix et al., 1999). In parallel, two independent sets of 2-week-old Arabidopsis ecotype Columbia (Col-0) seedlings were incubated with 10 μM flg22 for 30 min, and total RNA extracted. To confirm elicitation, the flg22-induced production of ethylene was measured (Fig. 1C). Moreover, reverse transcription (RT)-PCR of selected genes such as *AtWRKY29* (At4g23550), previously described to be rapidly flg22 inducible in Arabidopsis protoplasts (Asai et al., 2002), and *AtMPK3* (At3g45640), the Arabidopsis ortholog of *WIPK* (Romeis et al., 2000) that is rapidly induced in Cf-9-tobacco suspension cells upon Avr9 treatment, showed the flg22-inducibility of both systems (Fig. 1, B and D).

Identification and Classification of Early flg22-Regulated Genes

We used high-density oligonucleotide arrays (Affymetrix) to study early flg22-induced changes in gene expression and to identify flg22-rapidly elicited (*FLARE*) genes. The arrays contain probe sets for about 8,200 different Arabidopsis genes (Zhu and Wang, 2000). Biotin-labeled cRNA representing each time point was hybridized individually. To identify the induced or repressed genes in duplicate experiments, we used quantitative and qualitative criteria that were applied individually to the data set at each time point of the time course. Genes were considered as up- or down-regulated if their expression level deviated (positively or negatively) more than 2.5-fold upon elicitor treatment, and designated I for increase and D for decrease based on Wilcoxon's signed-rank test

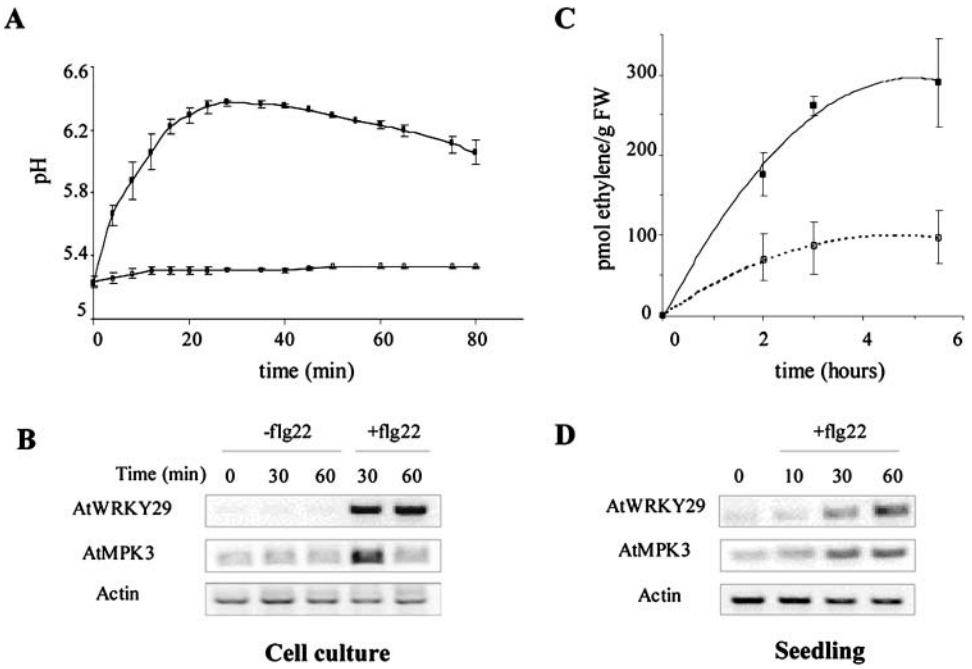

Figure 1. Responsiveness of Arabidopsis cell cultures and seedlings to flg22 elicitor. A, Extracellular medium alkalinization in Arabidopsis cell culture. The pH of the cell culture extracellular medium was measured with glass electrode. White boxes represent control cell cultures and black boxes represent flg22-treated cell cultures. Error bars correspond to SD observed in two independent experiments that were used for the microarray analysis. B, RT-PCR of *AtWRKY29* (At4g23550) and *AtMPK3* (At3g45640) in Arabidopsis cell culture. RT-PCR of a constitutively expressed actin gene (At5g09810) was also performed to control equal cDNA amount in each reaction (bottom lane). C, Ethylene production in Arabidopsis seedlings. Increase of ethylene was measured by gas chromatography. White boxes represent control seedlings and black boxes represent flg22-treated seedlings. Error bars correspond to SD. D, RT-PCR of *AtWRKY29* (At4g23550) and *AtMPK3* (At3g45640) in Arabidopsis seedlings. RT-PCR of a constitutively expressed actin gene (At5g09810) was performed to control equal cDNA amount in each reaction (bottom lane).

performed using Affymetrix software (see "Materials and Methods" for details and Liu et al., 2002).

In our L*er* cell culture assay, 225 c*FLARE* distinct genes (approximately 2.8%) showed significant changes in mRNA level over 60 min (see Supplemental Table I, which can be viewed at www.plantphysiol.org). Ninety-three genes were significantly induced, whereas only six genes were repressed at both time-points (see Supplemental Tables II and III). Analysis of our seedling data revealed 252 s*FLARE* distinct genes that were significantly altered upon flg22 elicitation (see Supplemental Table IV).

Overall, 80% of the *FLARE* genes are currently annotated as encoding proteins of known or predicted function. We functionally classified these as signal transduction-related, signal-perception-related, effector proteins, and others (see Supplemental Tables V–VIII and Fig. 2, A and B). Among the signal transduction-related genes, many are transcription factors, which represent 43% and 52% of the overall signaling class in suspension cells and seedlings, respectively, and include several WRKY transcription factors (Table I). Among those, we identified *AtWRKY6* (At1g62300; Robatzek and Somssich, 2002) as well as *AtWRKY22 and AtWRKY29* (At4g01250 and At4g23550), whose overexpression increased resistance to both bacterial and fungal pathogens (Asai et al., 2002). In addition, six additional WRKY transcription factors were newly identified as flg22-induced genes and are likely to be involved in plant defense.

A number of *FLARE* genes encode proteins involved in regulating protein turnover such as U-box and RING zinc-finger proteins (Table I). This is consistent with other results indicating an important role for protein turnover in derepressing plant defenses (Peart et al., 2002). Intriguingly, many auxin signaling-related genes were down-regulated during the flg22 response (Table I).

The group of signal-perception-related genes includes resistance-like genes and genes required for resistance (Table II). Among those, we identified *RPS2* that confers resistance to *P. syringae* carrying *AvrRpt2* (Kunkel et al., 1993). Strikingly, this class of *FLARE* genes also includes a large number of receptor like-kinases (RLKs) with various extracellular domains.

The full complement of *FLARE* genes also comprises some which might be directly involved in halting the growth of pathogens (effector class), e.g. enzymes involved in phenylpropanoid metabolism (see Supplemental Table VII).

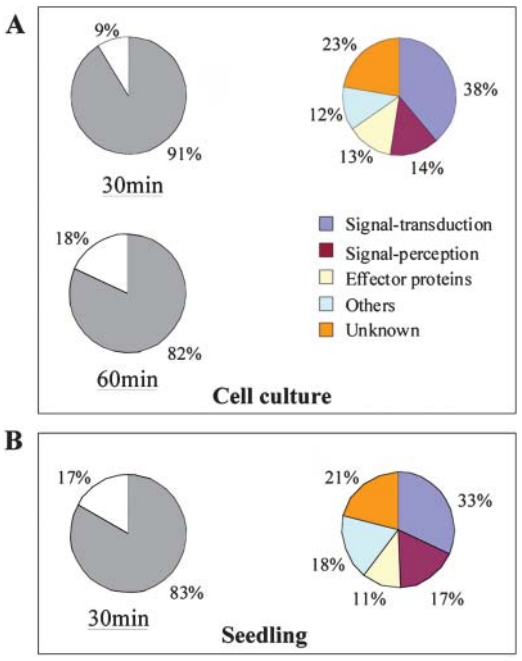

Figure 2. Abundance of flg22-regulated genes. Percentage distribution of Arabidopsis cell culture (A) and seedlings (B), flg22-activated (gray) and repressed (white) genes, and their classification in functional categories.

Differential Expression of *FLARE* Genes between Cell Cultures and Seedlings

We found approximately 70% of the c*FLARE* genes in 30-min treated cell cultures were also significantly induced in flg22 treated seedlings (see Supplemental Tables I and IV). In contrast, we observed that approximately 40% of the s*FLARE* genes identified in elicited seedlings were also up-regulated in the 30-min treated cell cultures highlighting a larger set of flg22 regulated genes in the seedling system (see Supplemental Table IX). Only one gene, encoding a putative calcium-dependent protein kinase (At1g08650), was down-regulated upon flg22 treatment in both Arabidopsis suspension cells and seedlings (see Supplemental Table X). Most auxin signaling-related genes revealed a similar repression profile in both systems, but none of these repressed genes were identical (Table I). These observations might not only be due to different flg22 concentrations used, but may also result from either the use of different ecotypes or different experimental systems. To address this, we performed RT-PCR on *PAL2* (At3g53260), *AtMYB2* (At2g47190), and *4CL* (At1g51680) on Col-0 cell cultures and L*er* cell cultures elicited with 100 nM of flg22 peptide over a 1-h time course. These genes were chosen based on their high inducibility in treated L*er* suspension cells and no transcript change in treated Col-0 seedlings. Our results showed a similar pattern of induction in both Col-0 and L*er* cell cultures (Fig. 3). In addition, no transcript alteration of these genes was detected in L*er* seedlings treated with 10 μM flg22 peptide (data not shown). These data suggest that the differences in gene expression between L*er* suspension cells versus Col-0 seedlings are mostly due to differences between cell cultures and seedlings rather than to differences between ecotypes.

Comparison of *ACRE* and *FLARE* Gene Complements

Both *FLARE* genes and *ACRE* (Durrant et al., 2000) genes comprise approximately 1% of expressed genes after 30-min treatment with flg22 in Arabidopsis and Avr9 in tobacco cell cultures. Moreover, in both systems we observed that more transcripts are induced than repressed (data not shown). To more precisely compare the rapid transcript alterations, we concentrated on flg22-induced expression changes of probable Arabidopsis orthologs of *ACRE* genes (*AtACRE* genes). Twenty full-length *ACRE* cDNA sequences were used to search for Arabidopsis orthologs, of which 10 *ACRE* genes were derived from cDNA library screening (Durrant et al., 2000) and the remainder from 3′ and 5′ RACE amplification (O. Rowland, A.A. Ludwig, C. Merrick, F. Baillieul, F. Tracy, W. Durrant, H. Yoshioka, and J.D.G. Jones, unpublished data). We also included *NtCDPK2* that was induced 15 min after elicitation of Cf9-tobacco cell cultures with Avr9 peptide (Romeis et al., 2000). Whereas in some cases single putative Arabidopsis orthologs could be identified, such as *AtACRE276*, other tobacco *ACRE* cDNA sequences revealed homologies to several Arabidopsis counterparts (Table III). For example, the tobacco *ACRE189* full-length cDNA displayed a high sequence similarity to 4 putative *Arabidopis* F-box genes, any of which could represent the functional Arabidopsis ortholog. The identities of the *AtACRE* candidates were confirmed using the TBLASTN program from The Institute for Genomic Research (TIGR) orthologous gene alignment database (http://www.tigr.org/tdb/toga/toga.shtml). Seventeen out of 21 tobacco full-length cDNAs showed high homology with either a single or several Arabidopsis counterparts. In total, these genes represent 32 putative *AtACRE* candidates. Since one-third of the Arabidopsis genome is covered in the Affymetrix GeneChip Arabidopsis genome array, only 14 out of the 32 *AtACRE* genes were present on the array, and their expression patterns were further studied. The remaining *AtACRE* candidates were profiled using semi-quantitative RT-PCR.

With the exceptions of tobacco *ACRE137*, *ACRE141*, *ACRE216*, and *ACRE275*, at least one of the Arabidopsis *ACRE* orthologs was induced in flg22-elicited Arabidopsis suspension cells (Fig. 4A). The overall expression analysis revealed 13 rapidly and transiently flg22-induced genes and 5 progressively induced genes (Table III; Fig. 4A). Whereas *CPK1* (At5g04870) was not induced based on our microarray analysis filters, we observed a slight induction of this gene in elicited cell cultures (Fig. 4A). In elicited seedlings, most of the *AtACRE* genes displayed a very

Table I. *Highlights of FLARE genes with known or putative roles in signal transduction*

Average relative values of flg22-treated samples, compared to control samples, from two independent experiments. Numbers show the factor of change between control and treatments; positive values represent up-regulation (e.g. 5 = 5-fold increase), negative values down-regulation (e.g. −5 = 5-fold decrease). Expression changes of less than 2-fold between control and treatment are indicated by a dash (–).

Gene Description	AGI Number	Change after Treatment		
		Cells	Cells	Seedlings
		30 min	*60 min*	*30 min*
WRKY transcription factors				
AtWRKY29	At4g23550	6.1	44	4.7
AtWRKY53	At4g23810	22	9.8	34.6
AtWRKY28	At4g18170	–	–	32.2
AtWRKY22	At4g01250	24.5	14.4	24.1
AtWRKY33	At2g38470	4.6	12.3	28.6
AtWRKY11	At4g31550	5	7	13.0
AtWRKY15	At2g23320	–	2.7	4.3
AtWRKY6	At1g62300	–	2.7	7.3
AtWRKY7	At4g24240	–	–	3.1
Protein turnover				
RING-H2 finger protein, RHA3b	At4g35480	–	–	28.0
RING-H2 finger protein, RHA1b	At4g11360	–	9.6	4.6
AtRMA1 protein	At4g03510	–	8.3	–
AtPUB12	At2g28830	2.0	11.5	4.9
Putative RING finger protein	At2g42360	–	4.1	10.3
Putative RING finger protein	At3g16720	3.6	2.7	8.4
AtPUB5	At4g36550	4.5	2.5	5.1
Putative RING finger protein	At4g26400	–	–	4.9
Putative RING finger protein	At2g35000	2.6	3.2	3.9
Putative RING finger protein	At2g42350	–	2.8	–
Similar to RING Zn finger protein	At2g44410	–	2.7	–
RING-H2 finger protein, ATL6	At3g05200	–	–	4.0
Hormone signaling				
Axi 1-like protein	At2g44500	5.9	–	4.7
Putative auxin-regulated protein	At2g46690	−2.6	–	–
Auxin transport protein, PIN3	At1g70940	–	−3.3	–
Early auxin-induced, IAA13	At2g33310	−2.6	−3.2	–
Early auxin-induced, IAA5	At1g15580	–	−6.6	–
Putative auxin-induced protein	At2g16580	−2.1	−9.3	–
Similar to auxin-regulated gene	At4g34750	–	–	−2.7
SAUR-AC1	At4g38850	–	–	−8.0
Putative auxin-induced protein	At2g21210	–	–	−9.0
Auxin-induced protein-like	At4g38840	–	–	−14.2
Putative auxin-induced protein	At4g38860	–	–	−23.6

similar expression pattern to that in suspension cells (Table III; Fig. 4B). Besides the confirmation of our microarray data, these results revealed a substantial overlap between the Avr9 race-specific defense response in tobacco and the flg22-elicited innate immune response in Arabidopsis.

Clustering Analysis of *FLARE* Genes in Arabidopsis Suspension Cells

We identified 3 significant clusters of (1) progressively induced genes (110 genes), (2) transiently induced genes (44 genes), and (3) progressively repressed genes (31 genes; see Supplemental Tables XI–XIII). These clusters were identified by subjecting the absolute expression values of the overall *FLARE* genes over the time course to a self-organizing map (SOM) algorithm using 3 × 1 two-dimensional matrix (see "Materials and Methods" for details). Within the cluster of transiently induced genes, we found the Arabidopsis *ACRE* orthologs *AtACRE1a/b* (At5g47230, At4g17490), *AtACRE111* (At4g25470), *AtACRE132* (At3g16720), *AtACRE231b/c* (At1g70090, At1g24170), *AtACRE264a* (At2g05940), and *AtACRE284a/c* (At2g30020, At2g40180; see Supplemental Table XII). To gain more insight into the *FLARE* gene regulation, we inspected promoter sequences of genes that clustered together with the progressively induced *AtACRE31* ortholog (At4g20780). This task was performed using GENESPRING software and resulted in the identification of 48 candidates within the *AtACRE31* regulon (see Supplemental Table XIV). We

Table II. *FLARE genes with known or putative roles in signal perception*

Average relative values of flg22-treated samples, compared to control samples, from two independent experiments. Numbers show the factor of change between control and treatments; positive values represent up-regulation (e.g. 5 = 5-fold increase), negative values down-regulation (e.g. −5 = 5-fold decrease). Expression changes of less than 2-fold between control and treatment are indicated by a dash (–).

Gene Description	AGI Number	Change after Treatment		
		Cells	Cells	Seedlings
		30 min	*60 min*	*30 min*
Homologs of disease resistance genes				
Similar to TMV resistance protein (tobacco)	At1g65400	27.7	38.4	27.6
Putative nematode-resistance protein	At2g40000	7.5	7.5	22.7
RPS2	At4g26090	–	–	18.0
Similar to RPP8	At3g50950	2.5	6.1	7.8
Similar to TMV resistance protein (tobacco)	At4g36140	–	–	4.3
Similar to RFL1 disease resistance protein	At4g33300	–	–	4.1
Resistance protein RPP5-like	At4g19520	–	–	3.5
TIR Toll/interleukin-1 receptor-like protein	At1g72930	–	–	2.5
Putative disease resistance protein	At2g19780	–	−6.1	–
Homologs of genes required for resistance				
Putative Mlo protein	At2g39200	8	34	13.9
Athsr4	At3g50930	10.9	22.2	9.1
Similar to Mlo protein	At1g61560	5.2	14.9	10.9
NDR1	At3g20600	–	–	6.3
Similar to EDS1	At3g52430	–	–	6.2
NDR1/HIN1-like protein	At2g27080	–	–	5.3
Hin1-like protein	At2g35980	2.5	2.8	–
NPR1	At1g64280	–	–	3.0
LSD1	At4g20380	–	–	2.5
Receptor-like kinases				
LRR-RLKs				
Receptor-like kinase (LRR5[a])	At2g31880	4.6	7.6	13.4
Receptor-like kinase (LRR22[a])	At5g25930	2.7	7.4	11.3
Receptor-like kinase (LRR17[a])	At2g02220	2.5	8.7	2.5
Receptor-like kinase (LRR10[a])	At4g39270	–	3.8	–
Putative-receptor-like protein kinase (LRR4[a])	At2g13790	–	2.7	5.4
Similar to CLV1 receptor kinase (LRR22[a])	At1g55610	–	−3.4	–
Receptor-like kinase (LRR6[a])	At4g22730	–	−4.7	–
Lectin-RLKs				
Receptor-like kinase (LEC[a])	At4g02410	–	3.7	7.6
LecRK1 receptor-like kinase (LEC[a])	At3g59700	2.8	6.9	2.7
Receptor-like kinase (LEC[a])	At1g70130	7.6	7	–
Receptor-like kinase (LEC[a])	At4g28350	–	5.5	2.5
Receptor-like kinase (LEC[a])	At4g29050	–	–	4.4
Lys-RLK				
Receptor-like kinase (Lys[a])	At2g33580	5.2	3.9	17.7
S-RLKs				
Receptor-like kinase (SD[a])	At2g19130	–	5.4	17.6
Receptor-like kinase (SD[a])	At4g32300	2.6	12.8	–
Receptor-like kinase (SD[a])	At4g21390	–	5.6	–
Receptor-like kinase (SD[a])	At1g61370	–	–	3.0
DUF26-RLKs				
Receptor-like kinase (DUF26[a])	At4g23220	–	–	33.2
Receptor-like kinase (DUF26[a]), RLK3	At4g23180	7	20.7	8.3
Receptor-like kinase (DUF26[a])	At4g23190	3.2	8.2	9.6
Receptor-like kinase (DUF26[a]), RKC1	At4g23280	2.7	10.5	6.5
Receptor-like kinase (DUF26[a])	At4g23250	–	5.4	–
Receptor-like kinase (DUF26[a])	At4g11890	–	–	7.5
Receptor-like kinase (DUF26[a])	At4g23270	–	–	3.9
Receptor-like kinase (DUF26[a])	At4g21400	–	–	2.5
K-RLKs				
Receptor-like kinase (K[a])	At2g17220	2.9	9.1	3.9
Receptor-like kinase (K[a])	At2g05940	10.7	5.3	3.0

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Gene Description	AGI Number	Change After Treatment		
Receptor-like kinase (RKF3L[a])	At1g11050	–	–	8.1
Receptor-like kinase (K[a])	At1g67470	–	–	5.7
Receptor-like kinase (K[a])	At2g47060	–	2.5	5.0
Receptor-like kinase (K[a])	At2g39660	–	–	4.1
Receptor-like kinase (K[a])	At3g09010	–	5.2	2.8
Receptor-like kinase (K[a])	At2g11520	2.5	–	2.6
Receptor-like kinase (K[a])	At2g40270	–	–	2.5
Receptor-like kinase (K[a])	At1g11140	–	−3.2	–
Receptor-like kinase (EXT[a])	At4g02010	–	–	−2.4

[a]Extracellular domain. The abbreviations for the extracellular domains stand for: LRR, Leu-rich repeat, the numbers refer to the number of repeats; LEC, lectin; SD, S-locus glycoprotein; DUF26 domain of unknown function; K, sequence with no predicted signal motif; EXT, extension.

scanned 1.1-kb ATG-upstream sequences for 5 to 8 bp motifs that are over-represented within the *AtACRE31* regulon using GENESPRING (see ''Materials and Methods'' for details). As a result, we found a significant increase in the frequency of one of these motifs, namely TTTGAC(T/A), in 28 of the 48 promoters tested (data not shown); the TTTGACT sequence representing the consensus binding site for WRKY transcription factors (Eulgem et al., 2000). In contrast, no over-representation of cis-regulatory elements was detected when we analyzed the promoter sequences of genes that clustered together with the transiently induced *AtACRE1a* ortholog (At5g47230).

To further confirm this statistical analysis we inspected the promoter sequences of the entire set of genes within the *AtACRE31* regulon for over-representation of TTTGACT and TTTGACA sequences as well as other known regulatory elements as previously described (Maleck et al., 2000). Once again, only the W-box and W-box-like element frequencies were at least twice the statistically expected frequency that occurs within a set of 500 promoter sequences from flg22 non-regulated genes (Table IV). Taken together, our promoter analysis led to the identification of a subset of *FLARE* genes potentially regulated by WRKY transcription factors within the *AtACRE31* regulon and suggests common regulatory processes involved during early race-specific and innate immune responses.

Relationship between the *FLARE* Gene Set and Sets of Genes Regulated by *P. syringae* in Nonhost, Compatible, and Incompatible Interactions

To further analyze the relation between flg22-triggered early responses and basal or gene-for-gene resistance, we compared the *FLARE* genes to the set of genes regulated by different bacterial treatments in Arabidopsis (Tao et al., 2003). Pseudomonas type III effector proteins are delivered into the cytosol of the host cell after a lag of 2 h post inoculation (hpi; Huynh et al., 1989; Grant et al., 2000). Thus, the gene expression dataset from early 3/6 hpi with virulent/avirulent or nonhost *P. syringae* strains (Tao et al., 2003), is the best available dataset to compare with our *FLARE* gene set regulated within an hour after elicitation.

We decided to focus our comparative analysis on up-regulated genes and carried out a comparison with data sets derived from 3 hpi and 6 hpi of *P. syringae* pv *tomato* (*Pst*), *P. syringae* pv *phaseolicola* (*Psp*), and *P. syringae* pv *tomato* (*Pst*) carrying either *AvrB* or *AvrRpt2* bacterial strains.

As in the Tao et al. (2003) analysis, the ratio of the expression level for each probe set to that in the corresponding water control was calculated at each 3-h and 6-h timepoint. In addition, expression changes derived from plants treated with *Pst* carrying either *AvrB* or *AvrRpt2* genes were divided by expression changes from plants treated with *Pst*. This last selection allows the identification of genes specifically induced by either *AvrB* or *AvrRpt2*. We also selected genes with a minimum fluorescence value of 10 together with a 2.5-fold change ratio (see ''Materials and Methods'' for details). The overall induced gene sets in nonhost, compatible, and incompatible interactions were then compared to the set of flg22-induced genes derived from both elicited cell cultures and seedlings. For this comparative analysis, the same criteria were used to select flg22-induced genes.

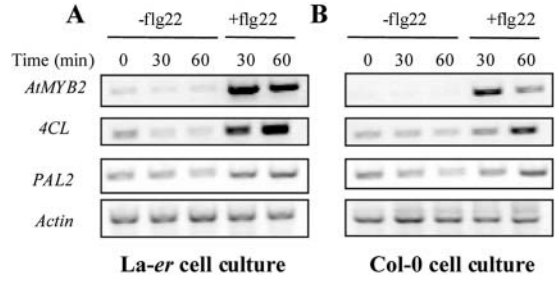

Figure 3. Comparison of flg22-regulated candidate genes in L*er* and Col-0 cell cultures using semiquantitative RT-PCR. Transcript profiling of *AtMYB2* (At2g47190), *4CL* (At1g51680), and *PAL2* (At3g5326) upon flg22 elicitation in (A) L*er* cell cultures and (B) Col-0 cell cultures. RT-PCR of a constitutively expressed actin gene (At5g09810) was performed to control equal cDNA amount in each reaction (bottom lane).

Table III. *Identification of putative Arabidopsis ACRE orthologs and summary of their transcription patterns in response to flg22*

ACRE Number	Genbank Accession Number	Arabidopsis *ACRE* Orthologs	AGI Number	BLASTX Results	TBLASTN Results (TOGA)	Transcription Patterns[a] Cells/Seedlings
1	AF211527	At-ERF5 ethylene responsive element binding factor	At5g47230	1.5 e^{-36}	12.3 e^{-36}	TI/TI
		At-ERF6 ethylene responsive element binding factor	At4g17490	4.1 e^{-36}	5.4 e^{-36}	TI/TI
4	AF211528	Putative disease resistance protein (TIR-NBS-LRR)	At5g17680	4.1 e^{-107}	7.1 e^{-99}	TI/PI
31	AF211529	Calcium-binding protein-like	At4g20780	1.0 e^{-48}	3.2 e^{-48}	PI/PI
		Calmodulin-like protein	At5g44460	1.7 e^{-46}	4.8e^{-46}	NC/NC
74	AY220484	U-box protein (AtPUB21)	At5g37490	1.0 e^{-80}	2.4 e^{-47}	TI/PI
		U-box protein (AtPUB20)	At1g66160	3.0 e^{-63}	3.8 e^{-63}	TI/PI
111	AF211530	DRE binding protein (DREB1A)	At4g25480	3.6 e^{-53}	3.8 e^{-52}	NC/NC
		DRE binding protein (DREB1B)	At4g25490	1.2 e^{-52}	1.4 e^{-52}	TI/TI
		DRE binding protein (DREB1C)	At5g51990	5.2 e^{-52}	1.8 e^{-52}	NC/NC
		DRE binding protein (similar to DREB1C)	At4g25470	8.5 e^{-52}	4.2 e^{-51}	NC/NC
126	AY220477	AtWRKY72	At5g15130	5.4 e^{-41}	7.1 e^{-49}	PI/PI
132	AF211532	RING-H2 zinc finger protein ATL3	At1g53820	6.5 e^{-34}	4.1 e^{-26}	NC/NC
		Putative RING-H2 zinc finger protein	At3g16720	2.9 e^{-26}	6.2 e^{-26}	TI/TI
137	AF211537	Hypothetical protein	At3g23160	1.5 e^{-43}	1.3 e^{-43}	NC/NC
141	AY220478	Putative ligand-gated ion channel	At2g29100	3.3 e^{-137}	2.1 e^{-131}	NC/NC
189	AY220479	F-box protein	At1g47056	3.4 e^{-158}	3.9 e^{-158}	NC/NC
		SKIP1 interacting partner 2 (SKIP2)	At5g67250	5.6 e^{-158}	5.2 e^{-158}	PI/PI
		F-box (AtFBL8/AtFBL24)	At4g07400	1.8 e^{-152}	4.0 e^{-152}	NC/NC
		F-box (AtFBL16)	At3g50080	4.1 e^{-146}	4.2 e^{-146}	NC/NC
216	AY220480	Putative protein kinase	At2g30260	3.6 e^{-131}	3.6 e^{-131}	NC/NC
231	AF211536	Glycosyl transferase-like	At3g28340	1.8 e^{-127}	2.9 e^{-127}	TI/TI
264	AY220481	Putative protein kinase	At2g05940	1.2 e^{-155}	1.2 e^{-155}	TI/TI
		Ser/Thr protein kinase	At5g35580	2.9 e^{-145}	1.1 e^{-143}	TI/NC
275	AY220482	Disease resistance protein (Cf-like)	At1g45616	5.0 e^{-46}	2.1 e^{-46}	NC/NC
276	AY220483	U-box protein (AtPUB17)	At1g29340	1.1 e^{-227}	9.7 e^{-228}	TI/TI
284	AY220484	Protein phosphatase 2C (PP2C)	At2g30020	4.7 e^{-104}	6.0 e^{-104}	TI/TI
		Protein phosphatase 2C (PP2C)	At4g08260	1.3 e^{-99}	2.0 e^{-99}	TI/TI
		Protein phosphatase 2C (PP2C)	At2g40180	3.9 e^{-95}	5.3 e^{-95}	PI/PI
NtCDPK2	AJ344154	Calcium-dependant protein kinase (CPK1)	At5g04870	4.1 e^{-239}	7.0 e^{-217}	PI/NC
		Calium-dependant protein kinase (CPK2)	At3g10660	9.1 e^{-233}	1.5 e^{-209}	NC/NC
		Calcium-dependant protein kinase (CPK20)	At2g38910	3.0 e^{-212}	1.5 e^{-166}	NC/NC

[a]Summary of the *AtACRE* transcription patterns in L*er* treated suspension cells and Col-0 treated seedlings. TI, transiently induced; PI, progressively induced; NC, no change.

In the nonhost interaction, we found that 12% of the genes induced after 3 hpi with *Psp* overlap with flg22-induced genes from both Arabidopsis elicited seedlings and cell cultures (Table V). Similar analysis at the 6 hpi timepoint revealed a more substantial overlap of 34% commonly induced genes between *FLARE* genes and genes induced by *Psp* bacterial treatment (Table VI). Highlights of these genes include 5 members of WRKY transcription factors, 16 receptor-like kinases, and 9 genes involved in the production of ROS (see Supplemental Table XVI). Although we did not have any data with *hrp* mutants from *Psp*, the majority of these genes might be induced in a PAMP dependent manner (Jakobek and Lindgren, 1993; Lu et al., 2001).

The analysis of genes induced in compatible interactions revealed a much smaller overlap with the *FLARE* gene set than did the nonhost interaction. Indeed, only 7% of genes were commonly induced upon flg22 treatment and in 6 hpi with compatible *Pst* (Tables V and VI). Because flg22 peptide derived from *P. syringae* pv *tomato* is a potent elicitor of defense responses in Arabidopsis (data not shown), this result suggests that some type III secretion proteins from *Pst* are potentially involved in repressing the flagellin-mediated response. To identify potential targets of these type III suppressor proteins, we selected genes that were both flg22- and *Psp*-induced but not up-regulated in *Pst* compatible interactions at 6-hpi timepoint. From this gene list, we also subtracted genes that were still induced in *P. syringae* pv *maculicola* at the same timepoint (data not shown). This allows the identification of candidates targeted by two different *P. syringae* pathovars. These genes are potentially involved in the nonhost resistance phenomenon observed in the Arabidopsis-*Psp* interaction. As a result of this analysis, we discovered 77 candidate genes including 11 transcription factors and 8 receptor-like kinases as examples (Table VII; see Supplemental Table XVII). Of these, 2 glycosyl-hydrolases (At3g13790, At3g54420) might be involved in cell wall synthesis, which is in agreement with recent report

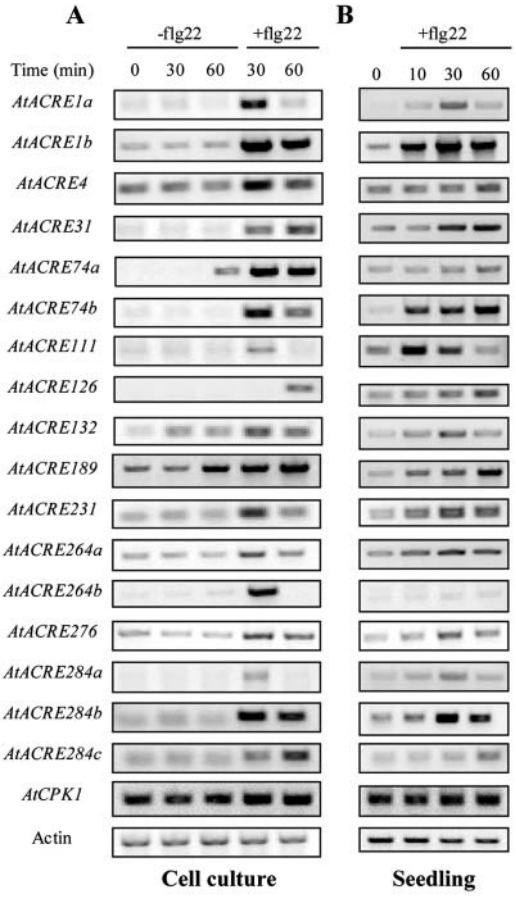

Figure 4. Temporal expression patterns of Arabidopsis *ACRE* orthologs. Semiquantitative RT-PCR transcript profiling of *AtACRE* genes of L*er* suspension cells (A) and Col-0 seedlings (B) challenged with ±flg22 peptide for 0, 30, and 60 min and for 0, 10, 30, and 60 min, respectively. *AtACRE* genes (from top to bottom): *AtACRE1a* (At5g47230), *AtACRE1b* (At4g17490), *AtACRE4* (At5g17680), *AtACRE31* (At4g20780), *AtACRE74a* (At5g37490), *AtACRE74b* (At1g66160), *AtACRE111* (At4g25470), *AtACRE126* (At5g15130), *AtACRE132* (At3g16720), *AtACRE189* (At5g67250), *AtACRE231* (At3g28340), *AtACRE264a* (At2g05940), *AtACRE264b* (At5g35580), *AtACRE276* (At1g29340), *AtACRE284a* (At2g30020), *AtACRE284b* (At4g08260), *AtACRE284c* (At2g40180), and *AtCPK1* (At5g04870). RT-PCR of a constitutively expressed actin gene (At5g09810) was performed to control equal cDNA amount in each reaction (bottom lane).

suggesting that *P. syringae* type III effectors might suppress cell wall based plant defense 12 hpi with virulent *Pst* DC3000 (Hauck et al., 2003).

We also identified a 1-aminocyclopropane-1-carboxylate synthase, termed *AtACS6* gene (At4g11280), which represents a key component of ethylene biosynthesis together with the ethylene responsive transcription factor *AtERF5* (At5g47230), suggesting that *Pst* might suppress some ethylene-related genes (see Supplemental Table XVII in information).

Moreover, of the three RING zinc finger genes that were induced upon both *Psp* and flg22 treatments, none was induced 6 hpi with either *Pst* or *Psm* treatments (Table VII; Supplemental Table XVII). This result is consistent with the involvement of protein turnover components in nonhost resistance (Peart et al., 2002).

Interestingly, although not present on this array, the nonhost resistance gene *NHO1* (At1g80460) is induced in Arabidopsis elicited cell cultures (data not shown) and the expression of this gene is also suppressed 6 hpi with *Pst* strain (Kang et al., 2003). Thus, this gene represents an internal control for the identification of potential targets of type III suppressor proteins.

Among the 77 candidate genes mentioned, 35 were induced specifically in interactions involving *AvrB* or *AvrRpt2* with the cognate R gene, suggesting that the R-gene/Avr-gene interaction negates the suppression effect mediated by virulent bacteria as suggested for *NHO1* gene (Kang et al., 2003).

In more general terms, we found that approximately 45% of the *FLARE* genes were also induced 3 hpi with *Pst* carrying either *AvrB* or *AvrRpt2* (Table V; Supplemental Table XV). Of these, approximately 30% are induced in an AvrB- or AvrRpt2-specific manner, based on *Pst* (*AvrB*) versus *Pst* and *Pst* (AvrRpt2) versus *Pst* comparisons (Table V; Supplemental Table XVI). This result suggests that Avr effector proteins might trigger a common gene subset very early after race-specific elicitor recognition and therefore enhance the PAMP-mediated innate immune response. At 6-hpi timepoint, we observed a decrease in the overlap between *FLARE* genes and genes up-regulated by AvrB and AvrRpt2 race-specific elicitors; only approximately 25% of overlap was found between the flg22-induced genes and genes induced by either AvrB or AvrRpt2 (Table VI). In addition, only approximately 20% of the *FLARE* genes were induced at 9 hpi of either *Pst* (A*vrB*) or *Pst* (*AvrRpt2*; data not shown). This last result suggests that the flg22 response and the AvrB/AvrRpt2-mediated defense responses might diverge at later timepoints explaining the different outcomes between these responses such as cell death in AvrB/AvrRpt2- but not in flg22-induced defense.

Effects of a Cycloheximide Treatment on *FLARE* Gene Expression in Arabidopsis Seedlings

The protein synthesis inhibitor CHX was used to assess whether the *FLARE* genes require de novo protein synthesis for their transcriptional activation. Arabidopsis seedlings were treated for 30 min with CHX prior to a 30-min treatment with flg22 peptide (see ''Materials and Methods'' for details). Transcriptional changes were then monitored by microarray and similar criteria were used to select differentially expressed genes as described before (see ''Materials and Methods'' for details). We found that approximately 70% of the overall *FLARE* genes displayed similar transcriptional changes in CHX/flg22 treated seedlings (see Supplemental Table XVIII). Moreover, by taking the *FLARE* induced genes as a baseline, we found that approximately 92% of the flg22-induced genes are up-regulated upon both CHX and flg22 (see Supplemental Table XIX). This result suggests that

Table IV. *Frequency of occurrence of conserved binding motifs for different types of transcription factors in the cluster containing AtACRE31 ortholog*

Transcription Factor Type	Motif Sequences	Frequency in flg22-Regulated Promoters (48 Promoters)	Frequency in Non-flg22-Regulated Promoters (500 Promoters)	Frequency Fold Change
AP2/EREBP (GCC-box)	GCCGCC	0.10	0.08	1.25
AP2/EREBP	ACCGCC	0.10	0.09	1.11
Myb	G(G/T)T(A/T)G(G/T)T	2.10	1.40	1.50
bZIP (TGA-type)	TGACG	1.27	0.88	1.44
bZIP (GBF-type)	CACGTG	0.20	0.15	1.33
bZIP (G/HBF-1 type)	CCTACC	0.12	0.12	–
EIN3/EIL	GGATGTA	0.06	0.04	1.5
WRKY (core)	TTGAC	4.10	2.05	**2.0**
WRKY (stringent)	TTGAC(T/C)	2.35	1.09	**2.35**
WRKY (stringent)	TTGACT	1.6	0.7	**2.3**
WRKY (stringent)	TTGACC	0.75	0.42	1.78
WRKY (stringent)	TTGACTT	0.69	0.28	**2.46**
W like	TTTGACA	0.60	0.30	**2.0**

In bold are the frequencies of over-representative elements that are at least twice the statistical expected frequency that occur within a set of 500 non-flg22 regulated promoters.

new protein synthesis is not required to induce the vast majority of the *FLARE* genes. On the contrary, the analysis of nonoverlapping genes revealed approximately 70% of genes predicted to be repressed by flg22 (see Supplemental Table XX). This observation suggests that the majority of flg22-repressed genes require de novo protein for their transcriptional inactivation.

Interestingly, when Arabidopsis seedlings were treated with CHX alone, 82% of the *FLARE* genes were induced (see Supplemental Table XIX). This result is consistent with the transcriptional activation of a large set of *ACRE* genes in Cf-9-tobacco cell culture challenged with CHX for 30 min (Durrant et al., 2000) and suggests that *FLARE* and *ACRE* genes are negatively regulated by rapidly turned over repressor proteins. It also confirms the key role played by protein turnover in the initiation of the plant defense response and suggests that relief of negative regulation is important to activate plant defense.

DISCUSSION

The innate immune response mediated by pathogen molecules, also referred to as PAMPs is shared between plants and mammals (Gómez-Gómez and Boller, 2002; Nürnberger and Brunner, 2002). In plants, the PAMP perception activates defense responses and so far little is known about the interplay between the PAMP response and compatible/incompatible plant/pathogen interactions. To address this we performed expression profiling of Arabidopsis cell cultures and seedlings challenged with flg22. We identified many components involved in signaling. Clustering analysis revealed three main groups of coregulated *FLARE* genes. A subset of progressively induced *FLARE* genes contains an over-representation of the W-box element and a W-box-like element within their promoters. The *FLARE* gene set was then compared to the set of *ACRE* genes previously identified as induced in Cf9-tobacco cell cultures challenged with the fungal derived Avr9 peptide. This revealed a substantial overlap between the *FLARE* and *ACRE* gene induction and highlights common defense processes shared between the bacterial PAMP response and fungal race-specific defense responses.

To further analyze the cross-talk between flg22-innate immune response, nonhost interaction, gene-for-gene, and compatible interactions, we compared our set of *FLARE* genes with genes up-regulated in *Pst*, *Pst* carrying either *AvrB* or *AvrRpt2*, and *Psp* inoculations. This comparative analysis suggests that (1) the flagellin response is likely to mimic nonhost defense responses, (2) *Pst* might suppress the expression of genes potentially involved in nonhost resistance as well as gene-for-gene resistance, and (3) incompatible

Table V. *Overlap between FLARE genes and genes induced after 3 hpi of different bacterial treatments*

Treatments	c*FLARE*		s*FLARE*	All *FLARE* Genes
	30 min	*60 min*	*30 min*	
Pst	12	14	8	8
Pst (*AvrB*)	63	64	65	49
Pst (*AvrB*) vs *Pst*	48	39	51	35
Pst (*AvrRpt2*)	55	54	60	44
Pst (*AvrRpt2*) vs *Pst*	34	23	41	25
Psp	21	21	14	12

Percentage distribution of *FLARE* genes that are commonly regulated in compatible (*Pst*), incompatible (*Pst* (*AvrB*), *Pst* (*AvrRpt2*), *Pst* (*AvrB*) vs *Pst*, *Pst* (*AvrRpt2*) vs *Pst*), and non host (*Psp*) interactions (compared to Tao et al., 2003). c*FLARE* genes signifies genes induced in cell cultures (30-min and 60-min timepoints); s*FLARE* genes signifies genes induced in seedlings (30-min timepoint); all *FLARE* genes signifies genes induced either in cell cultures (30-min and 60-min timepoints) or in seedlings (30-min timepoint).

Table VI. *Overlap between FLARE genes and genes induced after 6 hpi of different bacterial treatments*

Treatments	Cell Cultures		Seedlings	*FLARE* Genes
	30 min	*60 min*	*30 min*	
Pst	8	8	6	7
Pst (*AvrB*)	48	49	38	34
Pst (*AvrB*) vs *Pst*	36	42	29	27
Pst (*AvrRpt2*)	40	39	41	32
Pst (*AvrRpt2*) vs *Pst*	28	25	30	23
Psp	43	47	43	34

Percentage distribution of *FLARE* genes that are commonly regulated in compatible (*Pst*), incompatible (*Pst* [*AvrB*], *Pst* [*AvrRpt2*], *Pst* [*AvrB*] vs *Pst*, *Pst* [*AvrRpt2*] vs *Pst*), and non host (*Psp*) interactions (compared to Tao et al., 2003). c*FLARE* genes signifies genes induced in cell cultures (30-min and 60-min timepoints); s*FLARE* genes signifies genes induced in seedlings (30-min timepoint); all *FLARE* genes signifies genes induced either in cell cultures (30-min and 60-min timepoints) or in seedlings (30-min timepoint).

interactions mediated by either AvrB or AvrRpt2 might negate this suppression effect and thus promote resistance. We also identified potential targets for *P. syringae* pv *tomato* and *maculicola* suppressor type III proteins.

Highlights of *FLARE* Genes and Their Potential Role in Signaling Transduction

Treatment of Arabidopsis cell cultures and seedlings with flg22 elicitor results in the differential regulation of 3% of 8,200 genes within 60 min. None of these genes was induced or repressed in an *fls2-17* seedling mutant after flg22 treatment (Zipfel et al., 2004). Many induced genes encode signaling components, including transcription factors, protein kinases, and phosphatases and proteins that regulate protein turnover. Reversible phosphorylation is likely to play a role in the activation and inactivation of MAP kinases (MAPKs) in signaling pathways triggered by elicitors and stress signals. The identification of *FLARE* genes coding for protein phosphatase 2C suggests a possible role for these proteins as negative regulators of the flg22-activated MAPK cascade (Asai et al., 2002).

An interesting feature of the flg22/FLS2 response is the repression of auxin signaling-related genes in Arabidopsis treated cell cultures and seedlings, including genes encoding Aux/IAA proteins. Aux/IAA proteins were first isolated as members of a gene family that is rapidly induced in response to auxin (Abel et al., 1994). Upon flg22 treatment, the rapid repression of these auxin-related genes might contribute to the growth inhibition observed in flg22-treated Arabidopsis seedlings (Gómez-Gómez et al., 1999).

Involvement of Protein Degradation in the Plant Defense Response

Among the *FLARE* genes, several genes potentially involved in protein degradation were identified. In the early innate immune response in mammals, the proteolytic degradation of IκB via the proteasome leads to the translocation of the NF-κB transcription factors to the nucleus to activate transcription (Karin and Ben Neriah, 2000; Read et al., 2000; Silverman and Maniatis, 2001). In plant defense signaling, SGT1, an SCF-complex-associated protein, is required for protein turnover in the auxin response (Austin et al., 2002; Azevedo et al., 2002; Gray et al., 2002, 2003; Peart et al., 2002). In the auxin response, SCF^{TIR1} and related SCF complexes bind Aux/IAA proteins, leading to their degradation (Gray et al., 2001). *Aux/IAA* genes were reported to be induced upon CHX treatment, which is presumed to induce genes by preventing translation of mRNAs encoding rapidly turned over repressor proteins (Abel et al., 1995). Similarly, the transcriptional activation of the majority of *FLARE* genes upon CHX treatment suggests that accelerated proteolysis of repressors might be involved in activation of the plant immune response (see Supplemental Table XVIII). Such proteins are not necessarily direct transcriptional repressors; they could include other kinds of negative regulators of defense mechanisms.

Upon flg22 treatment, 10 genes encoding RING zinc-finger proteins were significantly induced (Table I). Such proteins are thought to have an E3-ligase activity and previous studies revealed their involvement in the elicitor response (Salinas-Mondragon et al., 1999; Takai et al., 2002). We also found induction of the U-box proteins *AtPUB5*, *12*, *17* (*AtACRE276*), and *20/21* (*AtACRE74*) upon flg22 treatment (Table I; Fig. 4). These genes encode proteins with a conserved U-box domain, which structurally resembles the RING finger domain (Aravind and Koonin, 2000; Ohi et al., 2003). In addition, we observed flg22 inducibility of a

Table VII. *Summary table displaying the proportion of genes potentially targeted by Pst and Psm type III secreted proteins*

Group	Function	*FLARE/Psp* Induced Genes Total Number	*FLARE/Psp* Induced Genes Minus *Pst/Psm* Induced Genes
	Cell wall modification	8	**2**
Effector	Hormone signalling	9	**5**
	Secondary product	5	**2**
	Ion responsive	6	**6**
	Kinase/Phosphates	4	**3**
Signaling	Protein turnover	3	**3**
	ROS production	9	**5**
	Transcription factors	19	**11**
Signaling/ recognition	Receptor-like kinases	16	**8**
	Resistance-related	3	**0**
Miscellaneous	Others	19	**15**
	Unknown	21	**16**

In bold are the number of genes in each functional category that are potentially targeted by *Pst* and *Psm* type III secreted proteins.

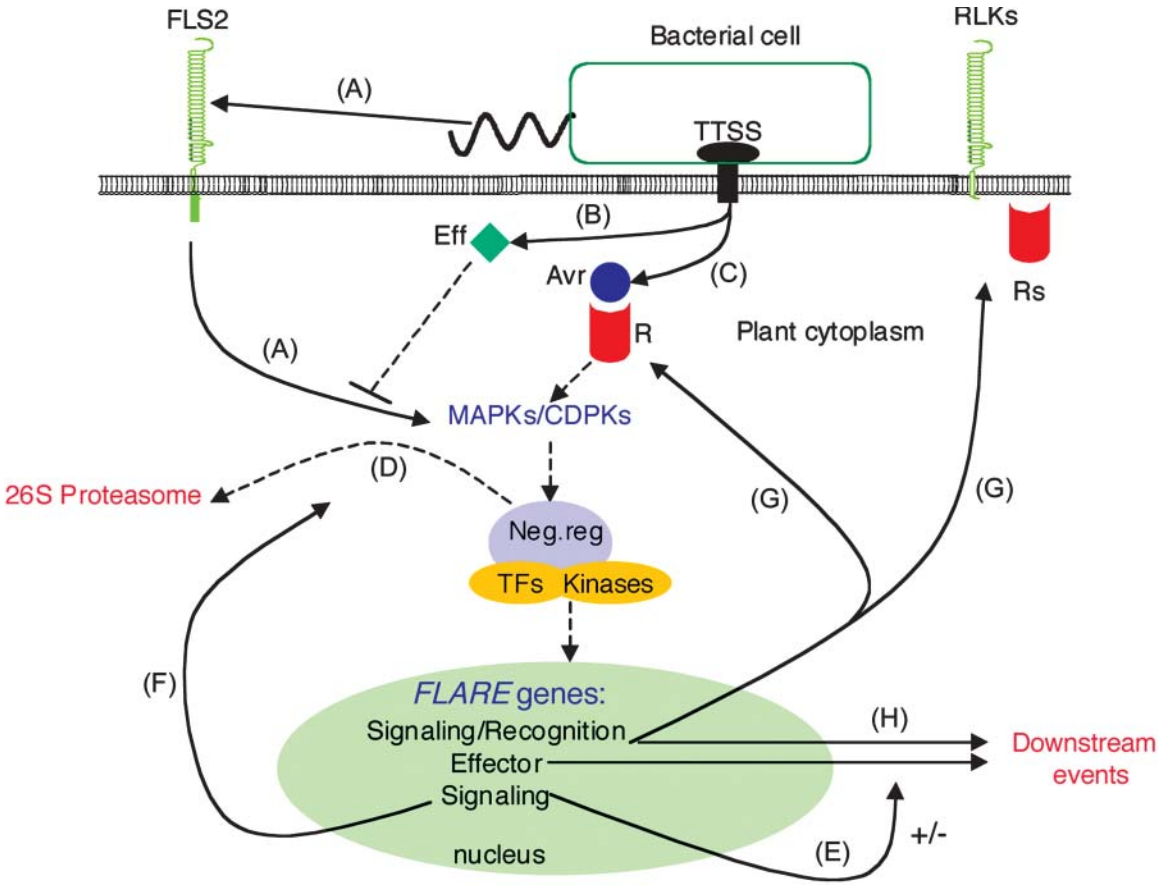

Figure 5. Model for the role of *FLARE* and *ACRE* genes in early plant defense processes. Dashed arrows indicate hypothetical processes. Plain arrows indicate the role that *FLARE* genes are likely to play according to our current survey and previous studies in plant defense signaling. Neg reg., TFs, TTSS, Eff, and Avr stand for negative regulator of defense, transcription factor, type III secretion system, virulent bacterial effector protein, and avirulent protein, respectively.

putative ortholog of the tobacco *ACRE189* gene termed *SKIP2* (At5g67250), which encodes an F-box protein with LRR domains. F-box proteins are components of the E3-ligase SCF complex and are involved in the delivery of appropriate targets to this complex for ubiquitylation followed by degradation in the proteasome (Deshaies, 1999; Kipreos and Pagano, 2000). Several negative regulators of plant defense responses have been previously reported (Dietrich et al., 1997; Li et al., 1999; Clough et al., 2000). As an example, *edr1* (*enhanced disease resistance*) was found to enhance disease resistance to the fungus *Erysiphe cichoracearum* (Frye and Innes, 1998). In addition, *SNI1* (*suppressor of npr1-1, inducible 1*) was found to suppress mutations in *NIM1/NPR1*, a positive regulator of the general plant defense systemic acquired resistance response (Li et al., 1999). These genetic studies suggest that the plant immune response is under negative regulation. Such negative regulators might be the targets of the *FLARE/ACRE* genes involved in 26S-proteasome pathways similar to the degradation of IκB, a negative regulator of NF-κB transcription factor, in animal systems. The identification of such putative negative regulators is a high priority for future studies.

Repertoire of *RLK/R FLARE* Genes and Their Potential Role in Resistance

We identified several resistance genes, putative resistance genes and *RLK* genes that are induced upon flg22 treatment. These genes were classified as signal-perception-related genes (Table II). The RLKs belong to various subclasses according to their extracellular domains and are likely involved in recognition of extracellular signals. For example, we found an *RLK* with a lysin extracellular domain (At2g33580). This conserved motif was originally identified in bacteria and is thought to function in general peptidoglycan binding (Ponting et al., 1999; Bateman and Bycroft, 2000). Elevated mRNA levels of genes encoding RLKs suggest that flg22 may enhance the sensitivity of plant cells to many different PAMPs. Therefore, the *FLARE RLK* genes are likely to represent components important for the perception of various general elicitors or even race-specific elicitors. Intriguingly, transcript elevation of several resistance genes as well as genes required for resistance were detected (Table II). Although flg22 is a bacterial PAMP, we identified *FLARE* genes coding for homologs of R proteins conferring

resistance to oomycetes, bacteria, fungi, nematodes, and viruses. So far, only the *R* gene *Xa1* was reported to be up-regulated by pathogen infection (Yoshimura et al., 1998), and none of the recent RNA profiling experiments have shown a differential expression pattern of these *R* genes (Maleck et al., 2000; Tao et al., 2003).

Suppression of PAMP Induced Genes by Virulent *P. syringae*

Nonspecific recognition of general elicitors produced by nonhost pathogens plays a major role in the nonhost inducible defense response (Jakobek and Lindgren, 1993; Lu et al., 2001). Consistent with this, we found that 34% of the *FLARE* genes were commonly induced in Arabidopsis-*Psp* interaction 6 hpi (Table IV). Because Arabidopsis is resistant to the *Psp* nonhost strain, PAMP-mediated response might significantly contribute to this resistance phenomenon. Whereas nonhost resistance remains poorly investigated, some components have emerged. As an example, *NHO1* was identified throughout a genetic screen for reduced nonhost resistance mediated by *Psp*. This Arabidopsis gene encodes a glycerol kinase homolog that is also involved in gene-for-gene interaction (Kang et al., 2003). *NHO1* is induced by *P. syringae* pv *phaseolicola*, *P. syringae* pv *syringae*, and *P. syringae* pv *tabaci* alike, suggesting that PAMPs shared between these bacteria are responsible for induction of this gene (Kang et al., 2003). Interestingly, we found this particular gene induced in Arabidopsis cell cultures challenged with flg22 peptide (data not shown). In this study, we report that only 7% of the flg22-induced genes were also induced upon 6 hpi of *Pst* bacterial strain (Table VI). This result suggests that some type III effector proteins might suppress the flg22-innate immune response and other PAMP-triggered responses, as suggested by recent work on the HopPtoD2 effector protein (Espinosa et al., 2003). We identified 77 potential targets for these *P. syringae* pv *tomato* type III suppressors (see Supplemental Table XVII). Like *NHO1* nonhost resistance gene, these candidate genes might play a crucial role in nonhost resistance.

Connection between PAMPs- and Race-Specific Defense Responses

The early transcriptional changes that occur in the Arabidopsis flg22/FLS2 response and the tobacco Avr9/Cf-9 responses display a striking overlap. For 13 out of 17 tobacco *ACRE* full-length cDNAs, we found that at least one representative of their orthologs was also induced in flg22-elicited suspension cells and seedlings (Table III; Fig. 4). We also identified *AtMPK3* (At3g45640) as flg22-induced (Fig. 1, B and D). This gene was reported to be involved in flg22 signaling (Nühse et al., 2000) and is orthologous to the tobacco *WIPK* gene that was rapidly induced by Avr9 peptide in Cf-9-tobacco suspension cells (Romeis et al., 2000). In addition, we observed that a large set of *FLARE* genes were rapidly elicited after infection 3 hpi with Pseudomonas strains carrying *AvrB* and *AvrRpt2* avirulence genes (Table V; Supplemental Table XV). Such overlap in response to a race-specific elicitor and a general elicitor highlights a conserved process of plant immunity and suggests that other pathogen-derived elicitors induce similar subsets of genes through different receptors. Moreover, this overlap suggests that race-specific resistance triggered by specific Avr genes may have evolved from mechanisms involved in recognition of PAMPs. Since plants lack mechanisms of acquired immunity, the evolution of polymorphism in recognition capacity for multiple pathogen-derived molecules could have led to the gene-for-gene interactions that we observe today (Dangl and Jones, 2001). Further investigation on the specificity of flg22/FLS2 and Avr9/Cf-9 transcript signatures will provide clues to explain the different outcomes of these responses such as the cell death observed in the Avr9-race-specific defense response, but not in flg22 innate immune response.

Model for Early Signaling Events in Arabidopsis Bacterial Response

We present here a model showing the interplay between flg22-triggered innate immune and early virulent and avirulent bacterial responses (Fig. 5). When potentially pathogenic *P. syringae* strains enter plant tissue, their PAMPs (such as flagellin) can elicit defenses through FLS2 and other receptors (arrow A). To suppress this elicitation, effector proteins are delivered into host cells through the type III secretion system (arrow B). In an incompatible interaction, some effector proteins (that can be recognized genetically as Avr proteins) interact with complexes containing host R proteins and elicit the defense response through *R* gene-dependent recognition (arrow C). This elicitation could occur through mechanisms that involve the central positive regulators of defense such as MAPKs or CDPKs that were targeted by the bacterial effector proteins.

After recognition, both race-specific and PAMP elicitors trigger similar responses such as ion fluxes, production of ROS, and activation of MAPKs and CDPKs (Felix et al., 1999; Gómez-Gómez et al., 1999; Grant et al., 2000; Asai et al., 2002). flg22 (and presumably other PAMP) elicitation leads to rapid and transient induction of signaling-related genes presumably through degradation of negative regulators of defense such as transcription factors and kinases (arrow D). The *FLARE* genes encoding proteins involved in protein turnover, such as RING finger and U-box proteins, are likely to be involved in ubiquitination of these negative regulators of defense (arrow F). Other induced signaling-related genes trigger the induction or repression of downstream components (arrow E). The progressively induced transcripts contain *RLKs* as well as some *R* genes, and point to a possible interaction between the innate immune

response mediated by PAMPs and sensitization of the cells for further pathogen recognition (arrow G). Other progressively induced transcripts encode components that might be involved more directly in plant defense processes such as antimicrobial proteins (arrow H).

Overall, then, these data suggest that PAMPs such as flagellin play an important role in plant/pathogen interactions. Their existence has led to selection for a large set of bacterial effector proteins that suppress PAMP-elicited pathways. PAMP elicitation leads to elevated levels of R proteins and of receptors for PAMPs. This complex evolutionary interplay still provides fertile ground for exciting new insights into the mechanisms that are involved.

MATERIALS AND METHODS

Cell Culture Materials and Elicitor Treatment

Cell cultures of Arabidopsis L*er* were maintained and used for analysis 7 d after subculturing as previously described (Felix et al., 1999). The pH of the cell cultures was measured with a small combined glass electrode (Metrohm, Basel). Elicitor peptide flg22 was synthesized by Sigma Genosys (St. Louis) diluted in dimethyl sulfoxide solvent and added to a concentration of 100 nM 75 min after transferring an aliquot of the cell cultures to a beaker on a rotary shaker. Cells were harvested by filtration, frozen in liquid nitrogen, and stored at −80°C. Cells of Arabidopsis Col-0 (Ferrando et al., 2000) were used 4 d after subculture and similar flg22 treatments were applied.

Seedling Materials and Treatments

After a 48-h treatment at 4°C, Arabidopsis Col-0 seeds were grown for 12 d on plates containing 1× Murashige and Skoog medium (Duchefa), 1% Suc, and 1% agar under continuous light conditions of 60 μE m^{-2} s^{-1} at 22°C. Seedlings were then transferred to liquid Murashige and Skoog medium (2 seedlings/500 μL of medium in wells of 24-well-plates). Two days after transfer the medium was supplied with flg22 peptide to a final concentration of 10 μM. Plantlets were collected 30 min after treatment, frozen in liquid nitrogen and stored at −80°C. In the case of the CHX experiment, 50 μM CHX was added 30 min prior to flg22 or water treatment.

For assaying ethylene production, 2-week-old seedlings, grown in liquid Murashige and Skoog medium, were transferred to 6-mL glass tubes (2 seedlings/tube) containing 1 mL of an aqueous solution of 10 μM flg22. Vials were closed with rubber septa and ethylene accumulating in the free air was measured by gas chromatography.

RNA Preparation and Microarray Processing

For cell cultures, total RNA was extracted using Trizol-Reagent (Sigma). RNA samples were cleaned over Qiagen RNeasy mini-columns (Valencia, CA). For seedlings, total RNA was extracted using RNeasy Plant Mini kit (Qiagen). Genome arrays, washing, staining, and scanning were carried out according to the manufacturer's suggestions (Affymetrix).

Transcript Profiling of *ACRE* Orthologs by RT-PCR

Total RNA from two independent cell culture experiments were extracted as described previously and pooled. Two micrograms of DNase-treated RNA were reverse transcribed for 90 min at 42°C in a 20-μL reaction volume containing 1 unit of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 250 μM each dNTP, 30 μM oligo(dT) 30 M primer, 20 units of RNase inhibitor, and 10 mM dithiothreitol. One microliter of the RT reaction was used for PCR in a 20-μL volume with 1 unit of Taq DNA-polymerase (Qiagen), 100 μM each dNTP, and 100 ng of each forward and reverse primers from *AtACRE* genes. PCR conditions were the following: 3 min, 94°C (first cycle); 30 s, 94°C; 30 s, 50°C; 1.5 min, 72°C (24–27 cycles); and 10 min, 72°C (last cycle). PCR products were separated on a 1% agarose gel and visualized after ethidium bromide staining. To control equal cDNA amount in each reaction, a PCR was performed with primers corresponding to the actin gene (At5g09810), which is constitutively expressed in vegetative structures *AC1* (5′-ATGGCAGACGGTGAGGATATTCA-3′) and *AC2* (5′-GCCTTTGCAATCCACATCTGTTTG-3′).

Identification of *FLARE* Genes

Genes were considered as up- or down-regulated if their expression level in elicited L*er* cell culture deviated (positively or negatively) more than 2.5-fold from that of the unelicited L*er* cell cultures in both independent experiments and if the genes were called I for increase and D for decrease as a result of the statistical comparative analysis performed using Microarray Suite Software MAS4 (Affymetrix). Before applying this filter, genes with an expression level above 10 (noise level of expression) were previously selected. For the Col-0 seedling assay, similar criteria were used to select flg22-regulated genes and the statistical analysis were performed using MAS5 (Affymetrix). To generate the list of *FLARE* genes with their appropriate annotation, the Affymetrix probe set-IDs for the flg22-regulated genes were collected and used to retrieve annotation and AGI numbers from the Salk Institute Genomic Analysis Laboratory database SIGnAL (http://signal.salk.edu/tabout.htm). Alternatively, when gene annotations were not found, their corresponding cDNA sequences were collected using the Julian Schroeder's database (http://www.biology.ucsd.edu/labs/schroeder/trendsreview.html) and searched against TIGR (http://tigrblast.tigr.org/er-blast/index.cgi?project=ath1) as well as the MIPS (http://mips.gsf.de/proj/thal/db/search/blast_arabi.html) Arabidopsis databases using a BLASTN program (Altschul et al., 1997). Additional annotations were identified from the ones associated with probe sets on the Affymetrix chip. Receptor-like kinases were classified according to the identity of the extracellular domains (Shiu and Bleecker, 2001), and the extracellular domain of each nonpreclassified RLK was identified using the SMART database (http://smart.embl-heidelberg.de/help/smart_about.shtml).

Comparative Analysis between *Flare* Genes and Genes Induced by Different Bacterial Treatments

Raw data derived from samples treated for 3 hpi and 6 hpi of water, *P. syringae* pv *tomato* (*Pst*), *P. syringae* pv *tomato* carrying either AvrB or AvrRpt2, and *P. syringae* pv *phaseolicola* (*Psp*) were used for analysis (Tao et al., 2003). Average from expression level of each probe set of a treatment was calculated. To select genes up-regulated in compatible interaction, average expression level from each probe set at each timepoint was divided by average expression level of the water treated samples at the corresponding timepoint. Similar selection was performed for the identification of genes induced in nonhost interaction mediated by *Psp*. For the identification of genes induced in incompatible interactions, average expression level from each probe set at each timepoint was divided by either average expression level of the water treated samples or *Pst* treated samples at each timepoint. This last selection allows the identification of genes specifically induced upon race-specific elicitors AvrB or AvrRpt2. Genes that deviate positively more than 2.5-fold change were then selected as significantly induced and compared to the flg22-induced genes derived from elicited cell cultures and seedlings. Moreover, we selected only probe sets with expression level equal or above 10 (noise level of expression). Similar selection criteria were used to identify flg22-induced genes.

Data Processing and Data Analysis

Global analysis of temporal gene expression pattern was performed by subjecting the absolute expression values of the overall *FLARE* genes over the time course to a SOM algorithm using 3 × 1 two-dimensional matrix with default SOM filters (DMT, Affymetrix). The sequences of the 5′ regions (up to 1,100 bp) were used to search for sequences (5–8 bp) that are over-represented within the progressively induced cluster (*AtACRE31* regulon) and the transiently induced cluster (*AtACRE1* regulon containg *AtACRE111/132/264*) compared with all genes outside of these clusters. This motif search algorithm was performed using GENESPRING software and only oligomers with *P* values below 0.05 cutoff were considered as significantly over-represented. For further promoter analysis, we extracted 1-kb promoter sequences from TAIR database (http://www.arabidopsis.org/tools/bulk/sequences/index.html) and analyzed the over-representation of this regulatory elements according to Maleck et al., 2000).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AF211527, AF211528, AF211529, AY220484, AF211530, AY220477, AF211532, AF211537, AY220478, AY220479, AY220480, AF211536, AY220481, AY220482, AY220483, AY220484, and AJ344154.

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Chapter 2

Bacterial disease resistance in *Arabidopsis* through flagellin perception

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Bacterial disease resistance in *Arabidopsis* through flagellin perception

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Plants and animals recognize microbial invaders by detecting pathogen-associated molecular patterns (PAMPs)[1–5] such as flagellin[6–10]. However, the importance of flagellin perception for disease resistance has, until now, not been demonstrated[7–11]. Here we show that treatment of plants with flg22, a peptide representing the elicitor-active epitope of flagellin[6], induces the expression of numerous defence-related genes and triggers resistance to pathogenic bacteria in wild-type plants, but not in plants carrying mutations in the flagellin receptor gene *FLS2*. This induced resistance seems to be independent of salicylic acid, jasmonic acid and ethylene signalling. Wild-type and *fls2* mutants both display enhanced resistance when treated with crude bacterial extracts, even devoid of elicitor-active flagellin, indicating the existence of functional perception systems for PAMPs other than flagellin. Although *fls2* mutant plants are as susceptible as the wild type when bacteria are infiltrated into leaves, they are more susceptible to the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 when it is sprayed on the leaf surface. Thus, flagellin perception restricts bacterial invasion, probably at an early step, and contributes to the plant's disease resistance.

In plants, disease resistance has been studied most thoroughly in cases that depend on the presence of specific resistance genes (*R* genes) conferring immunity to particular races of pathogens. The proteins encoded by *R* genes were shown to mediate specific recognition of factors specified by particular avirulence genes (*Avr* genes) in the pathogens[12,13]. In addition to *R*-gene-related mechanisms, plants have broader, more basal perception systems for patterns characteristic for entire groups or classes of microorganisms, so-called general elicitors[14], which are conceptually equivalent to PAMPs[1–5]. In contrast to *R*-gene-dependent defence, the responses to general elicitors do not always result in the cell death associated with the hypersensitive response, and the exact role of general elicitors in plant disease resistance is still unclear. PAMPs that act as general elicitors in plants include chitin[15] and ergosterol[16] from fungi, and flagellin[6] and lipopolysaccharides[17] from bacteria. Flagellin, the subunit building the filament of the bacterial flagellum, is also recognized as a PAMP in mammals, by way of the Toll-like receptor TLR5 (refs 9, 10). In *Arabidopsis*, perception of flagellin occurs by recognition of the most conserved domain in its amino terminus, represented by the peptide flg22 (ref. 6). Perception of this elicitor-active domain depends on the LRR-type receptor kinase FLS2 (flagellin sensing 2)[8] and activates a downstream mitogen-activated protein kinase pathway, composed of *At*MEKK1, *At*MKK4/*At*MKK5 and *At*MPK3/*At*MPK6 (ref. 7; the prefix *At* indicates *Arabidopsis thaliana*). flg22 induces numerous defence-related genes in *A. thaliana*, and the responses triggered by flg22 show great similarity to *R*-gene-mediated responses[18].

Here we studied the role of flagellin perception in bacterial disease resistance. In a first step we extended a previous transcriptional analysis[18] by comparing flg22-induced changes in intact wild-type and *fls2* mutant seedlings, using the full-genome Gene-Chip ATH1 (Affymetrix) of *A. thaliana* (about 23,000 genes). After a 30-min treatment of wild-type seedlings with flg22, 966 genes were categorized as upregulated, 625 of them more than 2.5-fold; and 202 were categorized as downregulated, 35 of them more than 2.5-fold (Supplementary Fig. 1 and Supplementary Table 1). In seedlings of the flagellin-insensitive mutant *fls2-17*, carrying a point mutation in the kinase domain of FLS2 (G1064R)[8], treatment with flg22 showed minor changes in six genes only (less than twofold changes). These genes do not belong to those regulated by flg22 in wild-type seedlings (Supplementary Table 1), indicating random fluctuations. This result demonstrates the validity of the criteria used to classify changes in the wild type as significant even below the 2.5-fold threshold, and it clearly shows that flagellin perception and signalling depend absolutely on the presence of a functional FLS2 receptor.

As well as a large group of genes with unknown functions (328 genes), a considerable number of the upregulated genes can be classified as being involved in signal perception (155 genes encoding receptor-like kinases (RLK) and *R* genes), signal transduction (145 genes), transcriptional regulation (87 genes), and potential antimicrobial action (29 genes) (Supplementary Table 2). Among the genes that are rapidly induced at the transcriptional level are the following genes (see MIPS database, http://mips.gsf.de/prog/thal/db/index.html): *FLS2* (At5g46330), *MEKK1* (At4g08500), *MKK4* (At1g51660), *MPK3* (At3g45640) and *WRKY22* (At4g01250). These genes encode elements that have previously been shown to be involved in the perception and transmission of the flg22 signal[7,8]. A similar positive feedback regulation with transcriptional activation of the components involved in the perception and signalling has been reported for the innate immune response in *Drosophila*[19,20]. Interestingly, our previous analysis of promoter sequences from flg22-induced genes revealed an over-representation of W-boxes[18], that is, *cis*-elements, which confer the specific binding of WRKY

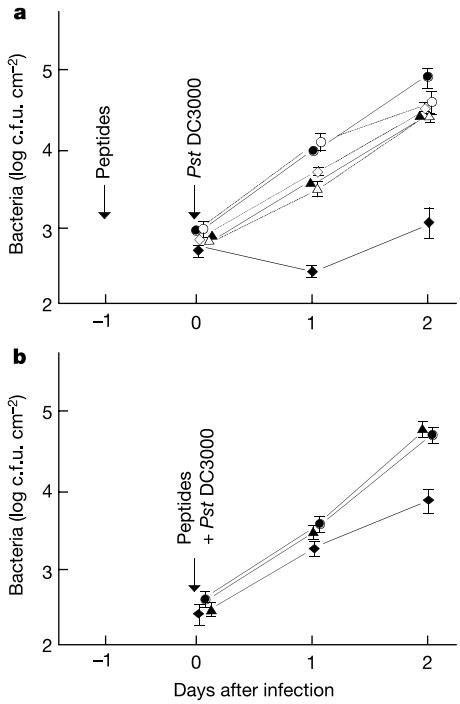

Figure 1 Treatment with flagellin limits *Pst* DC3000 growth. **a**, *Arabidopsis* wild-type L*er*-0 (filled symbols) and *fls2-17* (open symbols) plants were pretreated for 24 h by leaf infiltration with 1 μM flg22 (diamonds) or flg22$^{A.tum}$ (triangles). Subsequently, leaves were infected with 10^5 c.f.u. ml^{-1} *Pst* DC3000, and bacterial growth was assessed 1 and 2 days after infection. Control L*er*-0 and *fls2-17* plants (circles) were not pretreated before bacterial infection. **b**, L*er*-0 plants were infiltrated simultaneously with 1 μM flg22 or flg22$^{A.tum}$ and 10^5 c.f.u. ml^{-1} *Pst* DC3000. Controls were treated with bacteria only. Results shown are means ± s.e.m. ($n = 8$).

transcription factors[21]. A similar over-representation of W-boxes was found when promoter sequences from all flg22-induced *RLK* genes were analysed (Supplementary Table 4). Because several WRKY factors are among the strongly induced genes after 30 min, it will be interesting to test whether a self-amplification system leads to even more pronounced induction of these genes after prolonged treatment with flg22. The induced RLKs and *R* genes constitute one-sixth of all upregulated genes (Supplementary Tables 2 and 3). With regard to the numbers of genes comprising these families in the *Arabidopsis* genome, this indicates an over-representation of 4.3-fold for RLKs and 3.8-fold for *R* genes, respectively. In summary, as well as the induction of numerous elements of the defence response, flagellin treatment seems to induce factors with an important function in the amplification of the signal and factors leading to an enhanced sensitivity of the plant to further stimuli sensing the presence of invading microorganisms. In particular, one could speculate that some of the induced RLKs and *R* genes might be involved in the recognition of other, as yet unidentified, PAMPs or *Avr* signals.

Transient overexpression of constitutively active MEKK1, MKK4, or wild-type WRKY29 resulted in reduced disease symptoms after treatment with *Pseudomonas syringae* pv. *maculicola* ES4326 or *Botrytis cinerea*[7]. To test whether direct induction of this signalling chain by flagellin leads to increased plant resistance, growth of pathogenic bacteria *in planta* was addressed after pretreatment of leaves with the flg22 elicitor. Wild-type and *fls2-17* mutant *Arabidopsis* plants were pretreated either with flg22 or the inactive analogue[22] flg22$^{A.tum}$ by leaf infiltration 1 day before challenge with pathogenic *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) bacteria (Fig. 1a). In wild-type plants that received either no pretreatment (controls) or pretreatment with flg22$^{A.tum}$, bacteria multiplied at the same rate. However, bacterial growth was strongly decreased in plants pretreated with flg22 (about 100-fold difference at 2 days after infection). In *fls2-17* plants, pretreatment with flg22 did not lead to a decreased growth of bacteria (Fig. 1a). This result shows that the induction of resistance depends on a functional FLS2, and also that flg22 has no antimicrobial activity itself. Decreased bacterial growth was also observed, but less so, when flg22 peptide was applied concomitantly with the bacterial inoculum (Fig. 1b), indicating that exposure of elicitor-active flagellin present in the injected *Pst* DC3000 bacteria might be a limiting factor for efficient induction of the basal resistance, at least in the absence of *R*-gene-dependent detection of *Avr* factors.

Analysis of mutants and transgenic plants have revealed the importance of the salicylic acid, jasmonic acid and ethylene pathways in *Arabidopsis* resistance against pathogens[23]. We tested the requirements of flg22-induced resistance for these previously identified defence signalling elements in plants mutated in *NPR1*, *EDS1*, *SGT1*, *RAR1*, *ETR1*, *EIN2*, *JAR1*, *PAD2* or *PAD4*, or in plants overexpressing *NahG*. Whereas bacterial growth showed some accession-dependent and mutation-dependent variation, all mutants still exhibited a significant flg22-induced reduction in bacterial growth (Table 1). Thus, the genes tested are not required for flg22-induced resistance. It is surprising that *NPR1*, *EDS1* and *PAD4*, which are essential for salicylic-acid-mediated resistance[23] and are transcriptionally induced as rapidly as 30 min after treatment of seedlings with flg22 (Supplementary Table 2), are not involved in the observed flg22-induced resistance. Furthermore, *PR1*, whose induced expression through NPR1 is a marker for salicylic-acid-mediated resistance[23], is activated 24 h after treatment with flg22 (ref. 24). However, our findings are consistent with the fact that flg22- and chitin-induced phosphorylation of the ankyrin-repeat protein *At*Phos43 was independent of NPR1 and salicylic acid[25]. flg22 activates the production of ethylene and triggers a rapid oxidative burst[6]. We therefore propose that flg22 induces the activation of the salicylic acid, jasmonic acid and ethylene pathways in parallel and that knocking out a single pathway alone does not abolish the induction of resistance. Alternatively, signalling elements that are as yet unknown, or the induction of antimicrobials, reactive oxygen species or cell wall reinforcement, might be responsible for inhibiting bacterial growth.

To test the involvement of PAMP perception systems other than flg22–FLS2 in resistance, we pretreated wild-type and mutant *fls2-17* plants with crude extracts obtained from *Pseudomonas syringae* pv. *syringae* (*Pss*), *Pst* DC3000 and *Agrobacterium tumefaciens*. All bacterial extracts—even that from *A. tumefaciens*, a species with an inactive flg22 peptide sequence[22]—induced medium alkalinization of *Arabidopsis* cell cultures (data not shown) and therefore showed clear elicitor activity. Pretreatment of plants with all the bacterial extracts resulted in a decreased growth of the pathogenic bacteria *Pst* DC3000 in comparison with that in control wild-type plants (Fig. 2). The same effect was also observed in *fls2-17* plants, but to a smaller extent in the case of a pretreatment with *Pst* DC3000 extracts. Thus, extracts from the tested bacteria contain at least one elicitor of resistance distinct from flagellin, and we propose that *Arabidopsis* has additional detection systems for these, as yet undefined, PAMPs.

Because flg22 perception induced disease resistance in plants, we tested whether plants lacking flagellin perception are more susceptible to pathogenic bacteria carrying elicitor-active flagellin. Inter-

Table 1 **flg22-induced resistance in plants affected in salicylic acid, jasmonic acid and ethylene signalling**

	Bacterial count (log c.f.u. cm^{-2})	
Line	flg22$^{A.tum}$	flg22
Col-0	4.6 ± 0.2	3.4 ± 0.2
NahG	5.9 ± 0.01	4.7 ± 0.1
etr1-3	4.4 ± 0.3	2.7 ± 0.2
ein2-1	3.5 ± 0.25	2.7 ± 0.3
jar1-1	4.6 ± 0.15	3.5 ± 0.2
pad2-1	5.0 ± 0.1	3.9 ± 0.3
pad4-1	5.5 ± 0.2	3.6 ± 0.2
L*er*-0	5.1 ± 0.1	3.5 ± 0.1
fls2-17	4.8 ± 0.25	4.9 ± 0.1
eds1-2	5.6 ± 0.05	3.7 ± 0.2
sgt1b-3	5.0 ± 0.2	3.6 ± 0.05
rar1-13	5.2 ± 0.2	3.5 ± 0.2
No-0	5.0 ± 0.2	2.6 ± 0.25
npr1-5	4.5 ± 0.15	2.2 ± 0.05

The following were pretreated for 24 h with 1 μM flg22 or flg22$^{A.tum}$: *Arabidopsis* transgenic *NahG* and mutants *etr1-3*, *ein2-1*, *jar1-1*, *pad2-1* and *pad4-1* in a Col-0 background; mutants *fls2-17*, *eds1-2*, *sgt1b-3* and *rar1-13* in a L*er*-0 background; and mutant *npr1-5* in a No-0 background. Subsequent leaf infection with 10^5 c.f.u. ml^{-1} *Pst* DC3000 was performed, and bacteria were counted 2 days after infection as described in Methods.

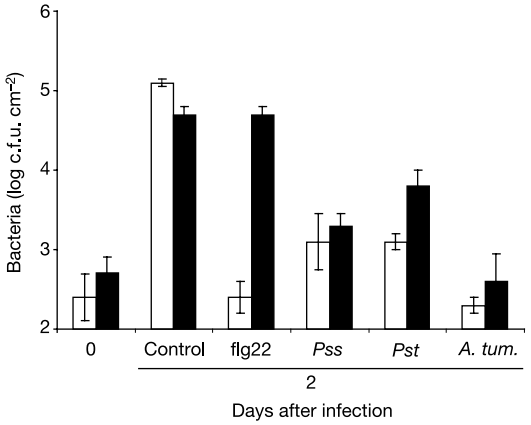

Figure 2 Treatment with different bacterial extracts limits subsequent growth of *Pst* DC3000 in L*er*-0 and *fls2-17* plants. L*er*-0 (open bars) and *fls2-17* (filled bars) plants were either left untreated, pretreated for 24 h with 1 μM flg22 or pretreated with one of the following bacterial extracts: *Pseudomonas syringae* pv. *syringae* (*Pss*) (3 mg ml^{-1}), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (3 mg ml^{-1}) or *Agrobacterium tumefaciens* (*A. tum.*) (10 mg ml^{-1}). Subsequent leaf infection with 10^5 c.f.u. ml^{-1} *Pst* DC3000 was performed, and bacterial growth was assessed 2 days after infection. Results shown are means ± s.e.m. ($n = 8$).

estingly, *Pst* DC3000 bacteria infiltrated directly into the intercellular leaf space grew at the same rate in *fls2-17* as in wild-type plants (Fig. 1a) and caused the same visible disease symptoms within the first week after infection (data not shown). In addition, various non-pathogenic or avirulent strains of *Pseudomonas* and *Xanthomonas* grew at the same, restricted, rate in both the *fls2-17* and wild-type plants (data not shown). Under natural conditions, *Pst* DC3000 enters host plants, usually the leaves, through wounds or natural openings such as stomata, and then spreads and multiplies to high population densities in intercellular spaces[26]. Thus, the infiltration of bacteria with a syringe might bypass the first steps of the natural infection process, notably the steps of invasion and spreading that probably rely on flagella-based motility. Bacterial motility might not be important within the intercellular spaces, because non-motile mutants of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *malvacearum* were similar to the parental strains in their ability to grow after vacuum infiltration into bean and cotton leaves, respectively[26]. We therefore infected *A. thaliana* plants by spraying *Pst* DC3000 bacteria onto leaf surfaces. Under these conditions, *fls2-17* plants showed a faster and more severe development of disease symptoms than wild-type plants (Fig. 3a). These stronger symptoms correlated with higher numbers of bacteria in *fls2-17* leaves (Fig. 3b), a difference that was particularly pronounced in younger leaves. Higher sensitivity of *fls2-17* mutants, compared to wild-type plants, was found in all of three independent experiments. Because *fls2-17* mutants originate from a population mutagenized with ethyl methane sulphonate, to exclude the possibility that the enhanced sensitivity of *fls2-17* was due to a genetic difference other than the one in the *FLS2* gene, we tested a second, independent, mutant affected in the *FLS2* gene in a Col-0 background. This mutant, carrying a T-DNA insertion in the promoter region abolishing expression of the *FLS2* gene (checked by reverse transcriptase polymerase chain reaction; data not shown), also showed enhanced sensitivity to *Pst DC3000* in comparison with its wild-type Col-0 background (Supplementary Fig. 2). In addition, the ecotype Ws-0 presents a flagellin-insensitive phenotype[22,24], formerly attributed to a mutation in a hypothetical *FLS1* gene[24] but recently shown to be a natural *fls2* mutant carrying a point mutation that resulted in a stop codon in the kinase domain of *FLS2* (S.R., unpublished observations). In comparison with the accession Col-0, Ws-0 plants exhibited faster and more severe development of disease symptoms after being sprayed with *Pst* (data not shown). However, Ws-0 plants transformed with a functional *FLS2* gene, under the control of its native promoter sequence, acquired responsiveness to flg22 (Supplementary Fig. 3) and became less susceptible to *Pst* DC3000 (Fig. 3c), indicating that the natural deficiency in flagellin perception in the ecotype Ws-0 can be complemented with the wild-type *FLS2* gene.

Enhanced disease susceptibility to airborne infection with *Mycobacterium tuberculosis* or *M. avium* has been observed in knockout mice lacking a single Toll-like receptor (TLR2). However, a more drastic effect on susceptibility was observed in mice lacking MyD88, a signal adaptor protein thought to be required for transfer of signals coming from all TLRs[27]. This indicates redundancy of the recognition process and the involvement of several TLRs in the innate immune system of animals. Interestingly, a common dominant TLR5 stop codon polymorphism abolishes flagellin signalling and is associated with susceptibility to Legionnaires' disease in humans[28]. Similarly, the results presented above provide a first example that perception of a single general elicitor or PAMP makes a difference for plant defence. Although the sensing of flagellin by FLS2 is an important initial checkpoint for controlling or restricting bacterial invasion in *Arabidopsis* leaves, it is not the only checkpoint; detection systems for additional bacterial PAMPs can be expected to have similar and complementary functions in controlling pathogen invasion at different steps of the infection process. The identification of these additional PAMP(s) and of the corresponding receptor(s) represents an exciting goal for the future. □

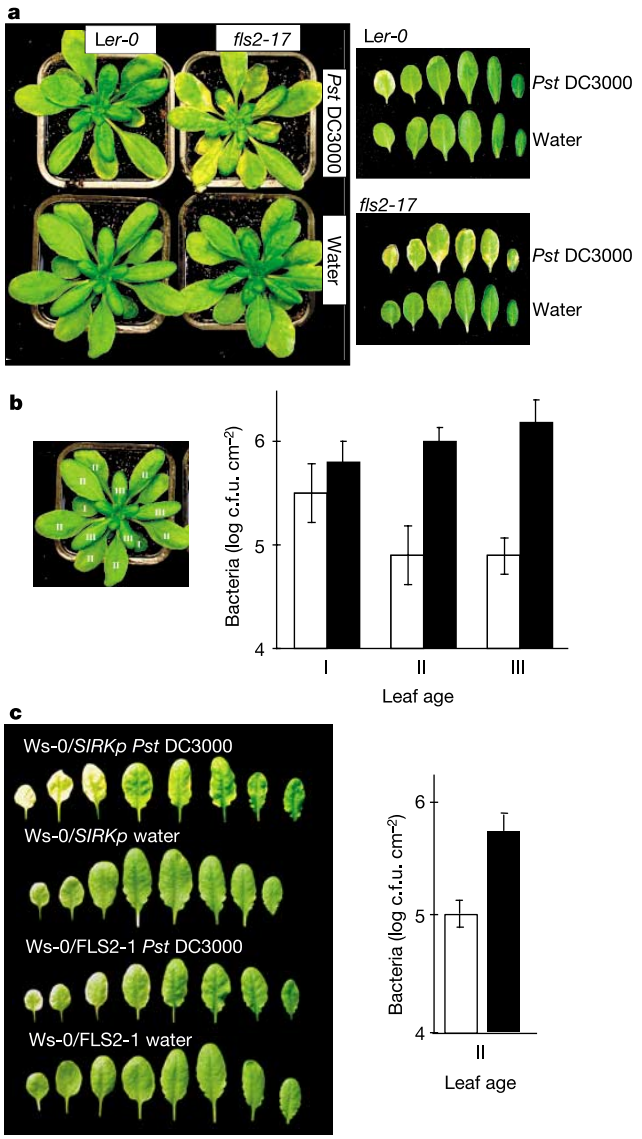

Figure 3 Bacterial disease resistance is determined by flagellin perception. **a**, A *FLS2* loss-of-function mutation, *fls2-17*, leads to enhanced disease susceptibility. Left: wild-type and *fls2-17* mutant plants were sprayed with *Pst* DC3000 bacteria or with water and photographed 4 days later. Right: symptoms after 4 days in a series of leaves of decreasing age. **b**, Number of *Pst* DC3000 bacteria extracted from wild-type (open bars) and *fls2-17* mutant plants (filled bars) 4 days after infection. Leaves were grouped by age as depicted on the left. **c**, A gain-of-function transgene of *FLS2* leads to decreased susceptibility in the accession Wassilewskaya (Ws-0), which lacks a functional *FLS2* gene. Ws-0 was stably transformed with *FLS2p::FLS2-3xmyc* (line FLS2-1; open bars), or *SIRKp::GUS* (line SIRKp; filled bars) as a control. Plants were sprayed with 5×10^8 c.f.u. ml^{-1} *Pst* DC3000, or water. Pictures were taken for a series of leaves of decreasing age (left), and bacteria were extracted and counted from leaves of age class II (right), 4 days after infection. Results are means ± s.e.m. ($n = 8$).

Methods

Plant material

All plants were grown at 20–21 °C with 65% humidity under light (about 100 μmol m^{-2} s^{-1}) in an 8 h light/16 h dark cycle in environment-controlled chambers. Plants aged 5–6 weeks were used for the infection experiments.

Flagellin treatment

Treatments with flg22 or flg22$^{A.tum}$ were performed by pressure infiltration (needle-less syringes) of 1 μM peptide solution into the leaves. For each treatment, four to eight plant replicates were used, and each experiment was repeated at least twice.

Bacterial growth assays

Bacterial strains used in this study were *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), *Pst* DC3000 *AvrRpm1*, *Pst* DC3000 *AvrRps4*, *Pst* DC3000 *AvrRpt2*, *Pseudomonas syringae* pv. *tomato* DC3000 *HrpS*$^-$, *Pseudomonas syringae* pv. *syringae* (*Pss*), *Pseudomonas syringae* pv. *tabaci*, *Pseudomonas syringae* pv. *glycinae*, *Pseudomonas syringae* pv. *phaseolicola*, *Pseudomonas fluorescens*, *Pseudomonas brassicacearum*, *Xanthomonas axonopodis* pv. *citri* and *Agrobacterium tumefaciens*. All strains were grown at 28 °C on King's B medium (40 g l^{-1} proteose, 20 g l^{-1} glycerol, 15 g l^{-1} agar) containing the appropriate antibiotics for selection. Syringe and spray inoculations, and bacterial growth *in planta*, were performed as described[29]. In brief, for syringe inoculation, the bacteria were scraped off a fresh plate, resuspended in sterile water to 10^5 colony-forming units (c.f.u.) ml^{-1}, and pressure-infiltrated into leaves with a needleless syringe. For spray inoculation, overnight *Pst* DC3000 cultures were collected, washed once and resuspended in sterile water. Plants were sprayed with a bacterial suspension containing 5×10^8 c.f.u. ml^{-1} bacteria with 0.04% Silwet L-77 (Lehle Seeds). Leaves were harvested and surface sterilized (30 s in 70% ethanol, followed by 30 s in sterile distilled water) for the spray inoculation method. Leaf discs from two different leaves were ground in 10 mM $MgCl_2$ with a Microfuge tube glass pestle. After grinding of the tissue, the samples were thoroughly vortex-mixed and diluted 1:10 serially. Samples were finally plated on NYGA solid medium (5 g l^{-1} bactopeptone, 3 g l^{-1} yeast extract, 20 ml l^{-1} glycerol, 15 g l^{-1} agar) supplemented with the appropriated antibiotic. Plates were placed at 28 °C for 2 days, after which the colony-forming units were counted.

Treatment with bacterial extracts

Extracts from *Pss* and *Pst* DC3000 were prepared as described[6], freeze-dried and dissolved in water (3 mg ml^{-1}). *A. tumefaciens* bacteria were harvested by centrifugation, washed once with water and lysed by incubation in lysozyme solution (0.2 mg ml^{-1}) for 30 min at 37 °C and homogenization with a Polytron. The soluble supernatant was freeze-dried and redissolved in water (10 mg ml^{-1}).

Generation of transgenic plants

Arabidopsis thaliana Ws-0 plants were transformed with a *FLS2p::FLS2-3xmyc* construct. The *FLS2* promoter up to −988 base pairs was amplified by polymerase chain reaction (PCR) and introduced into the *Eco*RI and *Hin*dIII sites of p*CAMBIA 2300* (www.cambia.org.au), additionally adding *Bam*HI and *Kpn*I restriction sites upstream of the *Hin*dIII site. The *FLS2* gene triple Myc-tag fusion was amplified by PCR and cloned into the *Bam*HI and *Kpn*I sites of p*CAMBIA*, and the construct was verified by sequencing. Stable transgenic lines were generated with the *A. tumefaciens*-mediated gene transfer procedure. Independent transformed plant pools were kept separate for the selection of independent transgenic lines based on their kanamycin resistance. Functional complementation of Ws-0 by *FLS2p::FLS2-3xmyc* was assayed with standard procedures[24]. Expression of FLS2-3xmyc protein was confirmed by western blot analysis with anti-Myc antibodies (Supplementary Fig. 3). For control transformation, a *SIRKp::GUS* construct[30] was used. Plants of the T_2 generation were chosen for the bacterial spraying experiments.

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Cdc42 and mDia3 regulate microtubule attachment to kinetochores

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During mitosis, the mitotic spindle, a bipolar structure composed of microtubules (MTs) and associated motor proteins[1,2], segregates sister chromatids to daughter cells. Initially some MTs emanating from one centrosome attach to the kinetochore at the centromere of one of the duplicated chromosomes. This attachment allows rapid poleward movement of the bound chromosome. Subsequent attachment of the sister kinetochore to MTs

Supplementary Methods

Experimental design: seedling materials and elicitor treatment for the microarray experiments. After a 48-hour treatment at 4°C, *A. thaliana* Landsberg *erecta* (Ler-0) and *fls2-17* seeds were grown for 12 days on plates containing 1x MS medium (Duchefa), 1% sucrose and 1% agar under continuous light (60 µE m^{-2} sec^{-1}, Biolux lamps) at 22°C. Seedlings were then transferred to liquid MS medium (two seedlings per 500 µl of medium in wells of 24-well-plates). Two days after transfer, the medium was supplied with flg22 peptide (10 µM final concentration). Plantlets were collected 30 min after treatment, frozen in liquid nitrogen and stored at -80°C.

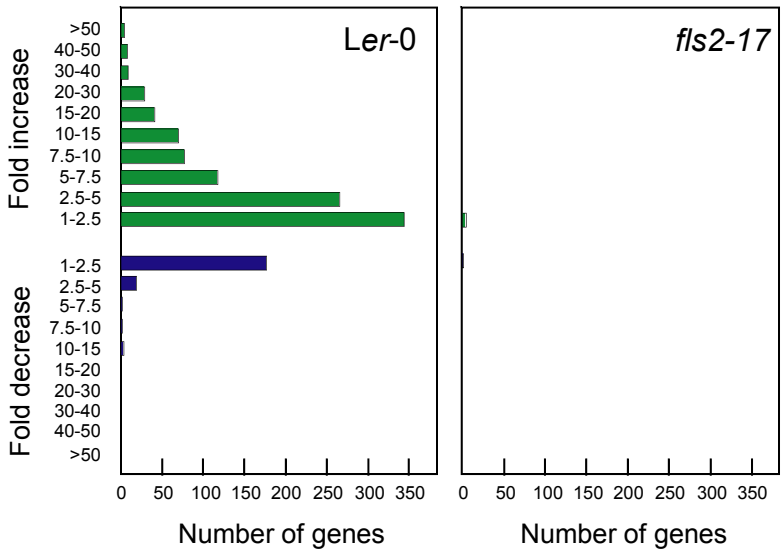
Samples used. Samples from 4 different wells were pooled. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen). Two independent experiments were performed in an interval of two weeks.

Extract labeling. Microarray analysis was performed using ATH1 GeneChips™ (Affymetrix). 10 µg of total RNA was reverse transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) according to the protocol recommended by Affymetrix. The oligonucleotide used for priming was 5'-ggccagtgaattgtaatacgactcactatagggaggcgg-(t)24-3' (Genset Oligo). Double-stranded cDNA was cleaned by phenol:chloroform extraction and the aqueous phase removed by centrifugation through Phase-lock Gel (Eppendorf). *In vitro* transcription was performed on 1 µg of cDNA using the Enzo BioArray High Yield RNA transcript labelling kit (Enzo Diagnostics). The cRNA was cleaned using RNAeasy clean-up columns (Qiagen). The cRNA was fragmented by heating in 40 mM Tris-acetate pH 8.1, 100 mM KOAc, 30 mM MgOAc. The Affymetrix eukaryotic hybridization controls were added to the sample prior to hybridization as per manufacturer's instructions.

Hybridization conditions. 10 µg of fragmented cRNA were hybridised (45°C, 16 hours). Hybridization was controlled by use of the GeneChip™ Eukaryotic Hybridization Control Kit (Affymetrix). Washing and staining was performed in a Fluidics Station 400 (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip scanner.

Microarray analysis. Chip analysis was performed using the Affymetrix Microarray Suite v5 (with a median target intensity of 500) and GeneSpring 5.1 (Silicon Genetics). Changes in gene expression were assessed using a signed Wilcoxon rank test. Genes were required to show the same direction of change in all replicate comparisons with a p-value cutoff of < 0.003 in each comparison. Any gene whose detection p-value was > 0.05 in all experimental conditions was discarded from the analysis as being unreliable data. The data were then further filtered using a one-way ANOVA (p-value < 0.05) with a Benjamini and Hochberg false discovery multiple testing correction.

Array design. Affymetrix ATH1 GeneChip



Supplementary Figure 1 Genome-wide analysis of flg22-dependent gene expression in Ler-0 and *fls2-17* seedlings. Expression values were derived from the average of 2 microarray replicates corresponding to two independent experiments and RNA extractions. Probe sets representing flagellin-regulated genes were defined as described in Methods. Briefly, we compared the expression level for each probe set of treated Ler-0 or *fls2-17* seedlings at 30 minutes with the one of the corresponding untreated seedlings. All probe sets called as “increased” or “decreased” by Affymetrix Microarray Suite v5 based on our “change” p-value threshold (see Methods) were considered, without fold-change cut-off, as being significant.

Supplementary Table 2. Resistance and resistance-associated genes induced upon flg22 treatment

ProbeSet ID	AGI	Description	Fold change
			L*er-0*
TIR-NBS-LRR			
249264_s_at	At5g41740	Disease resistance protein	12,4
260296_at	At1g63750	Similar to disease resistance protein (RPP1-WsC) (*Arabidopsis thaliana*)	8,8
259629_at	At1g56510	Disease resistance protein	5,5
249903_at	At5g22690	Disease resistance protein	5,2
247848_at	At5g58120	Similar to disease resistance protein RPP1-WsA (*Arabidopsis thaliana*)	4,1
245654_at	At1g56540	Disease resistance protein	3,8
249312_at	At5g41550	Disease resistance protein	3,5
248875_at	At5g46470	Disease resistance protein	3,2
249320_at	At5g40910	Disease resistance protein	2,5
249321_at	At5g40920	Disease resistance protein	2,4
250419_at	At5g11250	Similar to disease resistance protein RPP1-WsC (*Arabidopsis thaliana*)	2,4
245454_at	At4g16920	Similar to disease resistance protein RPP5 (*Arabidopsis thaliana*)	2,0
249029_at	At5g44870	Disease resistance protein	2,0
CC-NBS-LRR			
252126_at	At3g50950	Disease resistance protein	5,0
253997_at	At4g26090	**RPS2**	4,3
250829_at	At5g04720	Similar to disease resistance protein RPP8 (*Arabidopsis thaliana*)	4,0
258544_at	At3g07040	**RPM1**	3,7
253377_at	At4g33300	Similar to disease resistance protein RFL1 (*Arabidopsis thaliana*)	3,4
247065_s_at	At5g66900	Disease resistance protein	1,9
256425_at	At1g33560	Disease resistance protein	1,7
245219_at	At1g59124	Similar to viral resistance protein PRM1 (*Arabidopsis thaliana*)	1,5
TIR-NBS (TN)			
262382_at	At1g72920	Similar to virus resistance protein (*Nicotiana glutinosa*)	10,0
262381_at	At1g72900	Similar to virus resistance protein (*Nicotiana glutinosa*)	8,0
262383_at	At1g72940	Disease resistance protein	4,9
248845_at	At5g46480	Disease resistance protein	3,7
262384_at	At1g72950	Disease resistance protein	3,1
258537_at	At3g04210	Disease resistance protein	2,5
TIR (TX)			
264153_at	At1g65390	Similar to disease resistance protein RPS4 (*Arabidopsis thaliana*)	16,2
265597_at	At2g20145		6,5
249032_at	At5g44910	Disease resistance protein	6,3
265723_at	At2g32140	Disease resistance protein	4,8
NBS-LRR			
246406_at	At1g57650	Disease resistance protein	18,4
CC-NBS			
262126_at	At1g59620	Disease resistance protein	1,9
AtMlo			
265008_at	At1g61560	**AtMlo6**	13,6
266992_at	At2g39200	**AtMlo12**	9,8
262455_at	At1g11310	**AtMlo2**	3,2
264852_at	At2g17480	**AtMlo8**	2,2
Cf-like			
255319_at	At4g04220	Disease resistance protein similar to receptor protein kinases	6,7
259298_at	At3g05370	Similar to Cf-2 disease resistance protein (*Lycopersicon pimpinellifolium*)	6,3
249393_at	At5g40170	Similar to disease resistance protein Cf-4 (*Lycopersicon hirsutum*)	4,0
259952_at	At1g71400	Similar to disease resistance protein Cf-4 (*Lycopersicon hirsutum*)	3,9
256431_s_at	At3g11010	Similar to disease resistance protein (*Lycopersicon esculentum*)	1,8
Others			
251774_at	At3g55840	Similar to nematode resistance protein Hs1pro-1 (*Beta procumbens*)	20,5
267357_at	At2g40000	Similar to nematode resistance protein	20,1
265993_at	At2g24160	Disease resistance protein	6,2
267411_at	At2g34930	Disease resistance protein	5,8
245765_at	At1g33600	Disease resistance protein	4,0
245768_at	At1g33590	Disease resistance protein	3,2
262649_at	At1g14040	Similar to xenotropic and polytropic retrovirus receptor GB:4759334	2,4
Resistance-associated genes			
263948_at	At2g35980	similar to harpin-induced protein hin1	24,1
250676_at	At5g06320	**NHL3** (NDR1/HIN1-like 3)	7,4
257083_s_at	At3g20590	Similar to non-race specific disease resistance protein GB:AAB95208 (*Arabidopsis thaliana*)	6,3
252373_at	At3g48090	**EDS1**	4,0
246600_at	At5g14930	Similar to disease resistance protein EDS1	5,4
251879_at	At3g54200	putative protein hin1 protein	3,5
252060_at	At3g52430	phytoalexin-deficient 4 protein (**PAD4**)	2,6
248981_at	At5g45110	regulatory protein NPR1-like; transcription factor inhibitor I kappa B-like	2,4
259071_at	At3g11650	unknown protein similar to hin1	2,0
254477_at	At4g20380	**LSD1**	1,7
257868_at	At3g25070	**RIN4**	1,7
259764_at	At1g64280	**NPR1**	1,1
Potential antimicrobials genes			
251895_at	At3g54420	class IV chitinase (CHIV)	12,2
245038_at	At2g26560	Similar to latex allergen from Hevea brasiliensis	7,6
253284_at	At4g34150	Putative protein hydroxyproline-rich glycoprotein precursor, Nicotiana tabacum, PIR2:S06733	7,5
255595_at	At4g01700	Putative chitinase similar to peanut type II chitinase, GenBank accession number X82329	5,8
259443_at	At1g02360	Putative chitinase GI:1237025 from [Arachis hypogaea]	4,5
245034_at	At2g26390	Putative serpin	4,0
262731_at	At1g16420	Similar to gb|AF098458 latex-abundant protein (LAR) from Hevea brasiliensis	3,6
267335_s_at	At2g19440	Putative beta-1,3-glucanase	3,6
265648_at	At2g27500	Putative beta-1,3-glucanase	3,4
264365_s_at	At1g03220	Similar to gb|D14550 extracellular dermal glycoprotein (EDGP) precursor from Daucus carota	2,9
251804_at	At3g55430	Similar to beta-1,3-glucanase, Triticum aestivum, PIR:T06268	2,7
250323_at	At5g12880	Putative protein hydroxyproline-rich glycoprotein, kidney bean	2,7
260556_at	At2g43620	Putative endochitinase	2,7
264279_s_at	At1g78820	Similar to glycoprotein (EP1) GI:349436 from [Daucus carota]	2,1
248703_at	At5g48430	Dermal glycoprotein precursor, extracellular-like	1,7
255904_at	At1g17860	Similar to lemir (miraculin) GI:2654440 from [Lycopersicon esculentum]	1,7
254665_at	At4g18340	Simlar to endo-beta-1,3-beta-D-glucosidase, Nicotiana tabacum, PIR2:S46495	1,4

Supplementary Table 3. Receptor-like kinases induced upon flg22 treatment

ProbeSet ID	AGI	Description		Fold change
		Subfamily	ED	*Ler-0*
LRR-RLK				
263478_at	At2g31880	LRR	LRR5	11,1
259400_at	At1g17750	LRRXI	LRR25	10,6
246082_at	At5g20480	LRRXII	LRR21[a]	10,0
246858_at	At5g25930	LRR	LRR22	6,5
248698_at	At5g48380	LRRX	LRR4	5,1
260239_at	At1g74360	LRRX	LRR20	4,5
264107_s_at	At2g13790	LRRII	LRR4	4,2
246366_at	At1g51850	LRRI	LRR3	4,1
262360_at	At1g73080	LRRXI	LRR26	4,0
255116_at	At4g08850	LRRXI	LRR24	3,7
257902_at	At3g28450	LRRX	LRR5	3,6
260975_at	At1g53430	LRRVIII-2	LRR6	3,6
248895_at	At5g46330	LRRXII	LRR28[b]	3,5
246327_at	At1g16670	LRRVIII-2	K	3,5
252378_at	At3g47570	LRRXII	LRR21	2,9
256169_at	At1g51800	LRRI	LRR3	2,9
260345_at	At1g69270	LRRX	LRR2	2,9
256170_at	At1g51790	LRRI	LRR3	2,8
261161_at	At1g34420	LRRVII	LRR10	2,8
262082_s_at	At1g56120	LRRVIII-2	LRR7	2,7
266231_at	At2g02220	LRR	LRR17	2,7
259074_at	At3g02130	LRRX	LRR18	2,5
246529_at	At5g15730	LRRI	K	2,5
258616_at	At3g02880	LRRIII	LRR5	1,7
256547_at	At3g14840	LRRVIII-2	LRR9	1,6
253338_at	At4g33430	LRRII	TK[c]	1,6
264663_at	At1g09970	LRRXI	LRR19	1,5
260974_at	At1g53440	LRRVIII-2	LRR9	1,5
RLCKs				
247740_at	At5g58940	RLCKIV	K	15,1
258650_at	At3g09830	RLCKVII	K	8,2
256177_at	At1g51620	RLCKIV	K	5,7
247532_at	At5g61560	RLCKIX	K[d]	5,2
250990_at	At5g02290	RLCKVII	K	4,6
261526_at	At1g14370	RLCKVII	K	4,4
253147_at	At4g35600	RLCKVII	K	3,4
259887_at	At1g76360	RLCKVII	K	3,4
251494_at	At3g59350	RLCKVIII	K[e]	3,2
263274_at	At2g11520	RLCKIV	K	1,9
251789_at	At3g55450	RLCKVII	K	1,8
255716_at	At4g00330	RLCKIV	K	1,8
258463_at	At3g17410	RLCKVIII	K[e]	1,6
251742_at	At3g56050	RLCKI	K	1,5
DUF26-RLKs				
254897_at	At4g11470	DUF26	DUF26	10,2
254241_at	At4g23190	DUF26	DUF26[f]	9,3
254256_at	At4g23180	DUF26	DUF26	8,9
255654_at	At4g00970	DUF26	K	6,2
260206_at	At1g70740	DUF26	K	5,7
255344_s_at	At4g04540	DUF26	DUF26	3,5
254248_at	At4g23270	DUF26	DUF26	3,2
254243_at	At4g23210	DUF26	DUF26	2,8
260303_at	At1g70520	DUF26	DUF26	2,5
260362_at	At1g70530	DUF26	DUF26	1,9
254409_at	At4g21400	DUF26	DUF26	1,5
Lectin-RLKs				
258982_at	At3g08870	L-lectin	LEC	11,3
255502_at	At4g02410	L-lectin	LEC	11,1
251054_at	At5g01540	L-lectin	LEC	10,8
253819_at	At4g28350	L-lectin	LEC	9,1
251479_at	At3g59700	L-lectin	LEC[g]	4,6
251096_at	At5g01550	L-lectin	LEC	3,9
251910_at	At3g53810	L-lectin	LEC	3,8
251097_at	At5g01560	L-lectin	LEC	3,4
267550_at	At2g32800	L-lectin	K	2,3
267165_at	At2g37710	L-lectin	LEC	1,5
247617_at	At5g60270	L-lectin	LEC	1,4

ProbeSet ID	AGI	Description		Fold change
		Subfamily	ED	*Ler-0*
K-RLKs				
260477_at	At1g11050	K	RKF3L	7,6
263804_at	At2g40270	K	K	7,1
266749_at	At2g47060	K	K	6,7
266037_at	At2g05940	K	K	4,0
246943_at	At5g25440	?	K	3,8
263419_at	At2g17220	K	K	3,2
267624_at	At2g39660	K	K	3,1
248934_at	At5g46080	?	SK	2,7
264232_at	At1g67470	K	K	2,6
254063_at	At4g25390	?	TK	2,4
246146_at	At5g20050	?	SK	1,7
LRK10-like RLKs				
249550_at	At5g38210	LRK10L-1	LRKL	8,8
249553_at	At5g38260	LRK10L-2	LRKL	8,2
256366_at	At1g66880	LRK10L-1	LRKL	7,5
261718_at	At1g18390	LRK10L-1	LRKL	3,6
255740_at	At1g25390	LRK10L-1	LRKL	2,7
245760_s_at	At1g66920	LRK10L-2	LRKL	2,5
255913_at	At1g66980	LRK10L-2	LRKL[h]	2,0
249552_s_at	At5g38240	LRK10L-2	LRKL	2,0
S-RLKs				
254408_at	At4g21390	S	SD	7,4
264757_at	At1g61360	SD-1	SD	5,9
267490_at	At2g19130	S	SD	3,7
264756_at	At1g61370	SD-1	SD	2,4
264767_at	At1g61380	SD-1	SD	1,7
253911_at	At4g27300	SD-1	SD	1,5
WAK-like RLKs				
257478_at	At1g16130	WAKL	WAKL	7,4
257479_at	At1g16150	WAKL	WAKL	4,4
261394_at	At1g79680	WAKL	WAKL	2,5
267134_at	At2g23450	WAKL	WAKL	2,2
261402_at	At1g79670	WAKL	WAKL	1,7
CrRLK1-like RLKs				
249485_at	At5g39020	CRPK1L-2	CRPK1L	8,9
249480_s_at	At5g38990	CRPK1L-1	CRPK1L	2,5
266968_at	At2g39360	CRPK1L-1	CRPK1L	2,0
249486_at	At5g39030	CRPK1L-2	CRPK1L	1,8
LysM RLKs				
255844_at	At2g33580	LysM	LysM	13,3
267289_at	At2g23770	LysM	LysM	6,7
258173_at	At3g21630	LysM	LysM	2,4
CR4-like RLKs				
248775_at	At5g47850	CR4L	CR4L	41,5
251769_at	At3g55950	CR4L	CR4L	1,9
RKF3-like RLKs				
265772_at	At2g48010	RKF3L	RKF3L	2,1
PERK RLKs				
262228_at	At1g68690	PERKL	TK	5,2

Footnotes

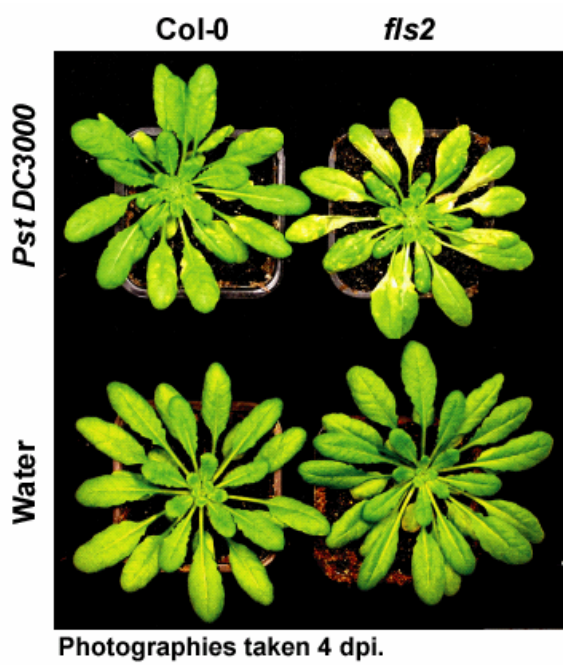
[a] similar to disease resistance protein kinase Xa21 (*Oryza sativa*)
[b] **FLS2** [c] **BAK1**
[d] similar to disease resistance protein kinase Pto (*Lycopersicon esculentum*)
[e] similar to Pto kinase-interactor 1 (*Lycopersicon esculentum*)
[f] **RLK3** [g] **LecRK1**
[h] similar to leaf rust resistance kinase Lr10 (*Triticum aestivum*)

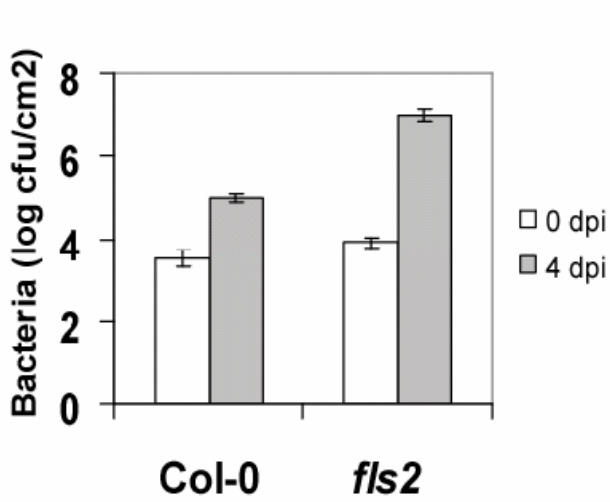
The abbreviations for the extracellular domains stand for: CR4L, Crinkly4-like; CrRLK1, Catharanthus roseus RLK1; DUF26, domain of unknown function; LEC, legume lectin; LRKL, wheat LRK10-like; LRR, leucin-rich repeat, the numbers refer to the number of repeats; LysM, lysine motif; RLCK, receptor-like cytoplasmic kinase; PERK, Proline Extensin-like Receptor Kinase; SD, S-locus glycoprotein-like domain; WAKL, wall-associated kinase like. K, TK, sequence with no predicted signal motif.

Supplementary Table 4. Frequency of occurrence of conserved binding motifs for different types of transcription factors in the *FLARE RLKs*

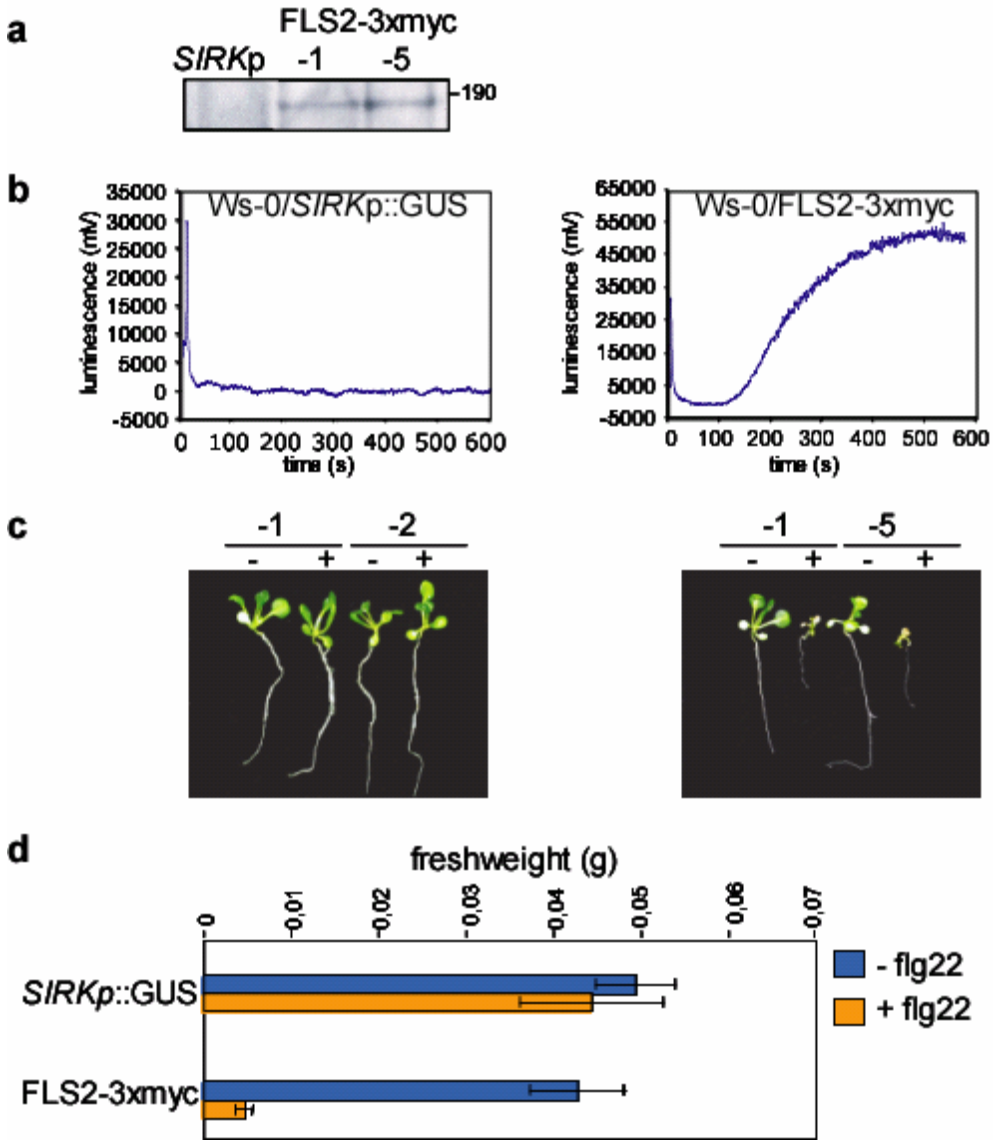
Transcription Factor Type	Motif sequences	Frequency in flg22-regulated promoters (105 promoters)	Frequency in non flg22-regulated promoters (500 promoters)	Frequency fold change
AP2/EREBP (GCC-box)	GCCGCC	0.10	0.08	1.25
AP2/EREBP	ACCGCC	0.08	0.09	0.88
Myb	G(G/T)T(A/T)G(G/T)T	1.66	1.40	1.18
bZIP (TGA type)	TGACG	1.13	0.88	1.28
bZIP (GBF-type)	CACGTG	0.12	0.15	0.80
bZIP (G/HBF-1 type)	CCTACC	0.11	0.12	0.92
EIN3/EIL	GGATGTA	0.05	0.04	1.25
WRKY (core)	TTGAC	4.00	2.05	1.95
WRKY (stringent)	TTGAC(T/C)	2.59	1.09	**2.38**
WRKY (stringent)	TTGACT	1.72	0.7	**2.46**
WRKY (stringent)	TTGACC	0.87	0.42	**2.07**

In bold are the frequencies of over-representative elements that are at least twice the statistical expected frequency that occur within a set of 500 non-flg22 regulated promoters. Analysis was performed as described in Navarro et al. (2004).





Supplementary Figure 2 Bacterial disease resistance is determined by flagellin perception. A null mutation of *FLS2* leads to enhanced disease susceptibility. Wild-type Col-0 and *fls2* null mutant (SAIL_691C4) plants were sprayed with $5x10^8$ cfu/ml *Pst* DC3000, or water. For bacterial counting, leaves corresponding to the class II were harvested. Results are averages ± s.e. (n=8).



Supplementary Figure 3. The flg22 insensitive phenotype of *Arabidopsis* ecotype Ws-0 is complemented by FLS2. **a**, Western analysis of representative transgenic Ws-0 lines expressing a FLS2-3xmyc fusion protein. 15 µg crude protein extracts of a control *SIRKp*::GUS line and selected independent T_1- transgenic lines (-1, -5) were loaded in each lane. Detection of FLS2-3xmyc fusion protein was done with 1:1000 α-myc and 1:30000 α-rabbit alkaline phosphatase conjugated antibodies. **b**, Flg22-induced oxidative burst is gained in FLS2-3xmyc transgenic Ws-0. Luminescence control *SIRKp*::GUS and *FLS2p::FLS2-3xmyc* leaf pieces in a luminol and peroxidase containing solution after treatment with 10 µM flg22. **c**, Seedling growth of FLS2-3xmyc transgenic Ws-0 is inhibited in response to flg22. 1-week-old seedlings of each two independent control *SIRKp*::GUS-1, -2 and *FLS2p::FLS2-3xmyc*-1, -5 lines were transferred to liquid MS medium and further incubated for 1 week in the absence (-) or presence (+) of 10 µM flg22. **d**, Statistical analysis of seedlings growth in presence or absence of 10 µM flg22 (n=6).

Chapter 3

Identification of new PAMP receptors in Arabidopsis

Chapter 3.1

Generation of an LRR-RLK Arabidopsis mutant collection

Introduction

Previous gene expression studies in Arabidopsis revealed that many genes encoding RLKs were rapidly induced following flagellin (flg22) treatment (Navarro et al., 2004; Zipfel et al., 2004). The expression of 106 RLK genes among the 610 genes present in the Arabidopsis genome was induced in seedlings as early as 30 minutes after flg22 treatment (Zipfel et al., 2004). Most of the RLK subfamilies defined in Arabidopsis (Shiu and Bleecker, 2001) have at least on representative gene induced. Interestingly, *FLS2* (At5g46330) was itself induced. We hypothesized that at least some of the induced RLKs might represent PRRs, and that flg22 perception is enhancing the "awareness" of the plant cells to further PAMPs.

To test this hypothesis and to identify new PRRs in Arabidopsis, we decided to generate mutants for the induced RLKs and to test them for their sensitivity to purified elicitors and/or susceptibility to diverse pathogens. Several collections of insertional Arabidopsis mutants have been developed over the last years that allow the rapid identification of a mutation in a gene of interest (Alonso et al., 2003; Sessions et al., 2002; Samson et al., 2002; Rosso et al., 2003).

Results and discussion

As a starting point, we only focused on the set of induced LRR-RLKs (28 genes) (Table 1) (Zipfel et al., 2004). This choice was based on several (biased) reasons. The LRR motif, thought to mediate protein-protein interaction, seems to be widely used in different kingdoms for (direct or indirect) microbe detection (Hoffmann, 2003; Bell et al., 2003; Dangl and Jones, 2001). The only known PRR in Arabidopsis, FLS2, is an LRR-RLK and directly interacts with the peptidic PAMP flg22 (Gòmez-Gòmez and Boller, 2000; Chinchilla et al., submitted). We suspect that other PAMPs

perceived by Arabidopsis are of proteinasceous nature, and that their recognition would therefore involve LRR-RLK, such as FLS2. In addition, the identification of other LRR-RLK PRRs in Arabidopsis could allow sequence comparisons with FLS2 in order to define the different ligand-binding specificities.

Table 1. Flg22-induced LRR-RLKs

AGI number	Name	Subfamily	Number of LRRs	Number of exons	Gene	Protein
At1g09970		LRR XI	19	4	3451 bp	976 aa (107 kD)
At1g16670		LRR VIII-2	-	6	2428 bp	390 aa (43 kD)
At1g17750		LRR XI	25	2	3462 bp	1088 aa (119 kD)
At1g34420		LRR VII	10	2	2981 bp	966 aa (106 kD)
At1g51790		LRR I	3	12	3854 bp	881 aa (93 kD)*
At1g51800		LRR I	3	12	3820 bp	894 aa (100 kD)
At1g51850		LRR I	3	13	3934 bp	865 aa (96 kD)
At1g53430		LRR VIII-2	6	19	5919 bp	1030 aa (114 kD)
At1g53440		LRR VIII-2	9	20	5601 bp	1035 aa (115 kD)
At1g56120		LRR VIII-2	7	19	5782 bp	1045 aa (115 kD)
At1g69270	*RPK1*[1]	LRR X	2	1	2377 bp	540 aa (60 kD)
At1g73080		LRR XI	26	2	3686 bp	1123 aa (123 kD)
At1g74360		LRR X	20	2	4282 bp	1106 aa (122 kD)
At2g02220		LRRX	17	1	2728 bp	909 aa (101 kD)*
At2g13790		LRR II	4	8	4956 bp	620 aa (69 kD)
At2g31880		LRR VII	5	1	2079 bp	641 aa (71 kD)
At3g02130		LRR X	18	1	3221 bp	985 aa (125 kD)
At3g02880		LRR III	5	3	2627 bp	627 aa (68 kD)
At3g14840		LRR VIII-2	9	23	5878 bp	988 aa (109 kD)
At3g28450		LRR X	5	1	2113 bp	605 aa (67 kD)
At3g47570		LRR XII	21	2	3420 bp	1010 aa (111 kD)
At4g08850		LRR XI	24	2	4145 bp	1045 aa (115 kD)
At4g33430	*BAK1*[2]	LRR II	4	11	4563 bp	615 aa (68 kD)
At5g15730		LRR I	-	6	2647 bp	434 aa (49 kD)
At5g20480		LRR XII	21	2	3391 bp	1031 aa (113 kD)
At5g25930		LRR XI	22	2	3359 bp	1005 aa (112 kD)
At5g46330	*FLS2*[3]	LRR XII	28	2	3808 bp	1173 aa (129 kD)
At5g48380		LRR X	4	2	2918 bp	620 aa (69 kD)

-, Although classified as LRR-RLK by Shiu and Bleecker (2001), these sequences do not contain any detectable LRR domain.
*, Differences in sequence annotation between databases has been observed for these genes.
1, Hong et al. (1997); 2, Li et al. (2002); Nam and Li (2002); 3, Gòmez-Gòmez and Boller (2000)

The hypothesis that induced RLKs are potential PRRs has a corollary. If flg22 treatment rapidly induces many RLKs, comprising its own receptor FLS2, then other PAMPs should also induce the expression of *FLS2*, as well as the other flg22-induced RLKs. We therefore retrieved publicly available lists of genes that were induced following diverse PAMP treatments (Ramonell et al., 2002; Molinier J. et al., 2005), or during bacterial infection experiments (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=120; http://affymetrix.arabidopsis.info/narrays/

Table 2. Gene expression of flg22-induced LRR-RLKs after different PAMP treatments

Source	Zipfel et al., 2004	NASC arrays-AtGenExpress: Pathogen infection (B)								Ramonell et al., 2002						Molinier et al., 2005		
Array type	Affy 24k array	Affy 24k array								AFGC array						Affy 8k array		
Material	Seedlings	Infiltrated leaves								Seedlings						Seedlings		
Treatment	flg22	HrpZ		NPP1		flg22		LPS		Chitin						Xylanase		
Concentration	10 µM	1 µM		2 µM		1 µM		100 µg/ml		100 mg/ml						20 µg/ml		
Duration	30 min	1h	4h	1h	4h	1h	4h	1h	4h	10 min	30 min	1h	3h	6h	24h	2h	6h	24h
Gene																		
At1g09970	1,5	-1,2	3,6	1,1	1,4	1,1	3,0	1,0	1,3							5,4	2,5	-1,3
At1g16670	3,5	3,3	2,1	2,6	1,5	3,7	1,4	1,1	1,0									
At1g17750	10,6	1,4	1,1	2,3	1,9	1,2	0,7	-1,1	-1,1									
At1g34420	2,8	1,0	1,4	1,4	1,4	1,3	1,5	1,1	-1,3									
At1g51790	2,8	2,7	7,6	1,3	1,9	3,6	9,3	1,2	1,6									
At1g51800	2,9	3,0	18,9	1,5	2,4	4,0	24,8	1,2	3,0									
At1g51850	4,1	4,3	130,1	2,9	2,2	8,7	263,8	1,1	8,3									
At1g53430	3,6	1,0	3,6	1,1	2,3	1,2	2,5	1,0	1,3	-1,1	1,7	2,8	-1,1	1,3	-2,0			
At1g53440	1,5	1,7	1,2	1,4	1,0	1,6	-1,1	1,1	-1,2									
At1g56120	2,7	3,4	6,7	2,6	2,7	4,3	5,0	1,3	1,4									
At1g69270	2,9	2,2	2,7	1,9	1,6	2,3	1,6	1,3	-1,2									
At1g73080	4,0	1,5	2,5	1,8	1,8	1,7	1,7	1,2	1,1									
At1g74360	4,5	3,3	14,4	1,8	5,3	3,3	4,3	1,3	1,4									
At2g02220	2,7	3,5	10,9	1,9	3,1	4,0	7,6	1,2	1,2							2,9	1,2	-1,3
At2g13790	4,2	-1,3	1,3	-1,2	1,2	-1,4	1,1	-1,4	1,1							8,4	1,8	-1,2
At2g31880	11,1	1,2	3,1	1,8	1,3	1,3	2,7	-1,1	-1,1							3,2	1,5	-1,5
At3g02130	2,5	-1,2	-1,2	1,0	-1,3	-1,4	-1,2	-1,1	-1,2									
At3g02880	1,7	1,3	3,8	1,0	1,6	1,5	3,5	1,1	1,4	-1,3	1,9	1,6	1,2	-1,3	-1,7			
At3g14840	1,6	1,7	1,5	1,2	1,5	1,7	-1,1	1,1	1,0									
At3g28450	3,6	1,2	2,5	1,3	1,7	1,4	1,7	1,1	1,0	1,5	3,2	2,4	1,9	2,0	-1,4			
At3g47570	2,9	1,0	1,5	1,0	1,0	1,4	1,3	1,0	-1,2									
At4g08850	3,7	1,8	4,4	1,3	1,8	2,1	4,1	1,0	1,2									
At4g33430	1,6	2,0	3,7	1,7	2,6	2,4	2,2	1,0	1,3							4,7	1,3	-1,2
At5g15730	2,5	1,6	3,5	1,5	2,2	1,7	2,7	1,0	1,1									
At5g20480	10,0	1,4	3,1	1,0	1,8	1,4	2,3	1,1	1,1									
At5g25930	6,5	1,4	8,8	1,3	5,2	1,8	4,1	1,0	1,2							17,7	6,6	-2,4
At5g46330	3,5	2,0	1,9	1,3	1,2	2,2	2,0	1,1	1,2									
At5g48380	5,1	1,7	3,2	1,6	1,3	2,1	2,1	1,2	1,1									

Fold changes

between 1.5 and 1.9
between 2.0 and 2.9
greater than 3.0

between -1.5 and -1.9
between -2.0 and -2.9
less than -3.0

Gene not present on the corresponding array.

Table 3. Gene expression of flg22-induced LRR-RLKs during bacterial infections

Source	Zipfel et al., 2004	NASC arrays-AtGenExpress: Pathogen infection (A)														
Array type	Affy 24k array	Affy 24k array														
Material	Seedlings	Infiltrated leaves														
Treatment	flg22	Pst DC3000	Pst Rpm1	Pst hrcC$^-$	Psp	$MgCl_2$	Pst DC3000	Pst Rpm1	Pst hrcC$^-$	Psp	$MgCl_2$	Pst DC3000	Pst Rpm1	Pst hrcC$^-$	Psp	$MgCl_2$
Concentration	10 µM	10^8 cfu/ml														
Duration	30 min	2h					6h					24h				
Gene																
At1g09970	1,5	1,1	-1,2	1,1	1,3	7,5	-1,1	1,4	1,4	1,2	5,1	3,6	2,5	3,8	3,0	1,1
At1g16670	3,5	1,4	1,7	1,4	2,0	1,3	-1,3	1,4	1,1	2,1	1,8	1,1	2,0	2,2	2,1	1,1
At1g17750	10,6	1,1	1,7	-1,3	1,5	1,4	1,0	2,3	1,1	1,6	1,9	2,3	2,5	1,5	1,5	-1,5
At1g34420	2,8	1,2	1,2	1,1	1,4	1,4	1,2	1,5	1,1	1,7	1,7	1,3	1,2	1,7	1,4	1,4
At1g51790	2,8	1,6	1,3	1,7	1,6	9,5	-1,5	-1,1	4,4	7,2	2,0	-2,9	1,4	6,2	5,8	1,4
At1g51800	2,9	1,9	1,3	1,6	1,8	24,3	-1,6	1,5	4,7	6,3	6,6	-1,2	2,8	11,2	8,9	2,8
At1g51850	4,1	4,9	4,2	6,4	5,0	216,0	1,0	-2,4	18,0	24,4	41,0	-2,4	-1,6	32,7	27,5	15,5
At1g53430	3,6	-1,1	1,0	1,1	1,3	2,3	-1,2	1,4	1,7	2,8	-1,2	-1,9	1,2	1,3	1,4	1,1
At1g53440	1,5	-1,4	1,0	1,1	-1,2	1,0	1,0	-1,5	1,5	1,2	-1,2	-2,9	-1,8	-1,1	-1,1	-1,3
At1g56120	2,7	2,3	2,2	2,6	2,9	2,6	-2,4	1,7	2,5	4,4	1,8	-1,2	2,2	3,9	3,3	1,4
At1g69270	2,9	1,0	1,5	1,2	1,3	1,1	-1,2	1,2	1,0	1,3	1,5	2,0	1,4	1,8	1,8	-1,1
At1g73080	4,0	1,1	1,2	1,0	1,9	3,4	-1,2	2,5	1,0	1,6	1,8	4,2	2,0	1,6	1,6	-1,4
At1g74360	4,5	1,6	2,0	1,5	3,1	6,2	-2,7	2,2	1,3	3,6	4,5	3,3	8,2	4,5	5,6	1,3
At2g02220	2,7	1,3	1,4	1,6	1,6	3,1	-1,7	1,5	2,4	4,8	1,4	1,2	1,5	4,0	3,8	1,2
At2g13790	4,2	1,1	1,0	1,0	1,1	1,0	1,1	1,1	-1,3	-1,1	-1,1	1,0	-1,1	1,2	1,0	-1,1
At2g31880	11,1	1,1	1,3	1,4	1,4	1,6	-1,8	1,1	1,4	1,8	1,7	1,3	2,6	3,2	2,8	1,1
At3g02130	2,5	-1,3	-1,2	-1,2	-1,3	-1,3	1,0	-1,2	1,1	-1,3	-1,4	-1,7	-2,2	-1,2	-1,3	-1,1
At3g02880	1,7	1,2	1,1	1,1	1,4	4,8	-1,6	1,2	1,0	1,8	2,4	1,6	2,3	1,7	2,2	-1,1
At3g14840	1,6	-1,3	1,0	1,3	-1,1	1,0	-1,4	-1,3	1,3	1,7	1,0	-2,8	-1,1	1,8	1,7	1,0
At3g28450	3,6	1,3	1,4	1,6	1,4	1,5	-1,5	1,2	1,3	2,2	1,4	1,2	1,5	1,8	1,7	1,1
At3g47570	2,9	-1,3	-1,1	-1,2	-1,3	1,0	-1,2	-1,4	1,1	1,2	1,2	-2,9	-1,4	1,0	-1,1	1,2
At4g08850	3,7	1,1	1,0	1,7	1,3	2,9	-3,1	-2,5	2,0	2,3	1,8	-2,0	1,2	3,1	3,2	1,3
At4g33430	1,6	1,8	1,9	2,1	2,2	2,1	-1,3	1,6	1,5	2,5	2,3	1,5	2,1	2,3	2,1	1,0
At5g15730	2,5	1,2	1,3	1,4	1,5	1,5	-1,6	1,2	1,4	2,4	1,7	1,3	1,6	1,7	2,0	1,1
At5g20480	10,0	-1,3	-1,3	1,1	1,0	6,1	-4,0	-1,4	1,4	1,7	2,4	-1,3	1,7	2,7	3,0	1,5
At5g25930	6,5	1,4	1,9	1,4	2,2	2,7	-1,8	4,6	1,4	2,9	1,7	2,4	2,8	1,8	2,0	1,0
At5g46330	3,5	1,1	1,0	1,6	1,4	3,8	-2,0	-1,7	1,6	1,2	1,7	-11,5	-2,6	1,1	-1,2	-1,1
At5g48380	5,1	1,1	1,0	1,2	1,3	2,1	-1,6	-1,1	-1,1	1,6	1,8	1,1	1,7	2,4	2,3	-1,1

Fold changes

between 1.5 and 1.9

between 2.0 and 2.9

greater than 3.0

between -1.5 and -1.9

between -2.0 and -2.9

less than -3.0

experimentpage.pl?experimentid=122). Interestingly, most of the flg22-induced LRR-RLKs were also induced after treatments with the bacterial PAMPs HrpZ, LPS, but also with the fungal and oomycete-derived PAMPs NPP1, chitin and xylanase (Table 2). The fact that fungal PAMPs induced the expression of the same set of LRR-RLKs as bacterial PAMPs do suggest that recognition of one PAMP not only triggers a response directed against one class of microorganisms, but rather induce a common set of responses against any invading microorganism. Therefore, our reverse-genetic approach could lead to the identification of receptors for bacterial, fungal, and oomycete PAMPs.

The data obtained from the AtGenExpress study (Table 2) was also a confirmation of our results obtained with flg22 treatment. However, differences could be observed that are likely due to the different concentrations, as well as the different plant materials used in these studies.

The set of flg22-induced LRR-RLKs was also induced during bacterial infections with virulent and avirulent *Pseudomonas syringae* pv *tomato* (*Pst*) (Table 3), consistently with the previous observation that bacteria-induced gene expression is overlapping with flg22-induced gene expression (Navarro et al., 2004). Interestingly, the virulent bacterial strain *Pst* DC3000 triggered a low gene induction 2 hours post-treatment, and this only for a few of the studied LRR-RLKs, whereas it suppressed LRR-RLK gene expression later (Table 3). In fact, it is believed that virulent bacteria secrete virulence factors, which suppress host basal defenses (Espinosa and Alfano, 2004). The down-regulation of LRR-RLK expression during *Pst* DC3000 infection suggest that these genes probably have an important biological function for the plant innate response.

In silico analysis of the T-DNA insertional lines from the SALK Institute (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al., 2003) revealed that potential insertion mutants for the 28 LRR-RLK genes were available in this collection. Three of these homozygous lines were kindly obtained from Prof. T. Nuernberger (University of Tuebingen, Germany). Lines for the remaining 25 genes were ordered and subjected to PCR-based genetic analysis (“genotyping”, see material and methods) to confirm the presence of the T-DNA in the gene of interest, and to identify homozygous plants for the mutation.

We have currently identified 22 homozygous lines out of the 28 flg22-induced LRR-RLKs (Table 4). For 6 genes, no homozygous mutants could be yet identified. This might be due to the fact that the potential insertional lines were in fact not “tagged” by a T-DNA, or that a homozygous mutation in the corresponding genes is lethal for the plant. However, additional potential insertional lines are available for these genes except for At5g25930 (Table 4, in grey, and http://signal.salk.edu/cgi-bin/tdnaexpress), and their future genetic analysis should allow the identification of the missing homozygous mutants, or confirm the possibility that a mutation in these genes is lethal. In addition, we should later check by RT-PCR, for example, if the presence of the T-DNA in the homozygous lines leads to a knock-out mutation in the tagged genes. This is likely the case for the insertions in exons. Southern-blot analyses of genomic DNA are also necessary to determine the number of existing T-DNA in the tagged lines.

Despite its preliminary stage, this collection already represents a powerful tool to decipher the involvement of LRR-RLKs in PAMP sensing in Arabidopsis. Flg22, and the newly discovered peptidic PAMP elf18 trigger growth inhibition of Arabidopsis seedlings (Gòmez-Gòmez et al., 1999; Kunze et al., 2004). This property was already used in a screen for flg22-insensitive mutants that led to the identification of the FLS2 receptor (Gòmez-Gòmez and Boller, 2000). FLS2 directly binds the flg22 ligand, but the possibility that this interaction, or the downstream signalling, require additional components, such as another RLK, is still an open possibility. Furthermore, the receptor for elf18 is different from FLS2, as *fls2* mutant plants still respond to elf18 (Kunze et al., 2004), and is still unknown. We therefore used the available collection to test if any of the LRR-RLKs would be required for the response to flg22 and/or elf18 using the seedling growth inhibition as bioassay (Table 5).

This initial analysis already revealed that lines mutated in the At5g20480 gene were completely insensitive to elf18 treatment, but still sensitive to flg22. In contrary, lines mutated in the At4g33430 gene were much less sensitive to flg22, but remained fully sensitive to elf18 in the growth inhibition assay. These two genes were therefore selected for further detailed analyses that are presented in the next two chapters.

Table 4. Flg22-induced LRR-RLK mutant lines

Gene	T-DNA line	Insertion site	Primer RP	Primer LP
At1g09970	SALK_056583*	+645 (exon 1)	ccaaactccagcggaatctca	cgacatggctccgtcgttaag
At1g16670	SALK_004253	+202 (exon 2)	tcctatgattcccctccacgc	gatcgcgtaaagccacatgct
At1g17750	SALK_098161	+409 (exon 1)	cctccctccaaggctgttgtt	gcgcgctctttttctctctca
At1g34420	SALK_030003			
	SALK_030002	+1881 (exon 1)	actcgcttgctggaaaaccct	cagcaaaacaagcttacgggaga
At1g51790	SALK_025181	+1087 (exon 2)	ttgaggcacacacacgtctcc	ttcgagaaacgagataccggg
At1g51800	SALK_137388	+2617 (intron 9)	acggttttgaacctcccgtta	caccattcgccatgaac
	SAIL_343_B11			
At1g51850	SALK_068036			
	SALK_068030			
	SALK_068022			
At1g53430	SALK_047602	+2757 (exon 9)	tgaaccgaaaggagggaaaaa	catcaatggaaggtccaattcc
At1g53440	SALK_130548	+4414 (exon 21)	aagccaatcctttcgcaatcc	tacgggcctctcatatccgct
At1g56120	SALK_004601			
	SALK_004593			
	SAIL_64_F08			
At1g69270	SALK_005054	3' UTR	gatggagccacaagggaaagg	tcacaactggtgtggctggaa
At1g73080	SALK_059281	+1192 (exon 1)	tgcattgggaaattgcagtagc	ccgtttaatgagttgaagccaaca
At1g74360	GABI_152F11			
	GABI_602G11			
At2g02220	SALK_008584			
	SALK_008585	+2338 (exon 1)	cggtttacacatatccaccggtc	cattggctgtggcgggtttgg
At2g13790	SALK_057955	+3877 (exon 10)	tcatcatcatttgcaagccga	cccattgaagctactttggctga
At2g31880	SALK_031580*	-376 (promoter)	gaaaccgggcttggatggatc	tccgacaacattccgatgacc
At3g02130	SALK_039514	+944 (exon 1)	actgggttacggaatttgcgg	cagacgacgggtcgctgtaag
At3g02880	SALK_040345			
	SALK_019840	+1027 (exon 1)	ggtccatcaagatcacaaactagca	cgtcgcttctcttcacgcttc
At3g14840	SALK_040386	+3721 (exon 17)	tgggtcttaagcagcctctcca	ttctcagctttctacggccttca
At3g28450	SALK_111475			
	SALK_111477	+7 (exon 1)	ccgggatttgacccgaaagccgg	ccttgcgacgaccgttcaacacg
At3g47570	SALK_150421			
	GABI_415H04			
	GABI_155E07			
At4g08850	SALK_061769			
	SALK_129546	-403 (promoter)	gtccgtgatagttgactatggg	gccatgttctgcttaggtgag
At4g33430	SALK_116202	+2418 (exon 9)	tgtctttgtctttgaaatgttattcaactg	ggcttcaaactcttcatccaacaaa
	SALK_034523*	+1020 (intron 4)	ctatttggcgacactactttctgac	ggtgcttcaaagttgggatg
At5g15730	SALK_103505	-49 (promoter)	tgccggatgcagatacagtga	ctttgcttacgctttcgcctg
At5g20480	SALK_044334	+1989 (exon 1)	gctgcagccacatatccagac	gggaagggtgccaacaacaggag
	SALK_044305			
	SALK_068675	+2460 (exon 1)	ggattgcttggccctgag	actagtagtctctcc
At5g25930	SALK_091274			
At5g46330	SALK_062054	+1046 (exon 1)	cggtgaaatgattcctcccaa	tgttgtccggtgatgttcctg
At5g48380	SALK_028071			
	SALK_008775	-475 (promoter)	ggcctgatccgcgtcaaccaagctcg	ttggtcaaaacaccaaaacc

	Homozygous
	Heterozygous
	Wild-type
	Not genotyped

* Homozygous seeds obtained from T. Nuernberger (University of Tuebingen).

The discovery of two genes those are likely to play an important role in the perception of two different PAMPs highlights the relevance of our collection. It is also a strong argument to perform additional functional tests on the collection in response to diverse purified PAMPs with different bioassays, such as ethylene production and/or oxidative burst. Additionally, as *fls2* mutants are more susceptible to bacterial infection, it would be also interesting to test the susceptibility of the mutants to different ranges of pathogens.

Table 5. Result of the initial growth inhibition screen

Gene	Homozygous line	Sensitivity to flg22	Sensitivity to elf18
At1g00970	SALK_056583	+	+
At1g16670	SALK_004253	+	+
At1g17750	SALK_098161	+	+
At1g34420	SALK_030002	+	+
At1g51790	SALK_025181	+	+
At1g51800			
At1g51850			
At1g53430	SALK_047602	+	+
At1g53440	SALK_130548	+	+
At1g56120			
At1g69270	SALK_005054	+	+
At1g73080	SALK_059281	+	+
At1g74360			
At2g02220	SALK_008585	+	+
At2g13790	SALK_057955	+	+
At2g31880	SALK_031580	+	+
At3g02130	SALK_039514	+	+
At3g02880	SALK_019840	+	+
At3g14840	SALK_040386	+	+
At3g28450	SALK_111477	+	+
At3g47570			
At4g08850	SALK_129546	+	+
At4g33430	SALK_116202	-	+
	SALK_034523	-	+
At5g15730	SALK_103505	+	+
At5g20480	SALK_044334	+	-
	SALK_068675	+	-
At5g25930			
At5g46330	SALK_062054	-	+
At5g48380	SALK_008775	+	+

Material and methods

Plant growth

Seeds of SALK lines were obtained from the Nottingham Arabidopsis Stock Center (NASC, http://arabidopsis.info/). Seeds were sown directly on soil, treated for 2 days at 4°C, and transfer to environment-controlled chambers at 21°C in short-day conditions (8h light/16h dark). Alternatively, seeds were first germinated 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, and plantlets then transferred to individual soil pots. Although, the T-DNA transgene present in the SALK lines harbours the *NptII* gene conferring Kanamycin resistance, no antibiotics were added to the medium, as silencing of the resistance gene has been reported (http://signal.salk.edu/tdna_FAQs.html).

Sequence analysis

The Flanking Sequence Tags (FSTs) for each line were retrieved through the SIGnAL T-DNAexpress webpage (http://signal.salk.edu/cgi-bin/tdnaexpress). These sequences were then searched against MIPS (http://mips.gsf.de/proj/thal/db/index.html) as well as TIGR (http://www.tigr.org/tdb/e2k1/ath1/index.shtml) Arabidopsis databases using a BLASTN program (Altschul et al., 1997) to determine the position of the putative insertion site.

The gene specific primers used for the genotyping were generated either by hand, or by using the SIGnAL iSect Toolbox (http://signal.salk.edu/isects.html).

The data presented in Table 1 are according to MIPS and TIGR Arabidopsis databases. The classification of the LRR-RLK subfamilies was done by Shiu and Bleecker (2001), and is similar to the one provided by the MIPS database.

Genomic DNA extraction

In initial experiments, plant genomic DNA was extracted using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Later a simpler and quicker procedure was used (Edwards et al., 1991). Small leaf pieces (about 0,5 cm^2) were frozen in liquid nitrogen, and immediately ground in 200 µl extraction buffer (200 mM Tris-HCl pH 7,5; 250 mM NaCl; 25 mM

EDTA; 0,5% SDS). After vigorous vortexing, the solution was centrifuged 5 min at 13000 rpm at room temperature. DNA from 150 μl of the supernatant was precipitated by adding 150 μl of isopropanol for 10 min on ice. After a 10 min centrifugation step at 13000 rpm, the supernatant was discarded and the pellet dried out using a Speed-Vac, and resuspended in 50 μl of water. All DNA samples were kept at -20°C.

Genetic analysis

To confirm the presence of a T-DNA transgene in the gene of interest, PCR analysis was performed on DNA extracted from individual plants. Two PCR reactions were performed: (i) one with two gene-specific primers (referred as RP and LP) (ii) one with a T-DNA-specific primer (referred as LBb1; present on the left-border of the T-DNA) and the RP gene-specific primer. The sequence of LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3') is specific for the binary vector used to generate the SALK lines (*pBIN-pROK2*). DNA from wild-type plants should only lead to one PCR product with RP+LP primers. DNA from heterozygous plants should lead to one PCR product with RP+LP primers and one product with LBb1+RP primers, whereas DNA from homozygous plants should only lead to one PCR product with LBb1+RP primers.
PCR was performed in the following conditions: 3 min, 94°C (1 cycle); [15 s, 94°C; 30 s, 65°C; 2 min, 72°C (2 cycles); 15 s, 94°C; 30 s, 63°C; 2 min, 72°C (1 cycle); 15 s, 94°C; 30 s, 62°C; 2 min, 72°C (2 cycles); 15 s, 94°C; 30 s, 60°C; 2 min, 72°C (1 cycle); 15 s, 94°C; 30 s, 59°C; 2 min, 72°C (2 cycles); 15 s, 94°C; 30 s, 57°C; 2 min, 72°C (1 cycle)] x 9 cycles; 15 s, 94°C; 30 s, 56°C; 2 min, 72°C (2 cycles); 15 s, 94°C; 30 s, 55°C; 2 min, 72°C (40 cycles); 5 min, 72°C (1 cycle). PCR products were then separated on a 1% agarose-TAE gel containing EtBr and visualized using a ultra-violet light table.

Growth inhibition assay

The growth inhibition assay was performed as described in Gomez-Gomez et al. (1999), except that the peptide concentration was here 1 μM, and that peptides were added in the liquid MS10 medium immediately after the seedling transfer.

Chapter 3.2

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

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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 signalling 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 et al., submitted). 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). Interestingly, 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 et al., submitted). 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 *efr-1*, for *EF-Tu response-1* (Fig. 1A and B). The growth inhibition triggered by flg22 treatment was identical in wild-type and in *efr-1* seedlings, suggesting that *efr-1* plants were specifically affected in EF-Tu responses (Fig. 1B).

To further characterize the *efr-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 *efr-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 *efr-1* mutant plants (Fig. 1E). The *efr-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, *efr-2* (*SALK_068675*), was isolated and proved as insensitive towards treatment with elf18 as *efr-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 *efr-1 and efr-2* mutants are impaired in all responses triggered by elf18, but not by flg22, show that EFR is specifically required for EF-Tu responses.

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 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 characteristics 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 et al., submitted). 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 labelled 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 correspond 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., submitted).

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 render 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 will 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 μg/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 LRR-RLKs (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 defence 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 μmol 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-µl 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 γ-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 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).

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 μM 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 μM 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.

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 μM 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 μM 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 μM 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 μM 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 μM 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).

Table 1 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 μl), *Xanthomonas campestris* pv *campestris* (*Xcc*, 10 μl), *Ralstonia solanacearum* GMI1000 (*Rs*, the asterisk indicates that the extract was boiled, 10 μl), *Pseudomonas syringae* pv *syringae* (*Pss*, 200 μg/ml), LPS from Pst DC3000 (100 μg/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.

Figure 1

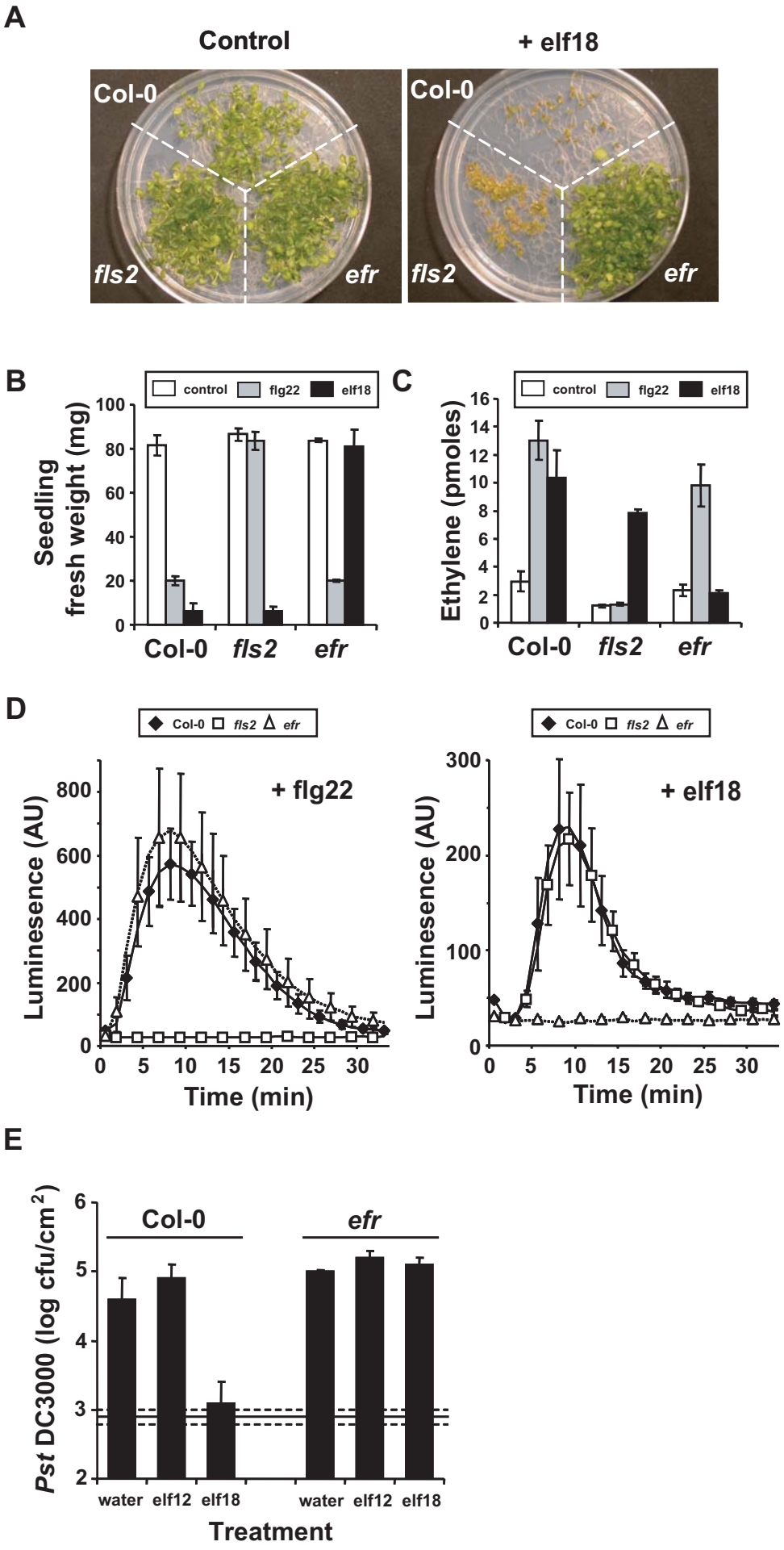
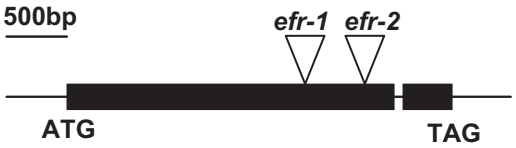
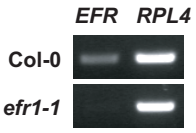

A
Control
+ elf18
Col-0
fls2
efr
B
control
flg22
elf18
Seedling fresh weight (mg)
Col-0
fls2
efr
C
Ethylene (pmoles)
D
Col-0
fls2
efr
+ flg22
+ elf18
Luminesence (AU)
Time (min)
E
Pst DC3000 (log cfu/cm2)
water
elf12
elf18
Treatment

Figure 2

A



B



C

a. MKLSFSLVFNALTLLLQVCIFAQA

b.
RFSNETDMQALLEFKSQVSENNKRE
VLASWNHSSPFCNWIGVTCGRRRER
VISLNLGGFKLTGVISPSIGN

c.
LSFLRLLNLADNSFGSTIPQKVGR
LFRLQYLNMSYNLLEGRIPSSLSN
CRLSTVDLSSNHLGHGVPSELGS
LSKLAILDLSKNNLTGNFPAS N
LTSLQKLDFAYNQMRGEIPDEVAR
LTQMVFFQIALNSFSGGFPPALYN
ISSLESLSLADNSFSGNLRADFGYL
LPNLRRLLLGTNQFTGAIPKTLAN
ISSLERFDISSNYLSGSIPLSFGK
LRNLWWLGIRNNSLGNNSSSGLEFIGAVAN
CTQLEYLDVGYNRLGGELPASIANL
STTLTSLFLGQNLISGTIPHDIGN
LVSLQELSLETNMLSGELPVSFGK
LLNLQVVDLYSNAISGEIPSYFGN
MTRLQKLHLNSNSFHGRIPQSLGR
CRYLLDLWMDTNRLNGTIPQEILQ
IPSLAYIDLSNNFLTGHFPEEVGK
LELLVGLGASYNKLSGKMPQAIGG
CLSMEFLFMQGNSFDGAIPDISR
LVSLKNVDFSNNNLSGRIPRYLAS
LPSLRNLNLSMNKFEGRVPTTGVF

LxxLxxLxLxxNxLxGxIPxxLGx

d.
RNATAVSVFGNTNICGGVREMQLKPC
IVQASPRKRKPLSVRKK

e. VVSGICIGIASLLLIIIVASLCW

f FMKRKKK

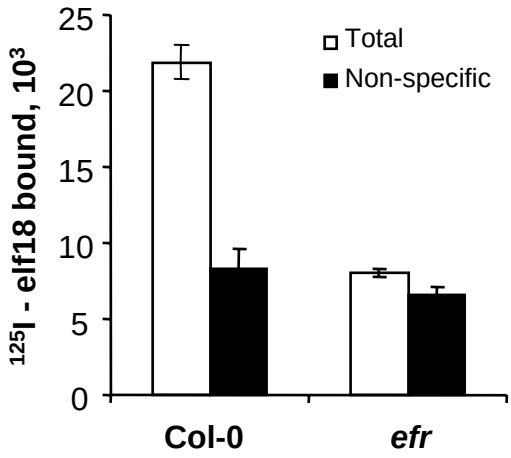
g. NNASDGNPSDSTTLGMFHEKVSYEELHSATSR

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

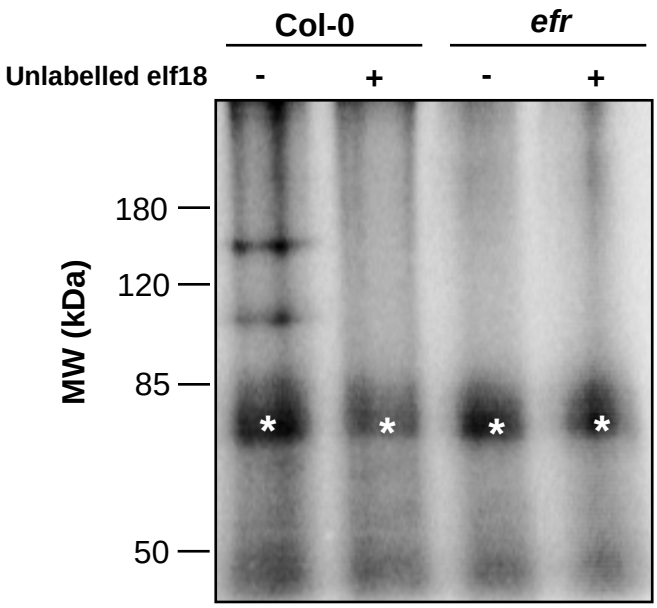
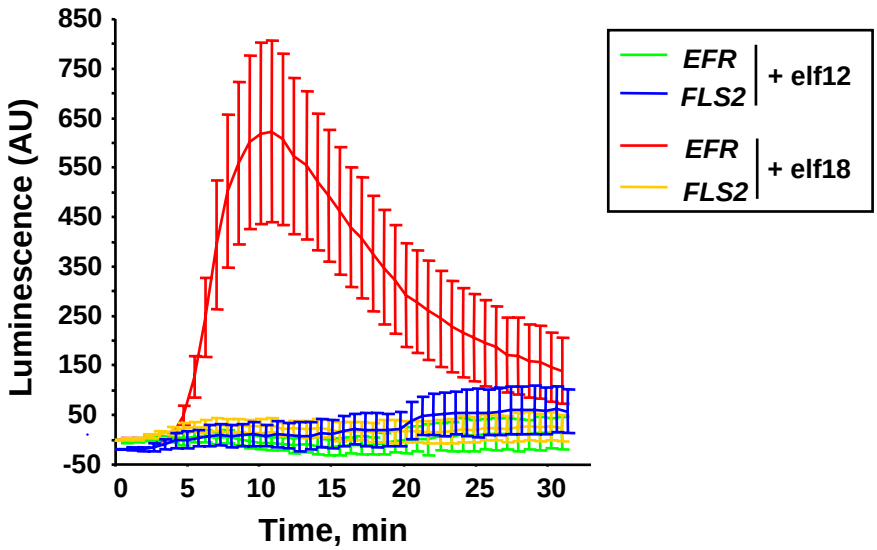

Col-0
efr
Unlabelled elf18
-
+
-
+
180
120
85
50
MW (kDa)
*
*
*
*

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

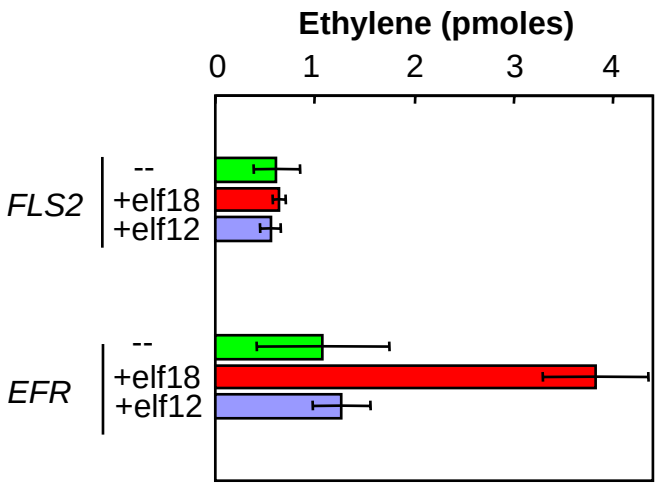

Ethylene (pmoles)
0
1
2
3
4
FLS2
--
+elf18
+elf12
EFR
--
+elf18
+elf12

Figure 5

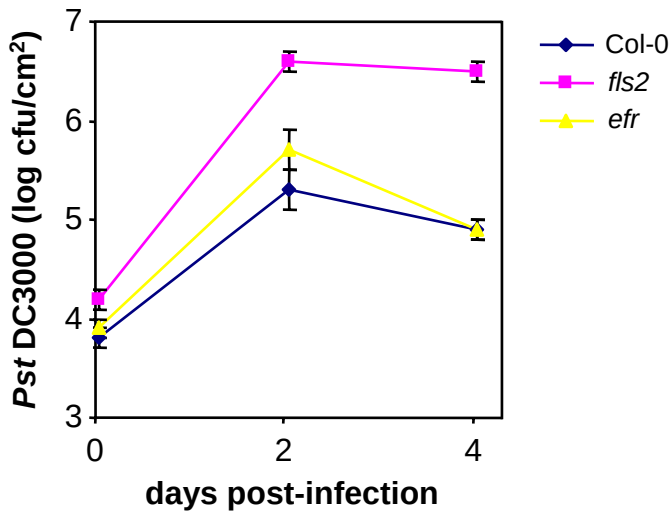
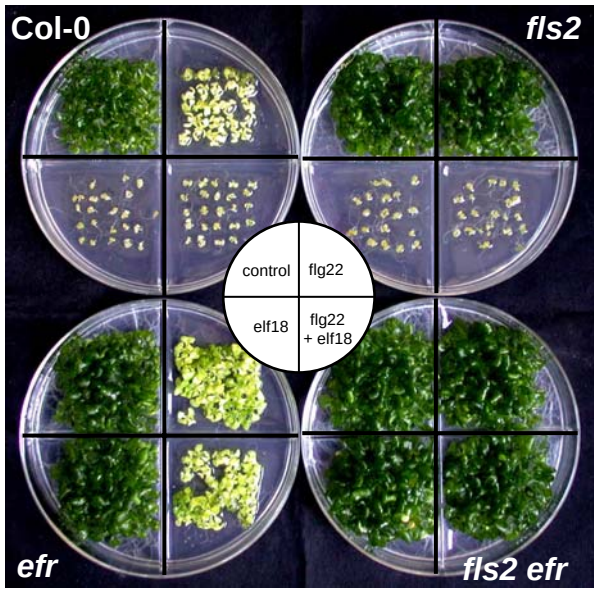

7
6
5
4
3
0
2
4
Pst DC3000 (log cfu/cm²)
days post-infection
Col-0
fls2
efr

Figure 6


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

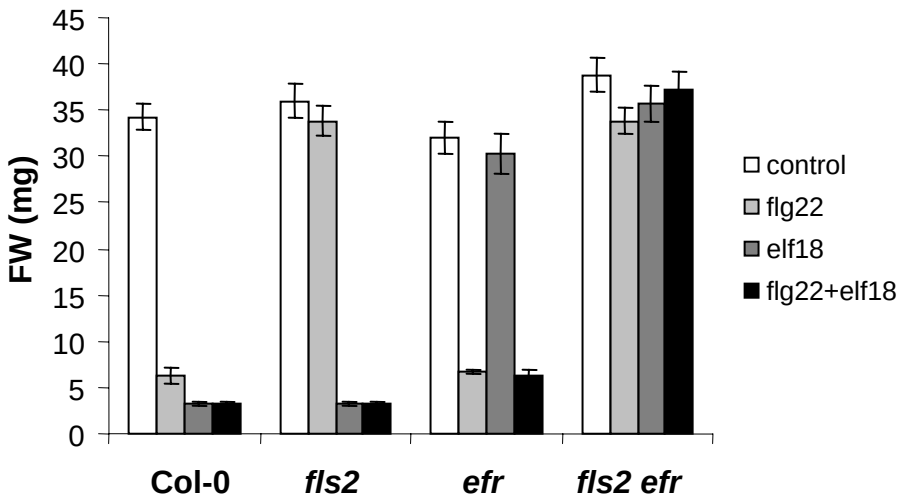
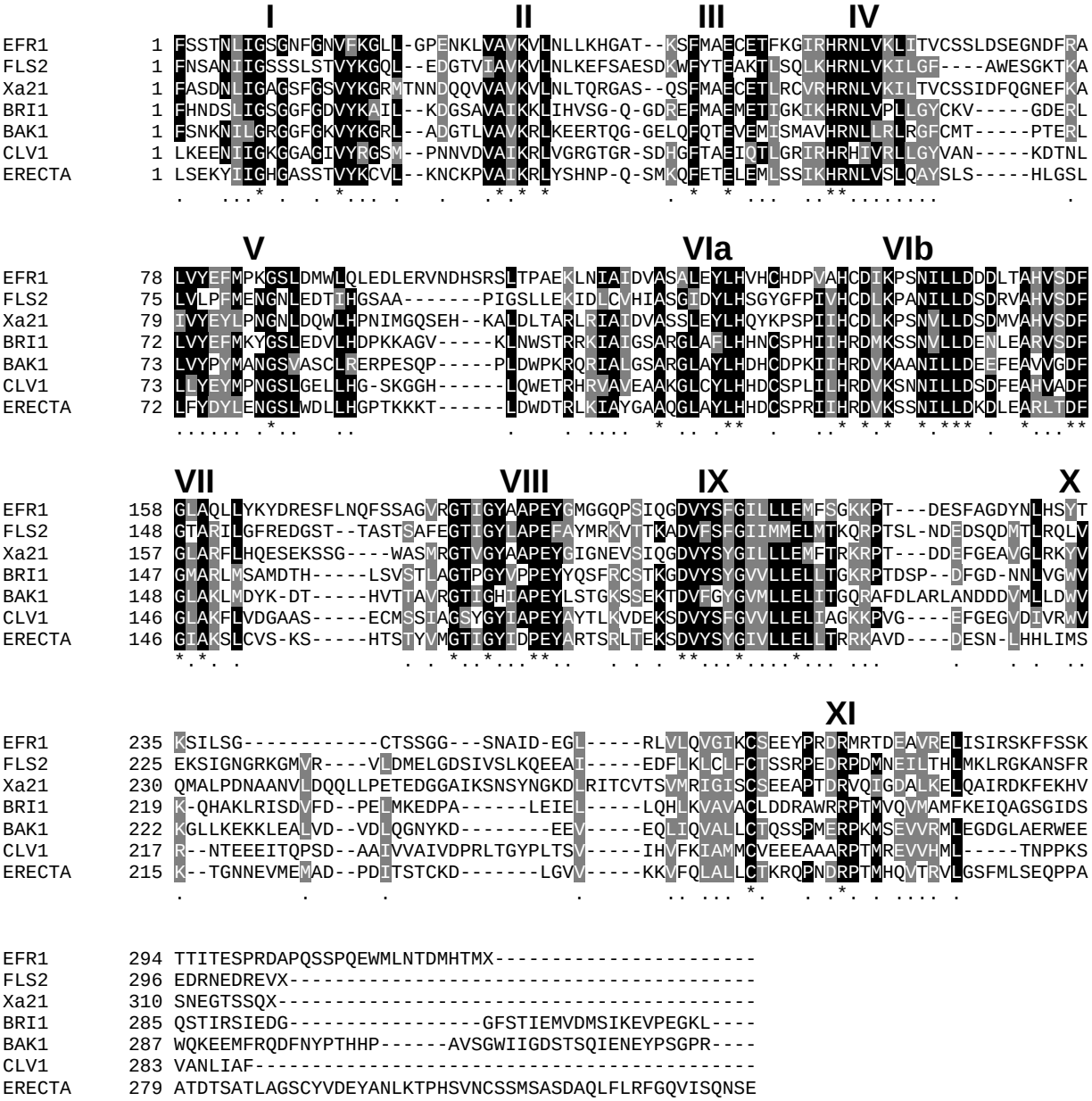

45
40
35
30
25
20
15
10
5
0
FW (mg)
Col-0
fls2
efr
fls2 efr
control
flg22
elf18
flg22+elf18

Table 1

Treatment	Ethylene production (pmoles/ml air)
Control	1.7 ± 0.4
Agrobacterium tumefaciens	7.3 ± 2.3
Agrobacterium tumefaciens (boiled)	4.7 ± 1.0
Xanthomonas campestris pv *campestris*	29.7 ± 4.1
Ralstonia solanacearum (boiled)	2.9 ± 0.3
Pseudomonas syringae syringae	5.8 ± 1.3
LPS	2.9 ± 0.0
HrpZ	2.2 ± 0.1

Figure S1


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

Chapter 3.3

The BRI1-associated receptor kinase 1 (BAK1) as a regulator of innate immune signaling

Cyril Zipfel, Delphine Chinchilla, Anne Caniard, Georg Felix, and Thomas Boller

Abstract

Flagellin and EF-Tu are conserved bacterial proteins that activated innate immune responses in Arabidopsis. Perception of these pathogen-associated molecular patterns (PAMPs) involves the two LRR receptor kinases FLS2 and EFR. In this chapter, we suggest that the LRR receptor kinase BAK1 protein, previously known to be involved in brassinosteroid response, may also play a role in flagellin and, to a lesser extent, EF-Tu signaling. In the process of screening a mutant collection of flagellin-induced LRR receptor-like kinases, we found that plants mutated in the *BAK1* gene were less sensitive to flagellin. Despite, its apparent initial wild-type sensitivity to EF-Tu, a detailed analysis of EF-Tu responses indeed revealed a weak effect of the *bak1* mutation. *Bak1* mutants exhibited normal binding activity to flg22, the elicitor-active epitope of flagellin, suggesting a role of BAK1 in signaling. In view of these preliminary results, we propose that BAK1 is a general regulator of LRR-RLK-dependent signaling in Arabidopsis.

Introduction

Detection of microbial pathogens by the innate immune system of animals and plants relies on an array of receptors that recognize conserved molecular patterns by large groups of microorganisms. These pathogen-associated molecular patterns (PAMPs) play key roles as activators of the innate immune response in animals (Janeway, Jr. and Medzhitov, 2002; Akira and Takeda, 2004; Philpott and Girardin, 2004) and, analogously, as “general elicitors” of defense response in plants (Nurnberger et al., 2004). Bacterial PAMPs recognized by Arabidopsis include flagellin, the main building block of the flagellum (Felix et al., 1999), the cell-wall component lipopolysaccharide (Zeidler et al., 2004) and the elongation factor EF-Tu (Kunze et al., 2004). The elicitor-active epitope of flagellin for many plants was identified as the 22-amino-acid peptide, flg22, representing a single stretch of the most conserved part in the N-terminus of the protein (Felix et al., 1999). Similarly, the N-acetylated first 18-amino-acid residues of the EF-Tu protein, corresponding to the elf18 peptide, are sufficient to induce defense reactions in plants belonging to the *Brassicaceae* family (Kunze et al., 2004).

Flg22 and elf18 perceptions lead to rapid ion fluxes, induction of ethylene biosynthesis, generation of reactive oxygen species in an oxidative burst, and changes in gene expression (Felix et al., 1999; Gòmez-Gòmez et al., 1999; Zipfel et al., 2004; Kunze et al., 2004). In addition, perception of flg22 activates a cytoplasmic MAP kinase pathway composed of MEKK1, MKK4/5 and MPK3/6 and is likely to involve WRKY transcription factors in Arabidopsis (Asai et al., 2002; Nühse et al., 2000). Phosphoproteomic approaches also revealed that flg22 treatment is leading to the rapid phosphorylation of many membrane-bound or cytoplasmic proteins, including the ankyrin-repeat AtPhos43 and the syntaxin AtSyp122 proteins (Peck et al., 2001; Nuhse et al., 2003; Nuhse et al., 2004).

A forward genetic screen for mutants affected in flg22 response in Arabidopsis resulted in the identification of the *FLS2* gene (*flagellin sensing 2*) encoding a transmembrane leucine-rich repeat (LRR) receptor kinase (Gòmez-Gòmez and Boller, 2000). Plants carrying mutations in the *FLS2* gene lack responses to flagellin and show impaired

binding to flg22 suggesting that FLS2 is a critical component for flagellin perception and signaling in Arabidopsis. Indeed, recent biochemical studies using cross-linking with radio-labelled flg22 and immuno-precipitation showed that flg22 interacts directly with FLS2, demonstrating that FLS2 is the *bona-fide* flg22 receptor in Arabidopsis (Chinchilla et al., submitted). We previously found that among flg22-rapidly induced genes, they were many genes encoding LRR-RLKs, including *FLS2* (Zipfel et al., 2004). We suggested that some of the proteins might be as well involved in PAMP perception. To test this hypothesis we generated a collection of insertional mutants for the induced LRR-RLKs. Our assumption was indeed confirmed with the discovery that a LRR receptor kinase, named EFR for EF-Tu receptor, is required for EF-Tu responses, and directly binds to elf18 (Zipfel et al., in preparation). Interestingly, FLS2 and EFR both belong to the LRRXII subfamily of LRR-RLKs. Despite the identification of their receptors, the mechanisms by which the extracellular flg22 and elf18 signals are transmitted into intracellular signaling events are unknown.

In this chapter, we present preliminary evidences that the LRR receptor kinase BAK1, previously known to be involved in brassinosteroid signaling, may be also involved in flg22, and, to a lesser extent, elf18 responses. A combination of physiological and biochemical analyses show that plants mutated in the *BAK1* gene are impaired in certain responses, but not in binding, to flg22. Plants mutated in *BAK1* also seem to have a reduced sensitivity to elf18 treatment.

Results

The growth of *bak1* mutant seedlings is not inhibited by flg22 treatment

In the course of the screen designed to isolate elf18-insensitive mutants, we also tested our collection of LRR–RLK mutants for their flg22 responsiveness. We found that two lines, *SALK_034523* and *SALK_116202*, were almost insensitive to treatment with 1 μM flg22 in the seedling growth inhibition assay (Fig. 1A). Interestingly, the effect of 1 μM elf18 treatment on seedling growth was similar in these lines compared to wild-type (Fig. 1A), suggesting that the mutations only impaired flg22 responses. These two lines harbour T-DNA insertions in the same gene, At4g33430

(Fig. 1 B). This gene has been previously described as a *bri1* (*brassinosteroid insensitive 1*) suppressor by an activation-tagging genetic screen (Li et al., 2002) and as a BRI1-interacting protein by a yeast two-hybrid screen (Nam and Li, 2002), and was therefore named BAK1 (BRI1-associated receptor kinase 1). We consequently renamed the allelic lines *SALK_034523* and *SALK_116202* as *bak1-101* and *bak1-102*, respectively (Fig. 1 B).

A role of BAK1 in Arabidopsis brassinosteroid (BR) responses was revealed by both gain-of-function and loss-of-function genetic studies (Li et al., 2002; Nam and Li, 2002). We were therefore wondering if the observed flg22 insensitivity of *bak1* mutants could be due to their reduced BR sensitivity. BR is known to regulate plant growth (Sasse, 2003). We measured the weight of *bak1* seedlings after a one week treatment with increasing concentrations of brassinolide (BL), the most active BR. Interestingly, we found that growth of *bak1* seedlings increased significantly in the presence of 100 nM and 1 μM BL, and this similarly as wild-type seedlings (Fig. 2A). A slight difference between *bak1* and wild-type seedlings would be observed at 10 nM, concentration that triggered a weak increase of wild-type, but not *bak1*, seedling fresh weight (Fig. 2A). An effect of 100 nM BL on seedling growth was also observed in the presence of 1 μM flg22 in both *bak1* and wild-type seedlings (Fig. 2B), suggesting that the flg22 insensitivity of *bak1* mutants is not due to reduced BR responses in seedlings.

bak1 mutants are probably impaired in both flg22 and elf18 responses

In the preliminary stages of their characterization, we found that *bak1* mutant lines were insensitive to flg22, but not to elf18, treatment in a seedling growth inhibition assay (Fig. 1A). In comparison to flg22, the inhibition of seedling growth provoked by elf18 is much more severe (Kunze et al., in preparation). Whereas 1 nM or 10 nM of flg22 hardly trigger any effect on Arabidopsis wild-type seedlings (Fig. 3A), similar concentrations of elf18 already reduce their growth of about 20 and 40%, respectively (Fig. 3B). The previous observation that *bak1* seedlings are not impaired in elf18 response could be due to a concentration effect, as a high concentration (1 μM) of elf 18 was initially used (Fig. 1A). Indeed, a dose-response experiment revealed that the growth of *bak1* seedlings was less affected by low doses (1-100 nM) of elf18, than wild-type seedlings (Fig. 3B), revealing a slight effect of the *bak1* mutation on elf18

responses. However, at the micromolar range, the effect of elf18 on *bak1* and wild-type seedlings was identical (Fig. 3B). The *bak1-102* allele appears to be more sensitive to elf18 that *bak1-101* (Fig. 3B). Future work should address whether the two alleles are impaired to a similar extent in *BAK1* expression. Noteworthy, no difference between bak1-101 and bak1-102 was noted in regard of their flg22 sensitivity. Consistently with Figure 1A, the growth of *bak1* seedlings was not affected for any of the flg22 concentrations tested (Fig. 3A). It should be noted however that a slight effect of the flg22 treatment could be observed on *bak1* seedlings at 10 μM (Fig. 3A).

Similarly to flg22, elf18 treatment induces numerous defense-related responses such as a rapid production of reactive oxygen species in an oxidative burst, and an increase in the production of the stress hormone ethylene (Kunze et al., 2004). To test whether BAK1 is only required for the seedling growth inhibition, or if it plays a more general role in signaling, we measured the oxidative burst generated by flg22 and elf18 in *bak1* leaf pieces. The oxidative burst triggered by both flg22 and elf18 treatments were strongly reduced in *bak1* compared to wild-type leaf pieces (Fig. 4A and B). Ethylene production in response to elf18 and flg22 might be slightly reduced in *bak1* leaves (data not shown), but these results require repetitions to be confirmed.

These results showed that *bak1* mutants are impaired in flg22-, and to a lesser extent, elf18-triggered seedling growth inhibition and oxidative burst. This suggests that BAK1 is a positive regulator of flg22 and elf18 responses.

BAK1 is not required for flg22 perception

Flg22 insensitivity could be explained by a defect in flg22 perception or in downstream signaling. To test whether BAK1 is required for flg22 perception and could act as co-receptor for FLS2, we performed binding assays using radiolabeled-flg22 in *bak1* plant extracts. The radiolabeled derivative of flg22, ^{125}I-flg22, bound specifically to wild-type plant extracts, and to a similar extent to extracts from *bak1* mutant plants (Fig. 3A), suggesting that BAK1 is not essential for flg22 binding. Another possibility could be that *bak1* mutants still exhibit flg22-binding, but that the affinity of the binding sites to flg22 would be reduced. However, we found that wild-type, as well as *bak1* binding sites showed a similar high-affinity to flg22, with an EC_{50} of 30-40 nM (Fig. 3B).

In addition, binding activity in *bak1* exhibited the same specificity and affinity for binding of flagellin as wild-type seedlings (data not shown).
All together, our results demonstrated that BAK1 is not required for proper flg22 perception, but rather might be involved in signaling downstream of FLS2.

Discussion

In this chapter we described that plants mutated in the LRR receptor kinase BAK1 (BRI1-associated receptor kinase 1) are impaired in their response to the bacterial peptidic PAMPs flg22 and elf18.

Are brassinosteroids playing a role in flg22 and elf18 responses?

BAK1 was initially identified and characterized for its role in brassinosteroid (BR) responses in Arabidopsis. BRs are a unique class of plant polyhydroxysteroids that are structurally related to the animal steroid hormones and elicit a plethora of physiological responses in plants (Sasse, 2003). Animal steroids are principally recognized by members of the nuclear receptor superfamily of transcription factors. In plants, BR perception is occurring via LRR receptor kinases (BRI1, BRL1 and BRL2) localized to the plasma membrane (Li and Chory, 1997; Cano-Delgado et al., 2004; Zhou et al., 2004). Direct binding of BR to the extracellular domain of the BRI1 was recently demonstrated (Kinoshita et al., 2005). BAK1 was independently identified as a *bri1* suppressor by an activation-tagging genetic screen (Li et al., 2002) and as a BRI1-interacting protein by a yeast two-hybrid screen (Nam and Li, 2002), hence its name. BAK1 is a much smaller receptor kinase than BRI1; it contains just five LRR motifs and lacks the second cysteine pair and the 70 amino-acid island that are characteristic of BRI1 and its homologs. A role for BAK1 in BR signaling was revealed by both gain-of-function and loss-of-function genetic studies (Li et al., 2002; Nam and Li, 2002). Overexpression of *BAK1* suppressed two weak *bri1* mutations and gave rise to a *BRI1*-overexpression phenotype in a wild-type background. By contrast, two null *bak1* mutants had a semi-dwarf stature and reduced BR sensitivity.
A role of BR in plant defense has been recently proposed in tobacco and rice, as plants treated with brassinolide (BL) are slightly more resistant to a wide range of pathogens (Nakashita et al., 2003).

We could therefore wonder whether the *bak1* phenotype observed in our study could be due to a defect in BR signaling.

We found that the effect of BL on seedling weight was similar in *bak1* and wild-type seedlings, and this even in the presence of flg22. However, the use in previous studies of two common assays of BR sensitivity - root length inhibition and decreased expression of the BR biosynthetic *CPD* gene - demonstrated that *bak1* seedlings were less sensitive to BR compared to wild-type (Li et al., 2002; Nam and Li, 2002).

In order to confirm that flg22- and elf18-responses are not indirectly depending on BR-signaling, we should test in the future flg22- and elf18-sensitivity of other mutants that are impaired in BR responses or biosynthesis, such as *bri1*, *ttl* or *bas1* (Li and Chory, 1997; Nam and Li, 2004; Neff et al., 1999).

Is BAK1 important for flg22-binding?

The fact that BAK1 interacts with BRI1 *in vitro* and *in vivo*, and that they phosphorylate each other *in vitro* suggested that BRI1 and BAK1 might function as a heterodimer to mediate plant steroid signaling (Li et al., 2002; Nam and Li, 2002). The perception of flg22 and elf18 depends on the two LRR receptor kinases FLS2 and EFR (Gòmez-Gòmez and Boller, 2000; Chinchilla et al., submitted). Direct interaction of FLS2 and EFR with their ligands was proven, but it is not known whether proper binding to flg22 and elf18 requires other proteins.

Binding of ^{125}I-flg22 in *bak1* seedlings was not affected, showing that BAK1 is not required for proper flg22 binding to FLS2. Although not tested, it is also probably the case for elf18 binding to EFR. Our results are coherent with the recent finding that a *bak1* null mutant is not impaired in binding of ^{3}H-BL to BRI1 (Kinoshita et al., 2005).

All together, these results suggest that BAK1 might act as a signal transducer after flg22 recognition by FLS2, elf18 recognition by EFR, as well as BL recognition by BRI1. Though, it is still unknown whether BAK1 also interacts with FLS2 and/or EFR.

BAK1 is one general component of a specific perception system

Our findings suggest that BAK1 is not only involved in BR signaling, but also in flg22-, and to a lesser extent, elf18-signaling. However, the physiological responses triggered by flg22 and elf18 treatments are different from the ones triggered by BR.

Other components, that would bring signal output specificity, are therefore likely to be involved in the early signaling events. This was indeed already suggested in the initial BAK1 characterization, as two null *bak1* mutations gave rise to only a weak *bri1*-like phenotype (Li et al., 2002; Nam and Li, 2002). Similarly, we observed that *bak1* plants were affected in their response, but were not completely insensitive to flg22 and elf18. Given that Arabidopsis has 13 BAK1-like proteins, it is possible that some BAK1-like proteins could interact with BRI1, FLS2 or EFR to contribute signaling specificity.

We therefore propose that BAK1 would be a common component of BRI1, FLS2 and EFR early signaling complexes but these receptor kinases could recruit in addition to BAK1 other components that are likely to confer specificity of the output signal. A similar mechanism was proposed to explain the discovery that the tomato BRI1 and the systemin receptor SR160 would be the same protein (Scheer and Ryan, Jr., 2002; Montoya et al., 2002; Scheer and Ryan, Jr., 2002). BRI1 might heterodimerize with different RLKs to mediate BR and systemin perception and/or signaling in tomato (Li, 2003).

Studies of various mutant alleles, as well as expression of a truncated LRR receptor kinase, provided circumstantial evidence that the LRR receptor kinases CLV1 and ERECTA may also function as heterodimers (Dievart et al., 2003; Shpak et al., 2003). Association of ERECTA with different partners could explain why this receptor kinase is likely to be involved in controlling organ size and shape (Shpak et al., 2003), but also in disease resistance against the pathogenic bacteria *Ralstonia solanacearum* (Godiard et al., 2003).

As elf18 responses were less affected than flg22 responses by the *bak1* mutation, it is likely that BAK1 requirement is less important for EFR than for FLS2. In fact, ongoing work is trying to decipher the involvement of *BAK1*-like genes in EFR- and FLS2-dependent signaling by testing *bak1*-like mutant lines for their elf18- and flg22-sensitivity (Delphine Chinchilla, unpublished data).

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). Brassinolide was purchased from Sigma and prepared as a stock solution of 10 mM in ethanol.

Plant growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) were grown in single pots at 20-21 °C with 65% humidity under ~100 μmol 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 *BAK1* T-DNA insertion lines SALK_034523 (*bak1-101*) and SALK_116202 (*bak1-102*) 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'-CTATTTGGCGACACTACTTTCTGAC-3' and 5'-GGTGCTTCAAAGTTGGGATG-3', 5'-TGTCTTTGTCTTTGAAATGTTATTCAACTG-3' and 5'-GGCTTCAAACTCTTCATCCAACAAA-3', were used for *bak1-101* and *bak1-102*, 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 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).

Binding assays

Five hundred milligrams of liquid nitrogen-ground leaves were resuspended in 500 µl of extraction buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 3 mM $MgCl_2$) and centrifuged at 14.000 rpm for 30 min at 4°C. Supernatant was discarded and pellet (P1) resuspended in 500 µl binding buffer (25 mM MES pH 6.0, 10 mM NaCl, 3 mM $MgCl_2$) and used for binding experiments. Aliquots of P1 were incubated in binding buffer in a total volume of 100 µl with ^{125}I-Tyr-flg22 (60 fmol in standard assays; >2000 Ci/mmol) for 30 min either alone (total binding) or with an excess (10 µM) of flg22 used as competitor (non-specific binding). Extracts were collected by vacuum filtration on chromatography paper (Macherey-Nagel , 2.5-cm diameter, pre-incubated with 1% bovine serum albumin, 1% bactotrypton, and 1% bactopepton in binding buffer) and washed for 10 s with 10 ml of ice-cold binding buffer. Radioactivity retained on the filters was determined by γ-counting.

Figure legends

Figure 1 *bak1* mutants are impaired in flg22-triggered seedling growth inhibition.
(A) Quantitative measurement of seedlings growth inhibition. Five-day-old wild-type Col-0, *efr-1*, *fls2, SALK_034523 (S_034523)* and *SALK_116202 (S_116202)* 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).
(B) Schematic representation of the *BAK1* gene. Exons are represented are black boxes. The start ant stop codons are indicated. The sites of insertion of T-DNA in the mutants *SALK_034523* (*bak1-101*) and *SALK_116202* (*bak1-102*) are shown by open triangles.

Figure 2 *bak1* seedlings are still responsive to brassinosteroid.
(A) Effect of brassinosteroid treatment on seedling growth. Five-day-old wild-type Col-0, *bak1-101* and *bak1-102* seedlings were transferred from solid agar plates to liquid medium alone, or supplemented with brassinolide (BL) at indicated concentrations. Seedling fresh weight was measured one week after treatment. Results are averages ± standard errors (n=6).
(B) Effect of brassinosteroid and flg22 treatment on seedling growth. Five-day-old wild-type Col-0, *bak1-101* and *bak1-102* seedlings were transferred from solid agar plates to liquid medium alone or supplemented with 1 μM flg22 alone, or 1 μM flg22 and 100 nM BL. Seedling fresh weight was measured one week after treatment. Results are averages ± standard errors (n=6).

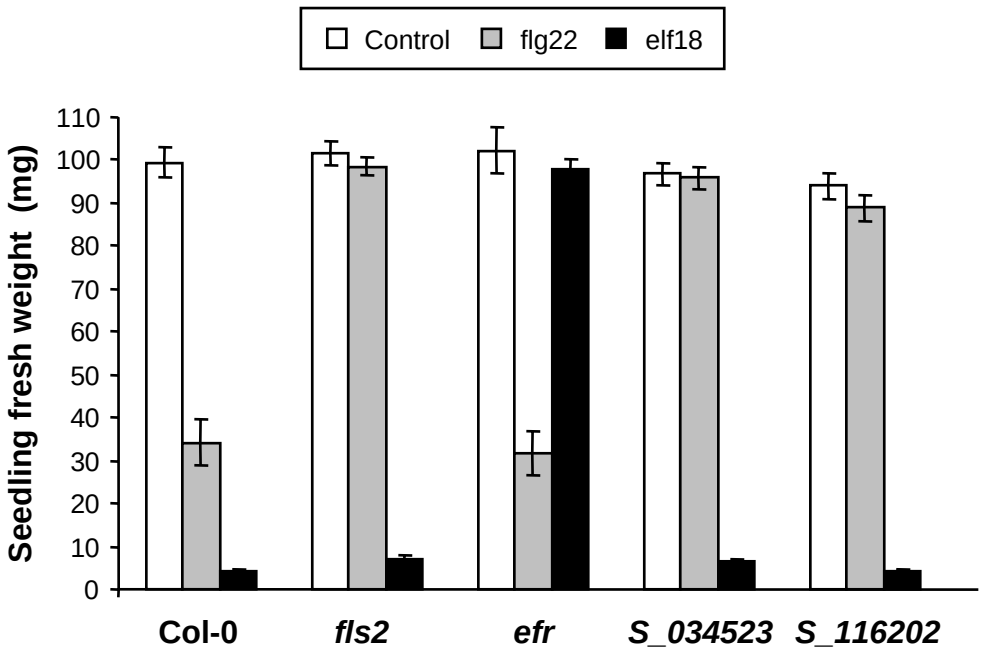
Figure 3 *bak1* mutants have a reduced sensitivity to flg22 and elf18.
Quantitative measurement of seedlings growth inhibition. Five-day-old wild-type Col-0, *bak1-101* and *bak1-102* seedlings were transferred from solid agar plates to liquid medium alone or supplemented with increasing amount of flg22 **(A)** or elf18 **(B)**. Seedling fresh weight was measured one week after treatment. Results are averages ± standard errors (n=6).

Figure 4 The oxidative burst triggered by flg22 and elf18 is also reduced in *bak1* mutants.
Oxidative burst in leaf tissues of wild-type Col-0 (right panel) and *bak-101* (left panel) plants. Luminescence of leaf slices in a solution with peroxidase and luminol was measured over the time after addition of flg22 **(A)** or elf18 **(B)** at indicated concentrations. Results are representative of 3 independent experiments.

Figure 5 Flg22-binding is not affected in *bak1.*
(A) Specific ^{125}I-flg22 binding is not impaired in *bak1* plants. Binding activity of wild-type Col-0 (filled circles) and *bak1-101* (opened circles) plant extracts was tested by adding ^{125}I-flg22 alone (total binding) or with 10 μM unlabeled flg22 as competitor (non-specific binding). Results are averages ± standard deviations (n=3).
(B) Affinity to flg22 is not altered in *bak1* plants. Radioligand binding was tested in the presence of increasing concentration of unlabeled flg22 required to reduce binding by 50% (IC_{50}).

Figure 1

A


Control
flg22
elf18
Seedling fresh weight (mg)
110
100
90
80
70
60
50
40
30
20
10
0
Col-0
fls2
efr
S_034523
S_116202

B

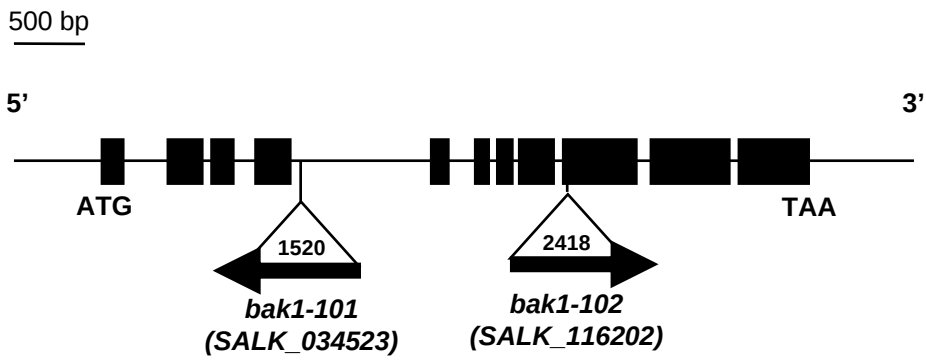

500 bp
5'
3'
ATG
TAA
1520
2418
bak1-101
(SALK_034523)
bak1-102
(SALK_116202)

Figure 2

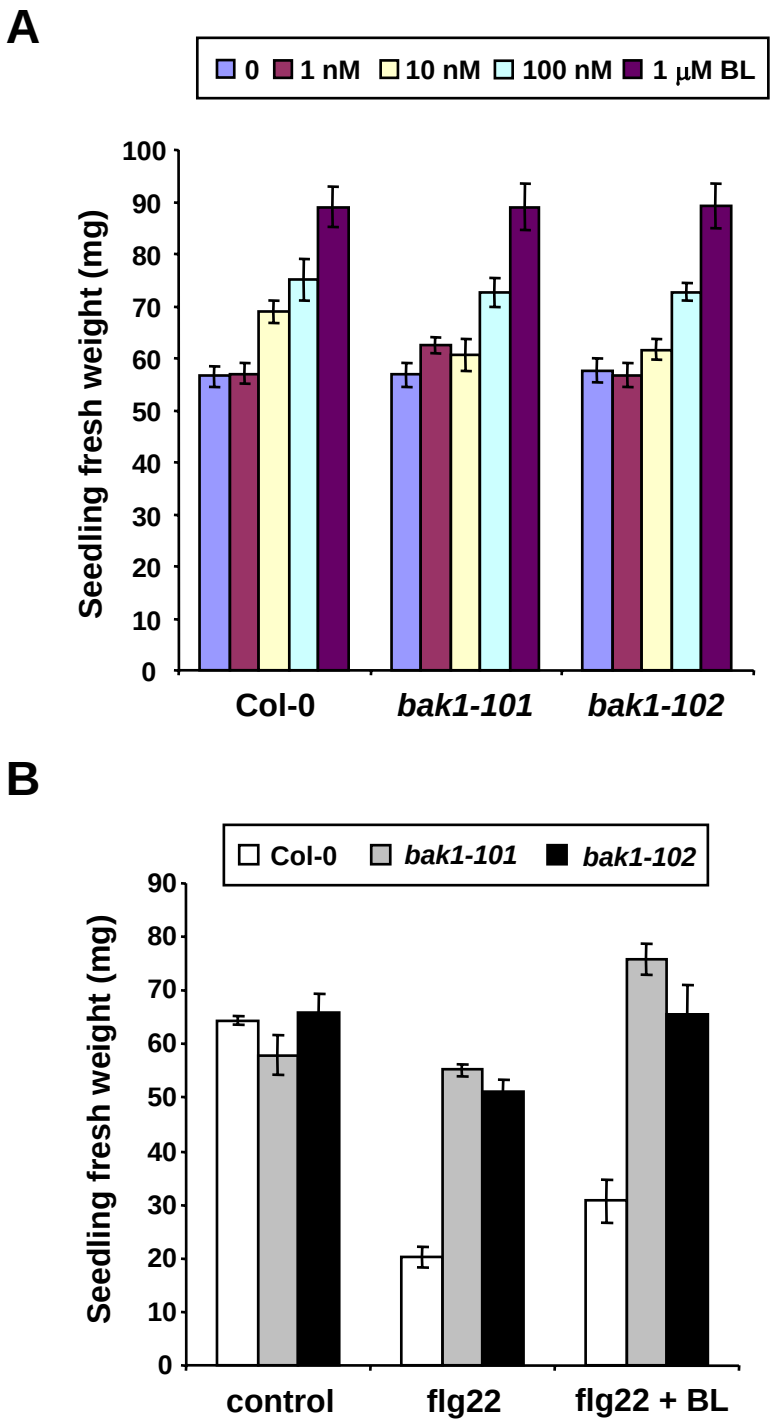

A
0
1 nM
10 nM
100 nM
1 μM BL
Seedling fresh weight (mg)
100
90
80
70
60
50
40
30
20
10
0
Col-0
bak1-101
bak1-102
B
Col-0
bak1-101
bak1-102
Seedling fresh weight (mg)
90
80
70
60
50
40
30
20
10
0
control
flg22
flg22 + BL

Figure 3

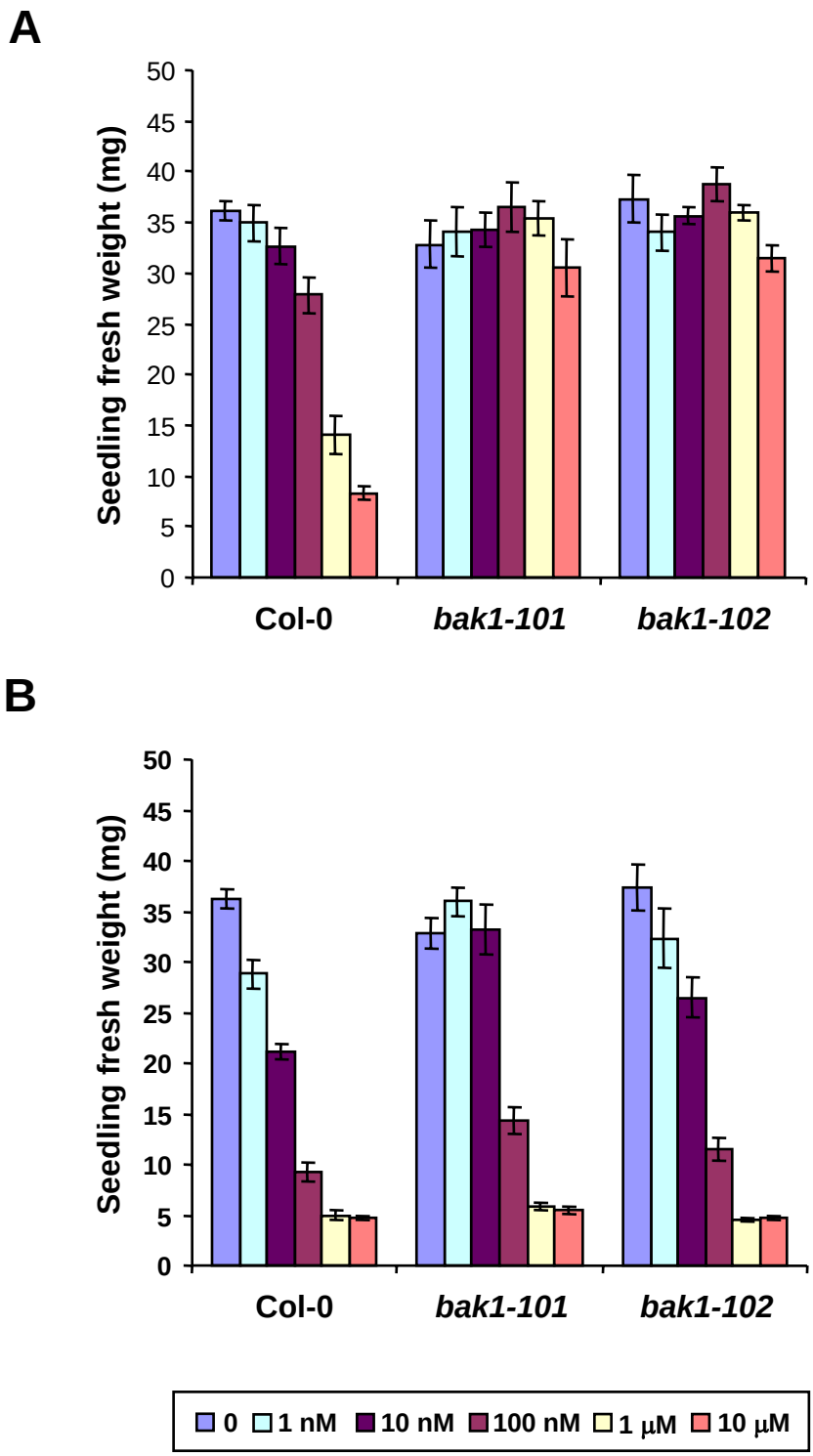
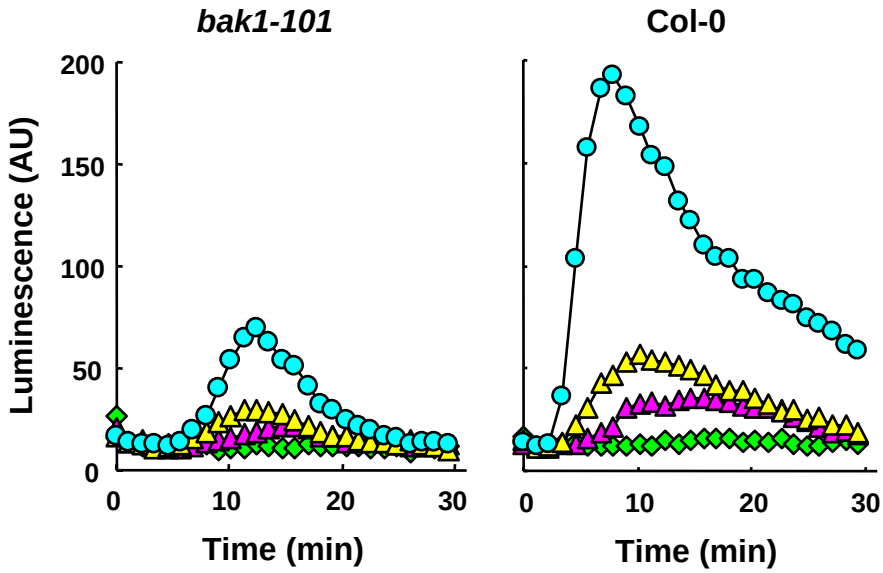
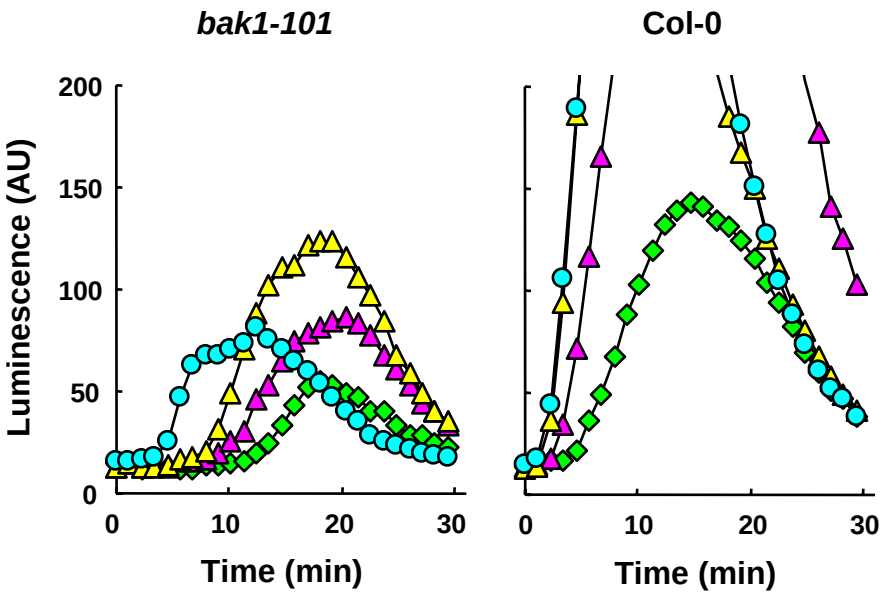

A
Seedling fresh weight (mg)
50
45
40
35
30
25
20
15
10
5
0
Col-0
bak1-101
bak1-102
B
Seedling fresh weight (mg)
50
45
40
35
30
25
20
15
10
5
0
Col-0
bak1-101
bak1-102
0
1 nM
10 nM
100 nM
1 μM
10 μM

Figure 4

A


bak1-101
Col-0
Luminescence (AU)
200
150
100
50
0
0
10
20
30
Time (min)

B


bak1-101
Col-0
Luminescence (AU)
200
150
100
50
0
0
10
20
30
Time (min)

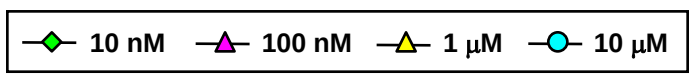
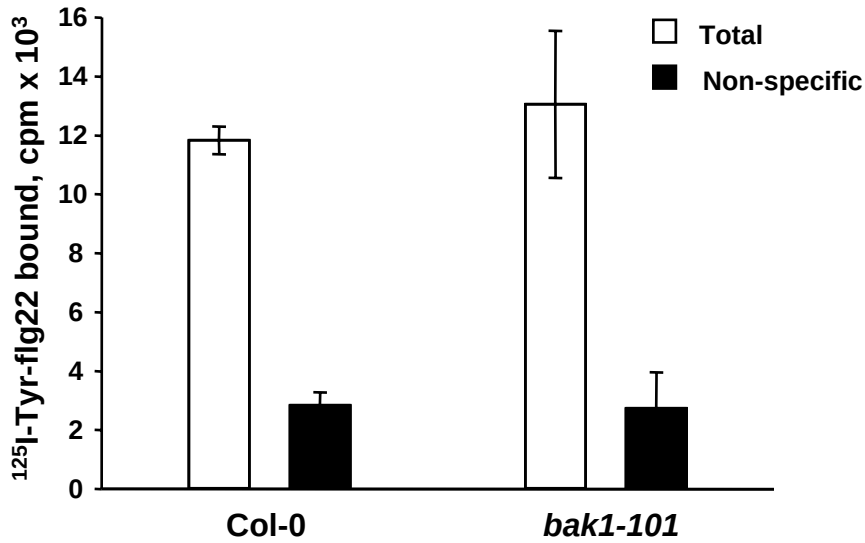
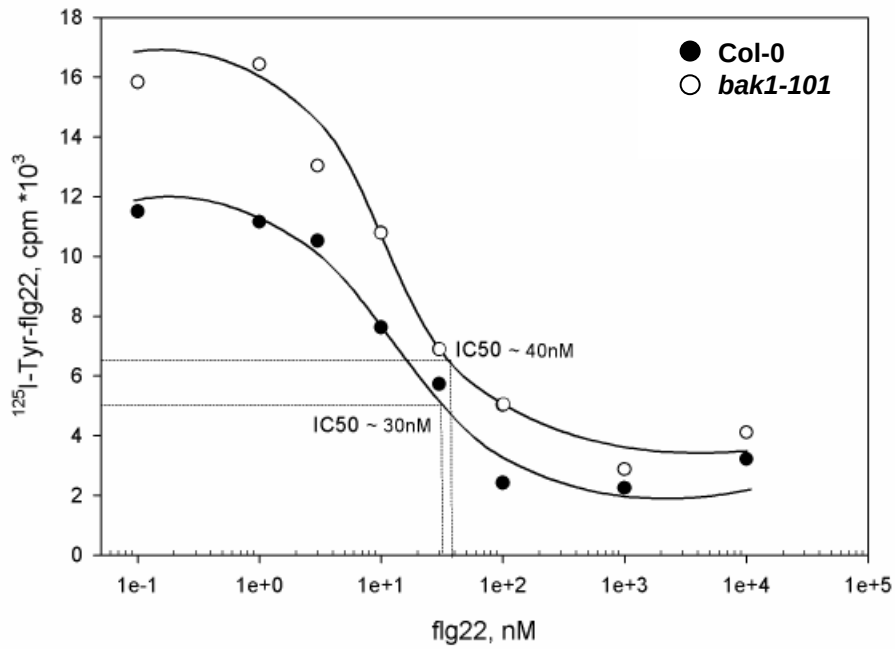

10 nM
100 nM
1 μM
10 μM

Figure 5

A


125I-Tyr-flg22 bound, cpm x 10^3
16
14
12
10
8
6
4
2
0
Total
Non-specific
Col-0
bak1-101

B


125I-Tyr-flg22, cpm *10^3
18
16
14
12
10
8
6
4
2
0
Col-0
bak1-101
IC50 ~ 40nM
IC50 ~ 30nM
1e-1
1e+0
1e+1
1e+2
1e+3
1e+4
1e+5
flg22, nM

Concluding remarks

The plant model *Arabidopsis thaliana* was used in this study to understand the basis of PAMP perception. Arabidopsis is a new player in the field of microbe sensing, and most of the knowledge accumulated on general elicitors since two decades mainly originated from studies on parsley, tobacco, tomato, soybean or rice. Interestingly, many of the known PAMPs are recognized in only a subset of plant species. For example, bacterial cold-shock protein (CSP) induces defense response at subnanomolar concentrations in tobacco and many other *Solanaceae*, but not in Arabidopsis (Felix and Boller, 2003). Similarly, Pep13, a peptide derived from the extracellular transglutaminase of oomycete pathogens, is active as a potent elicitor in parsley and potato but not in Arabidopsis (Brunner et al., 2002). During evolution specific lineages of plants have likely acquired distinct, diverse PAMP recognition capabilities, making difficult for a given microbe to avoid recognition in all plant species and thereby to become a “generalist” plant pathogen.

It is now clear that Arabidopsis plants respond to structures characteristic for bacteria, such as flagellin, LPS, harpins and EF-Tu (Felix et al., 1999; Zeidler et al., 2004; Dong et al., 1999; Kunze et al., 2004). With regards to fungi and oomycetes, Arabidopsis have evolved perception systems for chitin, xylanase, NPP1 and PaNie (Zhang et al., 2002; Molinier J. et al., 2005; Fellbrich et al., 2002; Veit et al., 2001).

On PAMP perception

Flagellin and LPS also act as PAMP in the innate immune system of mammals (Akira and Takeda, 2004). Mammalian innate immunity relies on structurally different transmembrane PRRs for detection of PAMPs (O'Neill, 2004; Fraser et al., 2004). The most prominent group of PRRs are the Toll-like receptors (TLRs), a family of a dozen transmembrane proteins with LRR ectodomains sensing bacteria, fungi, protozoa and viruses (O'Neill, 2004). Some TLRs of mammals may directly interact with their PAMP-ligands. The only proven example of direct recognition is TLR5, which binds flagellin (Hayashi et al., 2001; Smith, Jr. et al., 2003; Mizel et al., 2003). In Arabidopsis plants, flagellin perception also occurs via direct interaction with the transmembrane LRR receptor kinase FLS2 (Gòmez-Gòmez and Boller, 2000; Chinchilla et al., submitted).

Other than sharing the common feature of an extracellular LRR domain, there is no obvious sequence similarity between FLS2 and TLR5, suggesting a convergent evolution. Plants seem to have no clear homologs of TLRs but they have large gene-families of receptor-like kinases (Shiu and Bleecker, 2001; Shiu et al., 2004). Based on this observation and that the expression of many RLKs, including FLS2, was rapidly induced by flg22 treatment we postulated that some of these induced RLKs are involved in PAMP perception (Zipfel et al., 2004). Indeed, we identified by reverse-genetic the receptor for EF-Tu. The LRR receptor kinase EFR is required for EF-Tu responses and directly binds to the elf18 (Chapter 3.2). In addition, we proposed a new role for the previously described BAK1 LRR receptor kinase as a potential regulator of flg22 and elf18 downstream signaling (Chapter 3.3). The discovery of 2 RLKs involved in PAMP perception/signaling has confirmed the rationale of the reverse-genetic approach and our hypothesis that perception of one PAMP can potentiate the response to further PAMPs. FLS2 represented so far the only known PRR in Arabidopsis, and one can expect that the use of reverse-genetic tools will increase this number rapidly.

Our findings raise several questions: Does PAMP perception always occurs via direct interaction with a transmembrane receptor? Are all Arabidopsis PRRs LRR receptor-like kinases? Are all induced RLKs involved in PAMP perception/signaling?

The LRR domain is thought to act as a protein-protein interaction domain (Kobe and Kajava, 2001), fitting to the fact that peptidic PAMPs, such as flg22 and elf18, directly bind to FLS2 and EFR. However, the exact binding domains of FLS2 and EFR for flg22 and elf18, respectively, are not known, and one cannot exclude binding in an extracellular part outside of the LRR repeats. In fact, the steroid plant hormone brassinosteroid was recently shown to binds to an 70 amino-acid island domain located between the LRR repeats 21 and 22 of the BRI1 receptor kinase (Kinoshita et al., 2005). This discovery opens the possibility that LRR receptor-likes kinases not only recognize proteic ligands, but also ligands of diverse biochemical nature through non-LRR parts.

Receptor-like proteins (RLPs) are transmembrane proteins that possess extracellular LRRs but no intracellular kinase domain. Similarly to LRR-RLKs, they might also be involved in PAMP perception. Indeed, recent work on the tomato receptor for the fungal elicitor xylanase provides a first example for a RLP functioning as a PRR (Ron and Avni, 2004). Interestingly, our expression analyses revealed that 5 out of the 58 Arabidopsis RLPs were induced by flg22, suggesting that they might play role as PRRs (Zipfel et al., 2004). Because they lack obvious signaling domains RLPs have been proposed to interact with other proteins to signal, such as LRR-RLKs. In Arabidopsis meristem maintenance, for example, the LRR receptor kinase CLV1 may interact with the RLP CLV2 to form a binding complex (Jeong et al., 1999).

Alternatively, PAMP binding to the LRR domain might be indirect and require an additional PAMP-binding protein. An indirect mechanism is involved in LPS recognition in mammals where the soluble LPS-binding protein first interacts with LPS, allowing subsequent interaction of this complex with CD14 and MD-2 and, finally, with the transmembrane TLR4 (Miyake, 2004). Similarly, activation of the Drosophila Toll pathway by bacteria involves soluble peptidoglycan-recognition proteins (PGRPs), as well as Gram-negative bacteria binding-proteins (GNBPs) that mainly recognized peptidoglycans (Royet et al., 2005). A similar mechanism probably exists in plants. The first PRR protein identified in plants (Umemoto et al., 1997) is a soluble, cell-wall located protein that specifically binds the classic heptaglucoside elicitor from oomycetes (Umemoto et al., 1997). Recent data show that this glucan binding protein (GBP) has an intrinsic endo-β-glucanase activity (Fliegmann et al., 2004). Astonishingly, homologs of this GBP seem to be present in diverse plant species but high-affinity binding and elicitor response to the heptaglucoside is restricted to a few species of the *Fabaceae*. Thus, the receptor component involved in transmembrane signaling remains to be identified and might be a LRR-RLK.

Arabidopsis possess many RLKs that have versatile N-terminal ectodomains, other than LRRs, thought to act as the recognition sites for extracellular signals. More than 20 structurally distinct extracellular domains have been identified, and served as a basis for the classification of plant RLKs (Shiu and Bleecker, 2001). Interestingly, most

of the subfamilies of RLKs defined by their ectodomains have at least one member whose gene expression is induced by flg22 (Zipfel et al., 2004). Among them, the LysM subfamily should probably require more attention. The LysM motif is thought to act as general peptidoglycan motif (Bateman and Bycroft, 2000). However, in plants, LysM-containing RLKs have been recently shown to be involved in the response tof Nod factors during symbiosis between legumes and Rhizobiaceae (Madsen et al., 2003; Radutoiu et al., 2003; Limpens et al., 2003). Nod factors, secreted by the bacterial symbiont, are lipochito-oligosaccharides, consisting of substituted β,1-4-N-acetylglucosamine (chitin) backbones (Lerouge et al., 1990). The presence of LysM-RLKs in non-symbiotic plants (e.g. Arabidopsis) suggests that the LysM motif could be involved in the perception of the fungal cell-wall-derived general elicitor chitin, that is structurally similar to Nod factors.

Some of the induced LRR-RLKs might also recognize endogenous elicitors that signal danger upon infection. Several gene families coding for small-secreted peptides have been identified recently in Arabidopsis. Although many of these newly identified peptides may be involved in growth or developmental processes (Hobe et al., 2003; Casamitjana-Martinez et al., 2003; Fiers et al., 2004), others are likely to play roles in plant defense and are prime candidates for endogenous elicitors recognized by LRR containing proteins.

Homology searches revealed that they are 25 homologues of the *CLV3* gene, the CLE (*CLV3/ESR-related*) genes (Cock and McCormick, 2001; Sharma et al., 2003). The carrot PSK, known to promote dedifferenciation and cell division in plant cells, is recognized by the LRR receptor kinase PSKR (Matsubayashi et al., 2002). Four genes that encode precursor of the sulphated pentapeptide phytosulfokine (PSK) have been identified in Arabidopsis (Yang et al., 2001). In this context, it is interesting to note that the closest Arabidopsis homolog of the carrot PSKR is induced by flg22. Finally, a 5-kD peptide triggerring rapid alkalinization of the medium of tobacco cells, similarly to elicitors, has been isolated from tobacco, and named RALF (Rapid ALkalinization Factor) (Pearce et al., 2001). Interestingly, 34 RALF-like have been identified in Arabidopsis (Olsen et al., 2002). It would be now interesting to test if any of these peptides play a role in plant defense.

The existence of an Arabidopsis perception system for endogenous peptides activating plant defenses is further suggested by the *CDR1* gene that encodes an apoplast aspartic protease (Xia et al., 2004). Plants mutated in the *CDR1* gene are more susceptible to bacterial infections, suggesting that CDR1 directly or indirectly generates a mobile endogenous peptide elicitor to activate the basal defense mechanism.

In mammals, the repertoire of recognized PAMPs can be significantly enhanced through cooperative interaction of TLRs with other TLR or non-TLR pattern recognition proteins (Akira and Takeda, 2004). With >650 RLKs and RLPs, Arabidopsis has the potential to recognize many different ligands. The proportion of receptors recruited for growth and development, or defense is not easily predictable. However, based on our expression data, it is conceivable that ~20% of these receptors might be involved in defense. However, the finding that BAK1 is involved in flg22 and elf18 signaling suggests that some of the putative receptors indeed act as regulators of signaling, and that these regulators are shared between different physiological processes in plants. Interestingly, a rapid survey of the LRR-RLKs present in the Arabidopsis genome reveals that about half of them possess a low number of LRR repeats, as does BAK1. This suggests that half of the LRR-RLKs might indeed work together with other LRR-RLKs having a large number of LRRs to regulate perception and/or signaling. BAK1 is not required for brassinosteroid (Kinoshita et al., 2005), or flg22 binding (Chapter 3.3), suggesting that it rather plays a role in signaling. If our hypothesis is true, the efforts to identify LRR-RLKs mediating direct ligand recognition could be diminished by concentrating on proteins harbouring high number of LRRs.

PAMP perception is an ancient form of plant defense. This system has been overcome by successful pathogens or symbionts, either by avoiding recognition or by suppressing host defenses with effectors, some of which injected directly into host cells (Espinosa and Alfano, 2004; Dodds et al., 2004; Allen et al., 2004). In turn, some plant cultivars evolved R proteins to directly detect these effectors or rather the modifications triggered by them, as proposed by the “guard hypothesis” (Dangl and Jones, 2001; Van der Hoorn et al., 2002). Conceptually, R proteins are related to PRRs and one might think they form a rapidly evolving subfamily of them. Similarly, some PRRs might have

been recruited for the establishment of successful symbiosis. These ideas are supported by the observations that some classes of receptors involved in PAMP, Avr (Nimchuk et al., 2003) and symbiotic signals (Riely et al., 2004) perception are the same. For example, the Arabidopsis PRR FLS2 recognizing flagellin, the rice R protein Xa21 conferring resistance against *Xanthomonas oryzae* pv *oryzae*, and the lotus SYMRK required for successful bacterial and fungal symbiosis, are all LRR-RLKs (Gòmez-Gòmez and Boller, 2000; Song et al., 1995; Stracke et al., 2002).

Although a few characterized R proteins are transmembrane RLKs, such as Xa21 and Xa26 in rice (Song et al., 1995; Sun et al., 2004), or RLPs, such as the Cf proteins in tomato (Rivas and Thomas, 2002) or RPP27 in Arabidopsis (Tor et al., 2004), most are cytoplasmic proteins. The major classes of R proteins are TIR-NBS-LRRs and CC-NBS-LRRs (Nimchuk et al., 2003). Arabidopsis contains ~150 genes coding for NBS-LRRs proteins (Meyers et al., 2003). Recent data suggest that cytoplasmic proteins with a NBS (or NOD) domain act as PRRs for peptidoglycan perception in mammals (Philpott and Girardin, 2004). To date, there is no case reported on intracellular PAMP recognition in plants and one can wonder whether some members of the large and rapidly evolving NBS-LRR family might still function in sensing more general microbial patterns.

On the importance of PAMP perception for basal resistance

A common characteristic of microbe detection by the innate immune systems of plants and animals is the redundancy of PAMPs signaling the same type of microbe. For example, Arabidopsis can potentially recognize a flagellated Gram-negative bacterium through at least flagellin, EF-Tu and LPS perception. This redundancy probably ensures and potentiates efficiency of recognition. In addition, a growing amount of evidences suggests that early signaling events following bacterial and fungal infections, but also treatments with PAMPs or Avr proteins are similar (Romeis et al., 1999; Asai et al., 2002; Navarro et al., 2004; Pedley and Martin, 2004; Liu et al., 2004; Menke et al., 2004), indicating that perception of a single PAMP does not allow discrimination between different pathogens but rather indicates a general state of stress allowing to the plant to defend itself faster against a large range of pathogens.

Another aspect of this work was the contribution of PAMP perception in disease resistance. We have shown that Arabidopsis treatment with a single PAMP, such as flagellin or EF-Tu, triggers resistance against the virulent bacterial strain *Pst* DC3000 (Zipfel et al., 2004; Kunze et al., 2004). This was already suggested earlier in the case of LPS (Newman et al., 2002) or harpin (Dong et al., 1999). However, the question whether individual PAMP perception system plays a critical role during natural infection processes could never be answered before. We showed that plants mutated in the flagellin receptor FLS2 were more susceptible to *Pst* DC3000 infection, demonstrating for the first time an effective role of flagellin perception in natural basal resistance against bacteria (Zipfel et al., 2004). The discovery of the EF-Tu receptor EFR would now allow us to study the contribution of EF-Tu perception in Arabidopsis basal resistance against bacteria. Preliminary data yet indicate that EF-Tu perception is not a limiting factor for Arabidopsis basal resistance against *Pst* DC3000 (Chapter 3.2).

Here, we would like to discuss some points that explain, in addition to perception redundancy, the difficulty to clearly demonstrate a role of individual PAMP perception in, but paradoxically also reinforce the notion that PAMP sensing is important for disease resistance.

If plant PRRs are crucial for the host defense response against pathogens, it would be expected that pathogens would have mechanisms to manipulate PAMP perception by these PRRs.

PAMPs are conserved, essential structures of microbes that can be assumed intrinsically difficult to be modified without loss of functionality. Nevertheless, some sequence variations were observed in the flg22 or elf18 domains of some plant-associated or pathogenic bacteria. For example, the flg22 peptides derived from the plant-associated bacteria *Agrobacterium tumefaciens* and *Rhizobium meliloti*, or from the pathogenic bacteria *Ralstonia solanacearum*, are inactive (Felix et al., 1999; Pfund et al., 2004), but exhibit a highly active elf18 peptide (Kunze et al., 2004). Interestingly, elf18 peptides from some phytopathogenic bacteria, such as *Pst DC3000* and *Xylella fastidiosa*, also exhibit reduced activity as elicitors (Kunze et al., 2004). Some phytopathogenic strains such as *Xanthomonas campestris* pv *campestris* have both

reduced flg22 and elf18 activities (G. Felix, unpublished data). These results suggest that these bacteria species evolved to avoid flg22 and/or elf18 recognition. A similar observation was made in animals where flagellins (FlaA and FlaB) from *Helicobacter pylori* preserve motility properties but not stimulatory activity in human, in correlation with sequence variations in the domain recognized by TLR5 (Lee et al., 2003; Jacchieri et al., 2003).

Another way for the pathogen to escape recognition by the plant is to control PAMP expression. Flagellin based-motility is required for successful infection of host tissues, and therefore initially acts as a virulence factor (Ramos et al., 2004). However, this requirement seems to be only important for the initial steps of the infection. In plants, for example, flagella are necessary for initial colonization of roots and leaf surfaces by *Ralstonia solanacearum*, *Agrobacterium tumefaciens* and plant growth-promoting Pseudomonads, but not for endophytic multiplication (Tans-Kersten et al., 2001; Chesnokova et al., 1997; Lugtenberg et al., 2001). Flagellar biosynthesis is an energetically costly process that involves the coordinated expression of ~50 flagellar genes (Blocker et al., 2003). Several evidences in animals suggest that flagellin biosynthesis is tightly regulated by environmental factors, and might be switched off after initial host colonization (Krukonis and DiRita, 2003; Wolfgang et al., 2004).
The EF-Tu protein has been extensively studied for its essential function in protein translation (Krab and Parmeggiani, 2002), but seems to have additional unexpected roles. Recently, EF-Tu was located at the surface of *Mycoplasma pneumonia*, 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 johnosonii*, where it appears to mediate the attachment of these probiotic bacteria to human intestinal cells (Granato et al., 2004). Therefore, EF-Tu might be a new virulence factor required for adhesion to host cells, and one can wonder whether its expression, or secretion by a yet unknown mechanism, would be regulated during the infection process.

A growing amount of evidences suggests that pathogens are also able to suppress basal defense or Avr-mediated host cell death (Espinosa and Alfano, 2004). Type III protein secretion systems (TTSS) are central to the virulence of many bacteria,

including animal pathogens in the genera *Salmonella*, *Yersinia*, *Shigella* and *Escherichia*, and plant pathogens in the genera *Pseudomonas*, *Erwinia*, *Xanthomonas*, *Ralstonia* and *Pantoea* (Galan and Collmer, 1999). For example, *Pst* DC3000 mutated in their TTSS system are not able anymore to infect otherwise susceptible Arabidopsis plants (Roine et al., 1997). The TTSS in plant pathogens is known as the hypersensitive response and pathogenicity (Hrp) system because secretion mutants are unable to elicit the defense-associated hypersensitive response (HR) in non-host plants or establish a pathogenic relationship with hosts. The recent availability of several genomes from phytopathogenic bacterial strains has led to the identification of many candidate effectors (Chang et al., 2004). Two main virulence functions have been proposed for type III effectors: nutrient acquisition and suppression of host defenses. No reports have been published demonstrating a role for effectors in acquiring nutrients. The Hrp systems of *P. syringae* and *X. campestris* were implicated in the suppression of basal defenses long before these systems were shown to deliver effector proteins. For example, defense-related transcripts were found to be induced in bean by avirulent *P. syringae* and a *P. syringae hrp* mutant, but not by virulent Hrp-wild-type *P. syringae*, which suggested that the Hrp system suppresses basal defenses (Jakobek et al., 1993; Jakobek and Lindgren, 1993). In addition, hrp mutant bacteria induced formation of papillae at contact point between bacterial cells and the plant cell, which were not induced by virulent wild-type pathogens (Bestwick et al., 1995; Brown et al., 1995). More recently, the *Pst DC3000* effector AvrPto was demonstrated to suppress cell wall-based extracellular defense triggered by an avirulent *Pst DC3000 hrp* mutant, and allow normal growth of this strain, when overexpressed in transgenic Arabidopsis (Hauck et al., 2003). Similarly, wild-type *Xanthomonas campestris* pv *campestris* (*Xcc*) suppressed basal defenses induced by a *Xcc hrp* mutant, in which the main active elicitor was found to be LPS (Keshavarzi et al., 2004).

Finally, the *Pst* DC3000 effector HopPtoD2, suppressed *pathogenesis-related* (*PR*) gene expression, inhibited the HR triggered by an avirulent *P. syringae* strain in *Nicotiana benthamiana* and was required for full *Pst* DC3000 virulence in tomato and Arabidopsis. HopPtoD2 exhibits tyrosine phosphatase activity that is required for the suppression of plant defense, suggesting that it targets phosphoproteins or signal transduction cascades. Interestingly, the only tyrosine-phosphorylated proteins known

in plants are the MAP kinases (Tena et al., 2001). MAP kinase cascades are activated by a number of Avr proteins and by flg22 (Zhang and Klessig, 2001; Asai et al., 2002), opening the possibility that HopPtoD2 could target both Avr- and PAMP-dependent responses. Indeed, it seems that HopPtoD2 overexpression in Arabidopsis suppress some of the flg22-induced responses (James Alfano, University of Nebraska, pers. communication; Sheng Yang He, Michigan State University, pers. communication).
The study of pathogen effectors and the discovery of their plant targets represent a powerful and exciting tool for the future identification of key signaling component involved in plant basal defense.

Noteworthy, the first fungal and oomycete effectors (that act in these cases as Avr proteins) have been recently identified (Dodds et al., 2004; Allen et al., 2004). They localized into the plant cells, suggesting an unknwon protein transport pathway from the pathogen to the host. Their role as virulence factors was so far not defined.
In plant-virus interaction, a geminivirus nuclear shuttle protein (NSP) was identified through a yeast two-hybrid screen to interact with the kinase domain of a LRR-RLK in tomato and soybean (Mariano et al., 2004). A later study showed that the NSP protein also interacts with 3 LRR-RLKs (NIK-1 to -3) in Arabidopsis, and suppresses their kinase activity (Fontes et al., 2004). Loss of NIK1 and NIK3 function correlated with enhanced susceptibility to the geminivirus, corroborating a model in which NSP acts as a virulence factor to suppress NIK-mediated antiviral responses. Future studies should reveal whether NIK1 and NIK3 encode PRRs for yet unknown viral PAMPs.

If successful pathogens effectively evolved to avoid or suppress PAMP recognition, why could we find an enhanced disease susceptibility phenotype of the *fls2* mutant plants towards *Pst* DC3000 infection?
Flagellin seems to be the prominent PAMP recognized by Arabidopsis present in *Pst* DC3000. Whereas flagellin exhibits a high eliciting activity, EF-Tu, such as LPS derived from *Pst* DC3000 are weakly active (Kunze et al., 2004). *Fls2* mutants were as susceptible as wild-type plants when *Pst* DC3000 bacteria were injected into the leaf apoplast, suggesting that flagellin perception was limiting during the first steps of the infection (Zipfel et al., 2004). In fact, this is in good correlation with the requirement of

flagellin as a virulence factor for the initial colonization. Later, bacteria do not need flagellin anymore and probably stop its synthesis. Finally, the TTSS of *Pst* DC3000 is not immediately turn on during the infection, and recent gene expression data in Arabidopsis suggest that *Pst* DC3000 only starts to suppress plant defenses ~2 hours post-infection (Tao et al., 2003; de Torres et al., 2003).
This illustrates the necessity for future studies to consider different experimental conditions as well as different microbial strains in order to find the right limiting conditions that reveal the importance of a given PAMP perception system.

Plants, possess a large array of potential receptors, most of them orphan with respect to their functions or ligands. Ligand-receptor interaction and transmembrane signalling is a poorly understood phenomenon in plants and PAMP perception provides a well-suited experimental model system to study these processes. The combination of forward and reverse genetics with biochemistry should allow us to identify new receptors and to understand the molecular basis of PAMP perception. Most of the downstream signaling elements linking perception to defense gene expression are also unknown. Finally, the position of PAMP-based recognition in disease resistance of plants is not fully established but future work might further loosen boundaries between Avr and PAMP perception.

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Appendix 1

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

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The N Terminus of Bacterial Elongation Factor Tu Elicits Innate Immunity in Arabidopsis Plants

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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 thaliana* and other Brassicaceae. EF-Tu is highly conserved in all bacteria and is known to be *N*-acetylated in *Escherichia 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.

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 cross-linking 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 proinflammatory responses via the toll-like receptor TLR5 (Hayashi et al., 2001). However, whereas 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 thaliana* plants with crude bacterial extracts or with 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 Tu (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.

RESULTS

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

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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. By 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 endoprotease Glu-C (Figure 1A) and pronase (Figure 1B). These results indicate presence of a novel, proteinaceous factor in *E. coli* and other bacteria that elicits alkalinization in Arabidopsis cells.

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 proteins in fractions with elicitor activity were pooled and proteins precipitated by 80% acetone and separated by SDS-PAGE (Figure 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 (Figure 2A). Activity was observed to comigrate with the major polypeptide band of ~43 kD apparent molecular mass. The tryptic digest from the eluate with highest elicitor activity resulted in a mass fingerprint that covered 43% of EF-Tu (Figure 2B, underlined parts). Although demonstrating presence of EF-Tu, this result did not exclude the possibility that elicitor activity was attributable to a different, minor protein comigrating 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, NC). 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 (Figure 2C, shown for nontagged EF-Tu).

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 cold shock protein (Felix and Boller, 2003), we succeeded to localize elicitor activity to particular domains of the

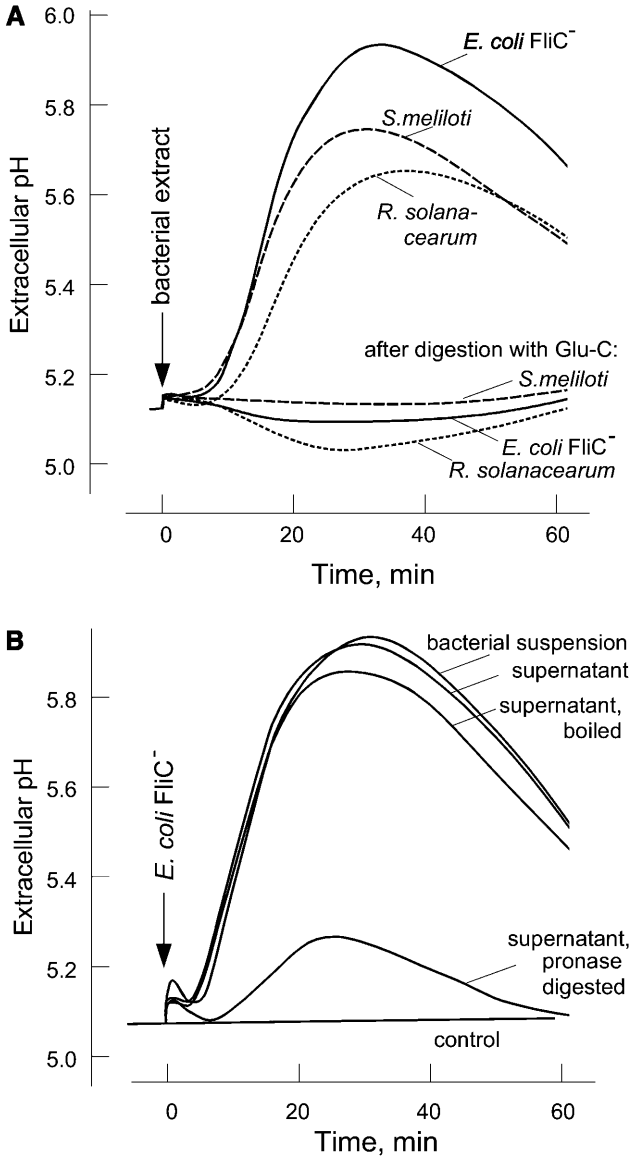

Figure 1. Induction of Extracellular Alkalinization by Bacteria and Bacterial Extracts.

(A) Extracellular pH in Arabidopsis cells after treatment with crude cell-free extracts from *E. coli* strain GI826 (FliC⁻), *R. solanacearum*, and *S. meliloti*. At $t = 0$ min, cells were either treated with 10 μL/mL of bacterial extracts or bacterial extracts that were preincubated 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).

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

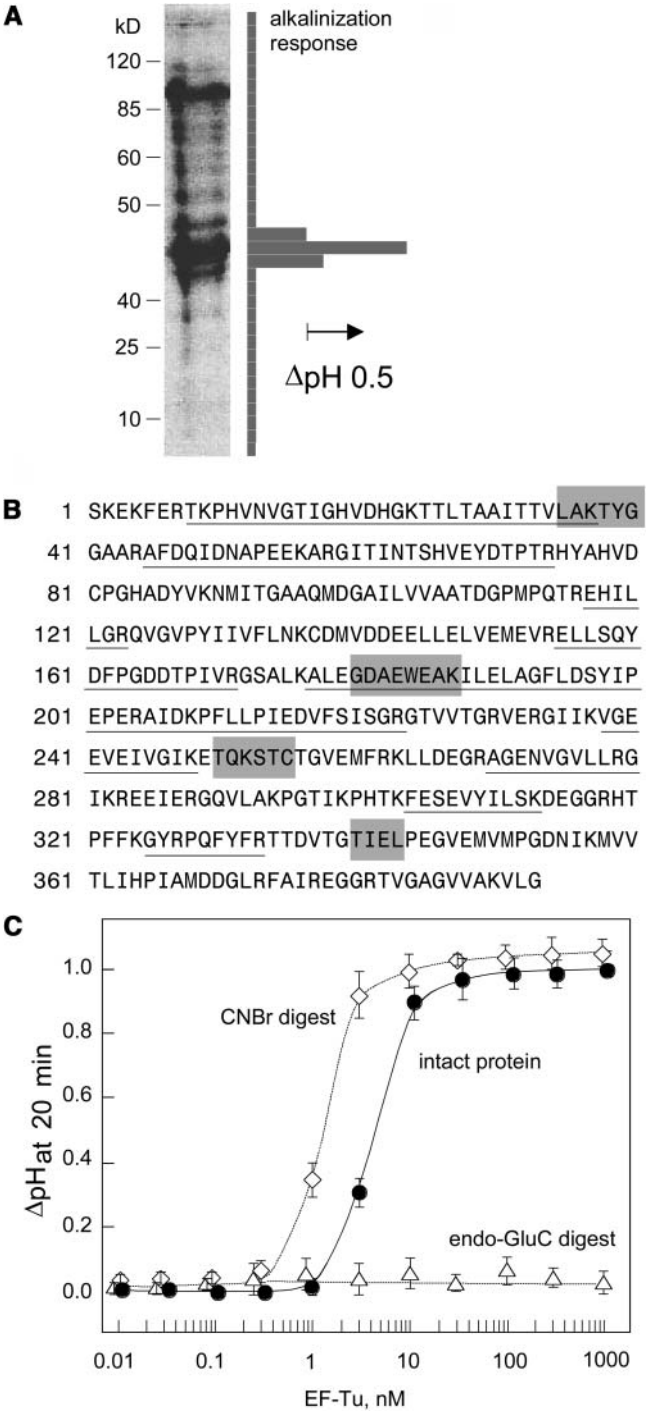

Figure 2. Identification of the Elicitor-Active Protein as EF-Tu.

(A) Alkalinization-inducing activity in extract from *E. coli* strain GI826 was prepurified on MonoQ-ion exchange chromatography and separated by SDS-PAGE. 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.

(B) 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 with 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).

(C) 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 three replicates.

from many different bacteria (Figure 2B). 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 Figure 2C). By contrast, chemical cleavage with cyanogen bromide (CNBr) at Met residues did not lead to inactivation but led to a slight increase in its specific activity (EC_{50} of <2 nM; Figure 2C). Thus, we concluded that the elicitor-active epitope of EF-Tu includes the amino acids Lys, Arg, Glu, and Asp but no Met. The CNBr fragments were separated on a C8 reverse-phase column, and the fractions containing activity were rerun on the column using a more shallow gradient (Figure 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 10,044 + n*28 (Figure 3B). This heterogeneity of mass, probably because of 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 to 91; Figure 3C), indicating that it is the N-terminal part of EF-Tu (Figure 3D) that is recognized by the plant.

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 (Figure 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; Figure 4). In early work on EF-Tu from *E. coli*, the protein was found to start with a Ser 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 an ~20-fold higher specific activity, inducing alkalinization

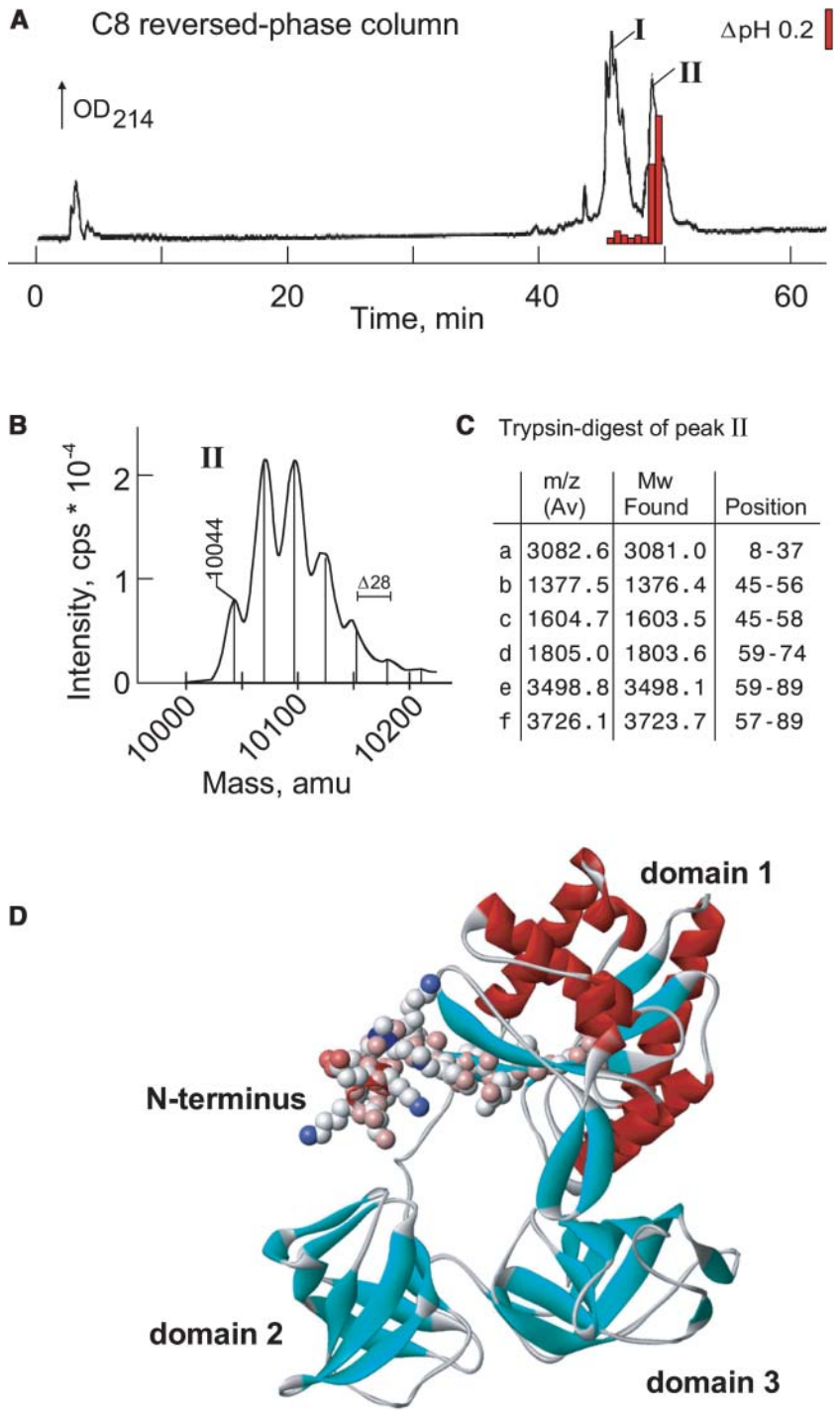

Figure 3. Identification of the CNBr Fragment Carrying Elicitor Activity.

(A) The CNBr digest of EF-Tu was separated on a C8 reverse-phase column. Fractions containing activity were rerun on C8 using a more shallow gradient, and eluate was assayed for UV absorption (OD_{214} 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 eight N-terminal amino acid residues. Ribbon model with the N-terminal part shown as ball and stick (drawn with WebLab ViewerLite; Molecular Simulations, Cambridge, UK).

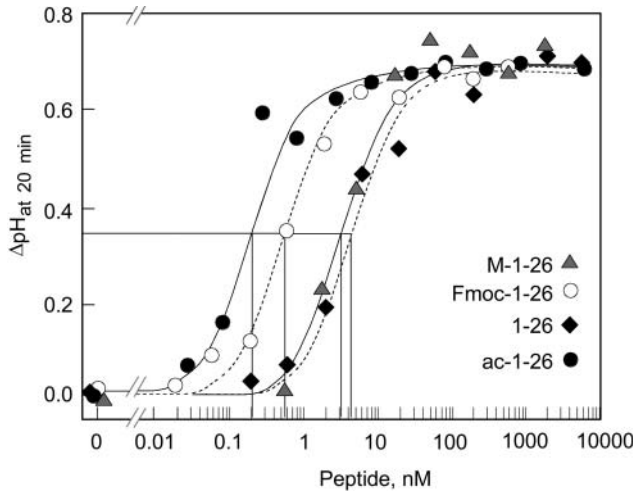

Figure 4. Elicitor Activity of Peptides Representing the N Terminus of EF-Tu.

Different doses of synthetic peptides representing the amino acids 1 to 26 of EF-Tu, either with the N-terminal NH_2-group left free (1-26) or coupled to an extra Met residue (M-1-26), an acetyl group (ac-1-26), or Fmoc 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; pH at the beginning of the experiment was 4.8.

with an EC_{50} of ~0.2 nM (Figure 4). By contrast, N-terminal prolongation by a formyl-group, a Met residue, or a formyl-Met residue group had little effect (Figure 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 elongation factor 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 (Figure 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 and 0.4 nM, indicating high reproducibility and robustness of the alkalinization assay. Because 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 a result of 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 (Figure 5).

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 and 0.6 nM (Figure 5). By 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 to 14) with GTI (position 15 to 17) strongly reduced activity (Figure 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 amino acid residues in *Agrobacterium tumefaciens* and *S. meliloti*, differing in positions 1, 3, 8, and 14, exhibited full activity. By contrast, peptides representing EF-Tu from *P. syringae* pv *tomato* DC3000 and *Xylella fastidiosa* showed reduced activity and EC_{50} value of ~15 and ~30 nM, respectively (Figure 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 homologs from Arabidopsis. In their nonacetylated forms, the peptides representing the cytoplasmic sequence exhibited no activity, whereas 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 (Figure 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 (Figure 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.

The Peptide elf12 Antagonizes Elicitor Activity of EF-Tu

Inactive, structural analogs of elicitors may act as specific, competitive antagonists for the elicitor from which they were derived. Examples for this include the oligosaccharide part of the glycopeptide elicitor (Basse et al., 1992) and C-terminally truncated forms of the flg22 elicitor (Meindl et al., 2000; Bauer et al., 2001). Indeed, elf12, which shows no elicitor activity even when applied at micromolar concentrations (Figure 5), exhibited antagonistic activity for EF-Tu–related elicitors but not for the structurally unrelated elicitor flg22 (Figure 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 (Figure 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 *A. tumefaciens* and *R. solanacearum*, indicating that EF-Tu was the predominant elicitor activity in these preparations.

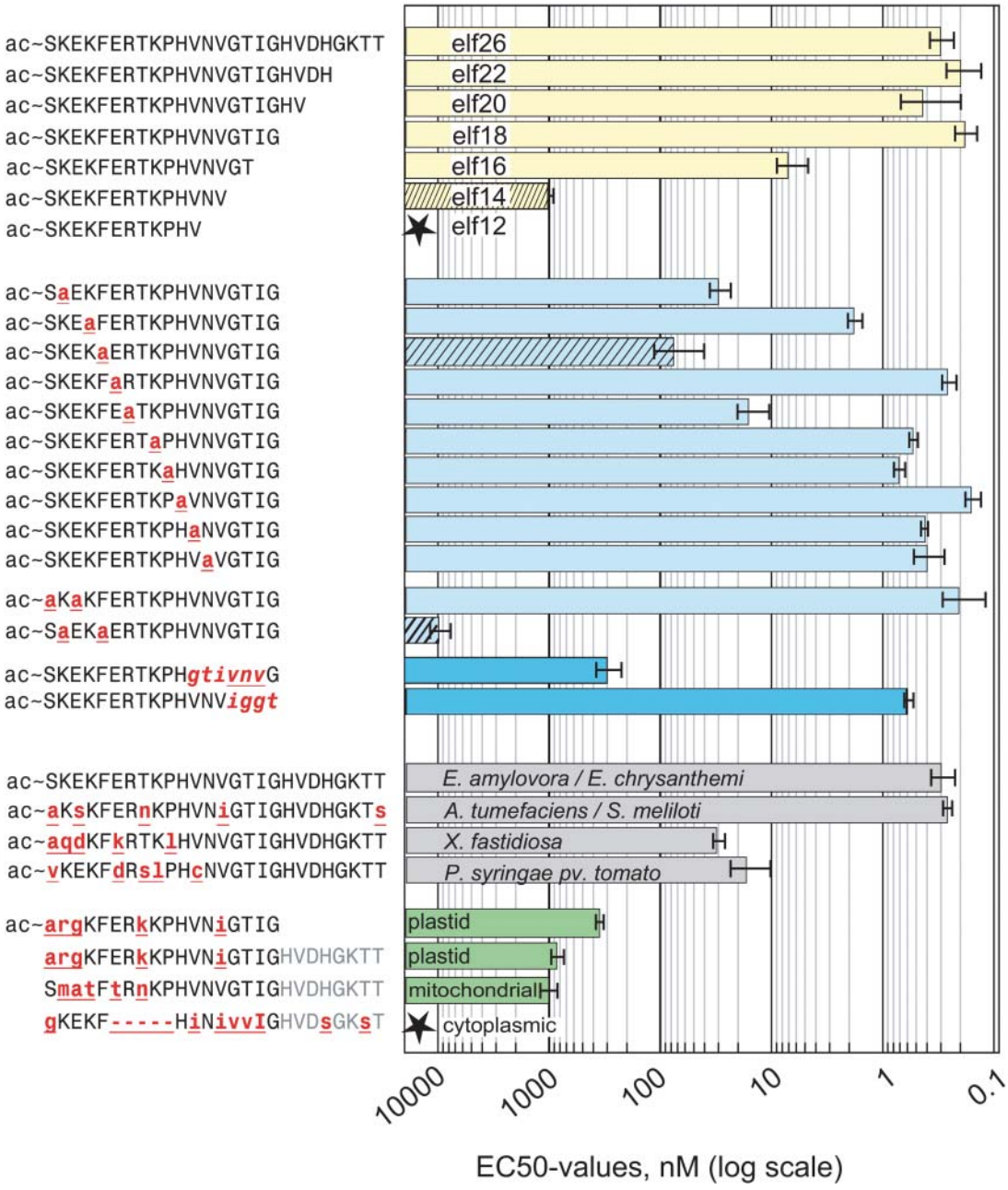

Figure 5. Alkalinization-Inducing Activity of EF-Tu N-Terminal Peptides.

Summary of EC_{50} values determined from dose–response curves with the different peptides. Peptide sequences and N-terminal acetylation (ac~) are indicated at the left. 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} >10^4 nM).

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 (Figure 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

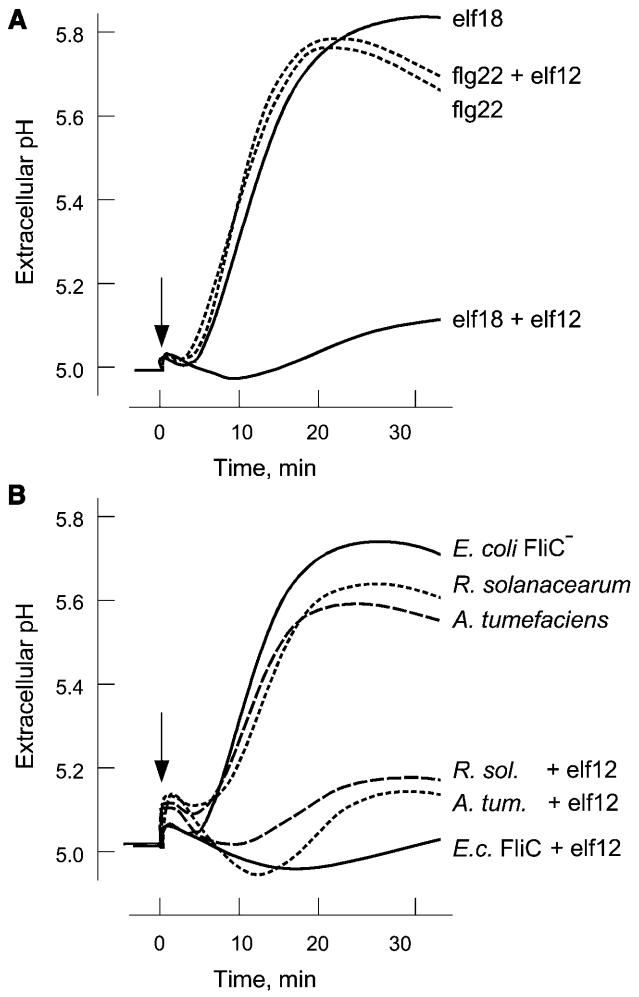

Figure 6. Antagonistic Activity of elf12 for EF-Tu–Related Elicitors.

(A) Alkalinization induced by 1 nM flg22 or 0.5 nM elf18 when applied alone or together with 30 μM elf12.

(B) Effect of 30 μM 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*.

tissue from other Brassicaceae, such as *Brassica alboglabra*, *B. oleracea*, and *Sinapis alba*, also responded to the EF-Tu peptides. By 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 (*Solanum tuberosum*), cucumber (*Cucumis sativus*), sunflower (*Helianthus annuus*), soybean (*Glycine max*), and *Yucca alifoli*, all of which showed enhanced ethylene biosynthesis when challenged with flg22 as a positive control (data not shown).

Arabidopsis accession Wassilewskija-0 (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 (Figure 7A) but also when assayed for induction of oxidative burst (Figure 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 (Figure 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; Robatzek and Somssich, 2002; de Torres et al., 2003). In Arabidopsis lines Ws-0 and Columbia-0 (Col-0) made transgenic for the β-glucuronidase (*GUS*) gene driven by the *SIRK* promoter, GUS activity was clearly induced at the sites in the leaves that were inoculated by pressure infiltration with 1 μM elf26 (Figure 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 (Figure 8A). In summary, these results show that Arabidopsis 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.

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 *P. 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 d before infection with *Pst* DC3000 (Figure 8B). In contrast with 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).

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-tRNAs (all except fMet-tRNA and selenocysteine-tRNA) and catalyzes 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 to 9% of total bacterial cell protein and thus is one of the most abundant proteins in bacteria. Because of its essential role in protein biosynthesis, the EF-Tu protein has been studied extensively at the biochemical and

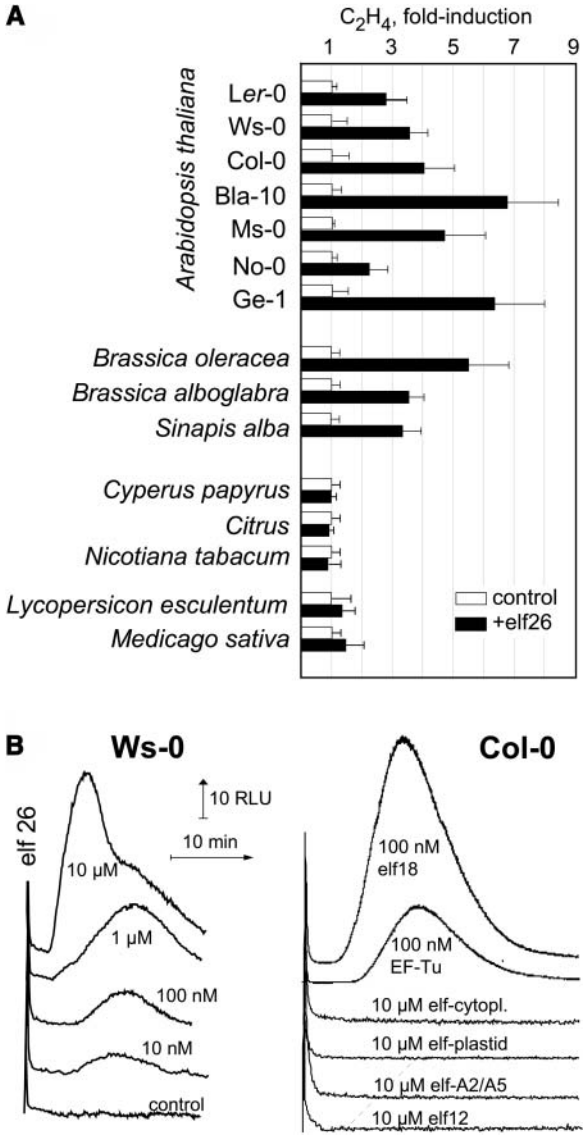

Figure 7. Induction of Elicitor Responses in Leaf Tissues of Different Plant Species.

(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 leaf 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 because of phosphorescence of the green plant tissue.

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 (Meindl et al., 2000; Bauer et al., 2001) and EF-Tu (Figure 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 they share common characteristics, the perception systems for flagellin and EF-Tu obviously involve different receptors because perception of flagellin requires the receptor kinase FLS2 (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004), whereas EF-Tu is also active in plants carrying mutations in FLS2 (Figures 7 and 8).

In our ongoing work, we further compare perception of EF-Tu and flagellin in Arabidopsis in more detail (our unpublished data). 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 nonredundant 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 (Figure 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*, *R. solanacearum*, and *A. tumefaciens*. By 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 *Pst* DC3000 and *X. 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

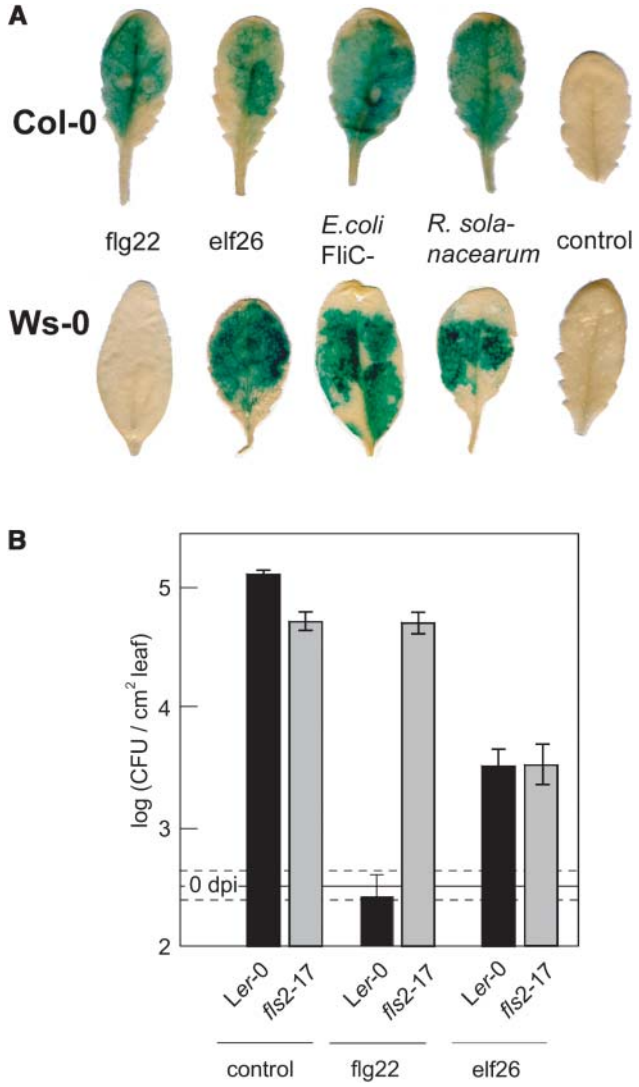

Figure 8. Induction of Defense Responses in Arabidopsis.

(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 μM flg22, 1 μM 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 Landsberg *erecta*-0 (L*er*-0) and fls2-17 plants were pretreated for 24 h with 1 μM flg22, 1 μM elf26, or water as a control. These leaves were subsequently infected with 10^5 colony-forming units (cfu)/mL *Pst* DC3000, and bacterial growth was assessed 2 d postinfection (dpi). Results show average and standard error of values obtained from four plants with two leaves analyzed each ($n = 8$). The solid and dashed lines indicate mean and standard deviation of cfu extractable from leaves at 0 dpi ($n = 12$).

to plants. Several of these bacteria carry sequence variations that renders 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 (Figure 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 Phe 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 (Figure 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 nondenatured EF-Tu is a highly active elicitor in tissue of intact plants and in cultured cells (Figure 2C).

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 ~20. EF-Tu is well known to be *N*-acetylated in *E. coli* (Laursen et al., 1981). Whereas *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 also unknown whether this modification has any functional significance. However, in view of the observation that PAMPs represent particularly conserved structures of a whole class of microbes, we predict 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 plasma membrane of plant cells. EF-Tu and cold shock protein 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 nonaccessible 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 *P. syringae* pv *tomato* (Zipfel et al., 2004). This provides 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 (Figure 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 report EF-Tu was also observed to act as a stimulator of a proinflammatory 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 hallmark protein.

Treatment of plants with crude bacterial extracts induces defense responses and leads to induced resistance (Jakobek et al., 1993; Zipfel et al., 2004). Whereas 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.

METHODS

Materials

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

Bacteria and Preparation of Bacterial Extracts

Escherichia coli GI826 was obtained from Invitrogen (Carlsbad, CA) and grown in LB medium at 37°C on a rotary shaker. *Agrobacterium tumefaciens* (strain C58 T), *Sinorhizobium meliloti*, and *Ralstonia solanacearum* (from DSM, Braunschweig, Germany) were grown in King's B broth at 26°C on a rotary shaker. Bacteria were harvested by centrifugation and washed and resuspended in water (~20 to 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 three cycles of freezing and thawing and subsequent incubation in lysis buffer (50 mM Tris-HCl, pH 8, and 0.2 mg/mL of 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, Madison, WI) 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 (Amersham Biosciences, Uppsala, Sweden) 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 of water 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 matrix-assisted laser desorption/ionization-time of flight analysis on a 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 reverse-phase chromatography on a C8 column (Grace Vydac, Hesperia, CA; 1 × 250 mm, 5 μm) at pH 3.5 (0.05% TFA in water as solvent A and 80% acetonitrile/20% water 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 5500 V for ionization and analysis in single quadrupole mode. The peptide masses were calculated using BioSpec Reconstruct (Applied Biosystems/MDS Sciex, Thornhill, Ontario, Canada). Peptides further digested with trypsin were analyzed by matrix-assisted laser desorption/ionization-time of flight analysis on a TofSpec 2E.

Plant Cell Cultures and Alkalinization Response

The *Arabidopsis thaliana* cell culture (May and Leaver, 1993) was maintained and used for experiments 4 to 8 d 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- to 6-week-old Arabidopsis plants grown in the greenhouse were cut into 2-mm slices and floated on water 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 of fresh weight) containing 0.1 mL of water supplied with 20 μM luminol and 1 μg of horseradish peroxidase (Fluka, Buchs, Switzerland). Luminescence was measured in a luminometer (LKB 1250; Wallac, Turku, Finland; TD-20/20; Turner Designs, Sunnyvale, CA) for 30 min after addition of elicitor. For assaying ethylene production, leaf slices (~20 mg of fresh weight per assay) were transferred to 6-mL glass tubes containing 1 mL of water 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 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 T3 generation were pressure infiltrated (needleless syringes) with 1 μM 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 with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucoronide cyclohexylammonium).

Infection of Arabidopsis Leaves with *Pseudomonas syringae* pv *tomato*

Pst DC3000 was grown at 28°C on King's B plates with 50 mg/L of rifampicin. Bacteria were resuspended at 1×10^5 cfu/mL of water and injected into leaves using a syringe without a 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 of rifampicin.

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 Molecular Modeling Database (9879) and Protein Database (1EFC).

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Abbreviations

Avr	avirulence gene or protein
BL	brassinolide
Bp	base pair
BR	brassinosteroid
CC	coiled-coil
Col-0	Arabidopsis ecotype Columbia
DTT	dithiothreitol
EDTA	ethylenedinitro-N,N,N',N'-tetraacetic acid
EF-Tu	elongation factor Tu
EGS	ethylene glycol-bis-(succinimidylsuccinate)
elf	EF-Tu peptide
flg	flagellin peptide
FLS2	flagellin sensing 2
FW	fresh weight
g	gramme
HR	hypersensitive response
kD	kilodalton
La-*er* (L*er*-0)	Arabidopsis ecotype Landsberg *erecta*
LP	left primer
LPS	lipolysaccharides
LRR	leucine-rich repeat
M	molar
MES	2-morpholinoethanesulfonic acid monohydrate
min	minute
MS	Murashige and Skoog medium
NOD/NBS	nucleotide oligomerization domain/nucleotide binding site
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PGN	peptidoglycan
PRR	pattern recognition receptor
R	resistance gene or protein
RLK	receptor-like kinase
RLP	receptor-like protein
RP	right primer
rpm	rotation per minute
RT	reverse-transcription
s	second
SDS	sodium dodecyl sulfate
T-DNA	transfer-DNA
TIR	Toll/Interleukin
TLR	Toll-like receptor
Tris	tris(hydroxymethyl)aminomethan
TTSS	type III secretion system
Ws-0	Arabidopsis ecotype Wassilevskaja
YEB	yeast extract broth medium

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Publications

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