Cell

Perception of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts *Agrobacterium*-Mediated Transformation

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DOI 10.1016/j.cell.2006.03.037

SUMMARY

Higher eukaryotes sense microbes through the perception of pathogen-associated molecular patterns (PAMPs). *Arabidopsis* plants detect a variety of PAMPs including conserved domains of bacterial flagellin and of bacterial EF-Tu. Here, we show that flagellin and EF-Tu activate a common set of signaling events and defense responses but without clear synergistic effects. Treatment with either PAMP results in increased binding sites for both PAMPs. We used this finding in a targeted reverse-genetic approach to identify a receptor kinase essential for EF-Tu perception, which we called EFR. *Nicotiana benthamiana*, a plant unable to perceive EF-Tu, acquires EF-Tu binding sites and responsiveness upon transient expression of *EFR*. *Arabidopsis efr* mutants show enhanced susceptibility to the bacterium *Agrobacterium tumefaciens*, as revealed by a higher efficiency of T-DNA transformation. These results demonstrate that EFR is the EF-Tu receptor and that plant defense responses induced by PAMPs such as EF-Tu reduce transformation by *Agrobacterium*.

INTRODUCTION

Recognition of microbes in higher eukaryotes depends on an array of pattern recognition receptors (PRRs). These PRRs recognize characteristic molecular structures shared by large groups of microbes, the so-called pathogen-associated molecular patterns (PAMPs). PAMPs play key roles as activators of the innate immune response in animals (Medzhitov and Janeway, 2002; Akira and Takeda, 2004) and, analogously, as elicitors of defense responses in plants (Nürnberger et al., 2004). While important for the innate immune system of plants and animals, the recognition specificities in the different kingdoms probably arose independently by convergent evolution to recognize highly conserved molecules (Zipfel and Felix, 2005; Ausubel, 2005). Perception of pathogens by plants can be divided into three main phases, which appear to reflect steps of coevolution in plant-pathogen interaction (Nürnberger et al., 2004). In a first phase, perception of PAMPs or "general elicitors" by the host leads to rapid activation of defense mechanisms such as cell-wall reinforcement by callose deposition and production of reactive oxygen species (ROS), and induction of numerous defense-related genes. In a second phase, virulence factors evolved by successful pathogens can inhibit these PAMP-elicited basal defences (Espinosa and Alfano, 2004; Nomura et al., 2005; Kim et al., 2005). In a third phase, plants have evolved resistance (R) proteins specialized to detect these pathogen-derived virulence factors or their effects on host targets. As a consequence of this R protein-dependent perception, a hypersensitive response (HR) occurs that includes localized cell death and leads to arrest of pathogen spread (Nimchuk et al., 2003; Jones and Takemoto, 2004).

PAMPs perceived by plants include structures characteristic for oomycetes like the cell-wall β-glucan, the pep13 epitope conserved in cell-wall transglutaminases, and secreted lipotransfer proteins termed elicitins (Nürnberger et al., 2004). Plants can also perceive structures made by true fungi like the cell-wall polysaccharide chitin and the fungal sterol ergosterol. Similarly, plants have been reported to recognize structures characteristic for bacteria-like lipopolysacharides (LPS), bacterial cold-shock protein (CSP), flagellin, and EF-Tu (Nürnberger et al., 2004; Zipfel and Felix, 2005). Some of these PAMPs are only perceived by a narrow range of plant species, whereas others trigger defense responses in many species. For example, flagellin induces responses in plants belonging to many different

orders, while perception of bacterial CSP and EF-Tu seemed to be restricted to the orders of *Solanales* and *Brassicales*, respectively.

Various microbial patterns act as PAMPs in plants, but the corresponding PRRs remain largely unknown. So far, receptor binding sites have been identified only for heptaglucan from oomycetes (Umemoto et al., 1997), fungal xylanase (Ron and Avni, 2004), and bacterial flagellin (Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006). Here, we report characterization of a high-affinity receptor binding site for EF-Tu, which is distinct from the flagellin receptor FLS2 but elicits a set of defense responses highly similar, if not identical, to that induced by flagellin. Based on the resemblance of the perception mechanisms for both PAMPs we hypothesized that EF-Tu might be perceived by a receptor kinase related to the flagellin receptor FLS2. By screening T-DNA insertion lines for various receptor-like kinases (RLKs) related to FLS2 we identified EFR (*EF*-Tu *r*eceptor) as required for perception of EF-Tu in *Arabidopsis*. *Nicotiana benthamiana* plants have no perception system for EF-Tu but gained the capacity to respond to this PAMP when transformed with *EFR*. We also show that *Arabidopsis efr* mutants lacking EF-Tu perception are more susceptible to transformation by *Agrobacterium tumefaciens*, thus revealing the functional importance of the EF-Tu perception system for plant defense.

RESULTS

A High-Affinity Binding Site Specific for EF-Tu on *Arabidopsis* Cells

In previous work we described a highly sensitive perception system for the acetylated N terminus of bacterial EF-Tu in *Arabidopsis* (Kunze et al., 2004). The sensitivity and specificity of *Arabidopsis* for this novel PAMP suggested perception via a surface receptor. To probe for this receptor site we used an elf26-derivative labeled with ^{125}Iodine (elf-^{125}I) in binding assays with intact *Arabidopsis* cells (Figure 1A). Binding occurred rapidly and reached a plateau within 25 min. Nonspecific binding in the presence of an excess of nonlabeled elf26 stayed low throughout the experiment (Figure 1A). Addition of 10 μM elf26 peptide 25 min after elf-^{125}I did not result in detectable displacement over the next 90 min, indicating essentially nonreversible binding of the ligand. Since binding assays were performed at 4°C, this nonreversibility was probably not due to an uptake process.

The affinity and the number of EF-Tu binding sites on intact cells were determined by saturation curves with increasing concentrations of labeled elf-^{125}I (Figure 1B). The values for specific binding accurately fitted to a rectangular hyperbola resulting in an apparent K_d of 0.8 nM and B_{max} of 2.1 pmol binding sites per g cells. Assuming ~4×10^7 cells/g fresh weight (Bauer et al., 2001), this corresponds to ~3×10^4 sites/cell.

The specificity of binding was tested in competitive binding assays with different EF-Tu-derived peptides and the structurally unrelated flg22 peptide (Figure 1C). Most effective competition, resulting in 50 % inhibition of radioligand binding at concentrations of ~10 nM (IC_{50} value), was observed for the fully active peptides elf26 and elf18. The C-terminally shortened peptide elf12 was inactive as inducer of PAMP responses but exhibited characteristics of a competitive antagonist. This peptide also competed binding, albeit with an IC_{50} of ~3000 nM. The peptide elf26-Pst, representing the N terminus of EF-Tu of the plant pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000), is a much weaker agonist than elf18 (Kunze et al., 2004) and also acted as a less efficient competitor in binding assays (IC_{50} of ~2000 nM; Figure 1C).

Affinity Crosslinking of elf-^{125}I Specifically Labels a Polypeptide of ~150 kDa

Covalent chemical affinity crosslinking with labeled ligands has been successfully used to characterize receptor binding sites in plants (Matsubayashi and Sakagami, 2000; Scheer and Ryan, 2002; Chinchilla et al., 2006). In experiments with intact cells of *Arabidopsis* we reproducibly observed specific crosslinking of elf-^{125}I to a polypeptide of ~150 kDa on SDS-PAGE (Figure 1D). Labeling was dependent on application of crosslinker (data not shown) and was suppressed by nonlabeled elf26 in a dose-dependent manner with 50% reduction (IC_{50}) at ~4 nM (Figure 1E). This is in good agreement with the IC_{50} value for elf26 in competitive binding assays (Figure 1C). Crosslinking experiments were performed without washing away unbound ligands, thus demonstrating a high selectivity of elf-^{125}I for the 150 kDa polypeptide. In some experiments, an additional, weaker band at ~100 kDa was visible (Figure 1D). Labeling of this band was also inhibited by nonlabeled elf26, indicating that it might be a truncated form of the major 150 kDa polypeptide.

To compare the binding sites for EF-Tu and flagellin, crosslinking assays were performed with elf-^{125}I and/or ^{125}I-flg on the same cells. Clearly, the 150 kDa polypeptide labeled by elf-^{125}I is different from the 175 kDa band labeled by ^{125}I-flg (Figure 1F), which was previously identified as the FLS2 protein (Chinchilla et al., 2006). In summary, EF-Tu interacts specifically with a high-affinity binding site migrating at ~150 kDa on SDS-PAGE.

EF-Tu and Flagellin Induce a Common Set of Responses

Although perceived by two distinct receptors, flagellin and EF-Tu appear to trigger the same set of responses. To test for interference or interaction of the two perception systems, we studied the effects of combined treatments with both PAMPs. Extracellular alkalinization, occurring as a consequence of altered ion fluxes across the plasma membrane, can serve as a rapid, robust bioassay to characterize qualitative and quantitative aspects of PAMP perception. When challenged with saturating doses of 100 nM of the peptides, flg22 induced alkalinization with a time lag of ~30 s, whereas the apparent lag-phase after treatment with elf18 lasted ~70 s (Figure 2A). In this batch

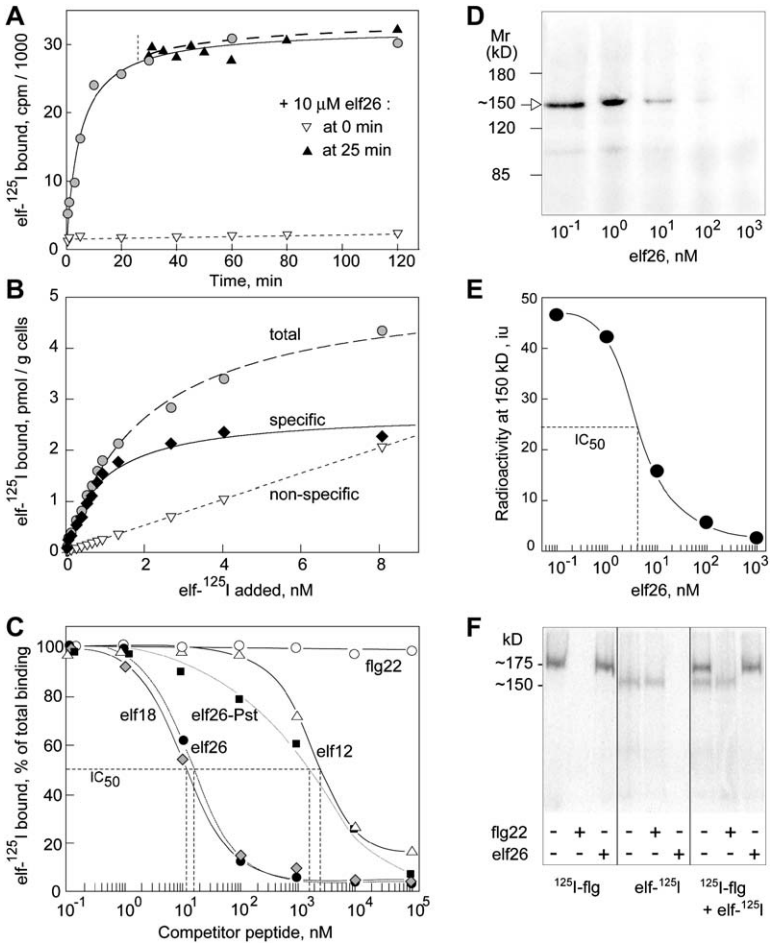

Figure 1. Intact *Arabidopsis* Cells Have High-Affinity Binding Sites Specific for EF-Tu

(A) Binding kinetics of the radiolabeled elf26-derivative elf-^{125}I to intact cells of *Arabidopsis*. Cells were incubated with elf-^{125}I in the absence (shaded circles) or presence of 10 μM unlabeled elf26-Tyr-Cys added at t = 0 min (nonspecific binding, open triangles) or at 25 min (solid triangles). Radioactivity retained on the cells was measured by γ-counting after washing the cells on filters. Results are representative of four independent series of experiments with different batches of cells.

(B) Saturation of binding. Binding to cells incubated with different amounts of elf-^{125}I (200 Ci/mmol) in the absence (total binding, shaded circles) or in the presence of 10 μM elf26 (nonspecific binding, open triangles). The values for specific binding (total binding minus nonspecific binding, closed diamonds) were fitted to a rectangular hyperbola (solid line) giving a B_{max} of 2.1 pmol/g cells and a K_d of 0.8 nM. Saturation was reproduced in an independent assay with a different batch of cells.

(C) Competitive binding assays with unlabeled flg22 and the EF-Tu-derived peptides elf18, elf26, elf26-Pst, and elf12. Results are presented as percentage of total binding which varied between 8,000 and 12,000 cpm in the different batches of cells used for these assays. Competitors were tested at least twice in independent assays.

(D) Chemical crosslinking of elf-^{125}I to *Arabidopsis* cells. After binding of elf-^{125}I in the presence of different concentrations of unlabeled elf26, crosslinking was initiated by the addition of EGS. Radiolabeled proteins were analyzed by SDS-PAGE and autoradiography on a Phosphor Imager. Equal loading was checked by Coomassie staining (not shown).

(E) Quantification (integration units, iu) of radioactivity in the band at 150 kDa (D).

(F) Crosslinking with elf-^{125}I and ^{125}I-flg in the presence of 10 μM unlabeled flg22 or elf26 as indicated.

of cells, flg22 reproducibly induced a slightly higher pH increase than elf18, and cotreatment with flg22 and elf18 resulted in a response that was not significantly different from the treatment with flg22 alone (Figures 2A and 2B). In some other batches of the *Arabidopsis* cell culture, elf18 induced a slightly higher maximal pH shift than flg22. However, the lag-phase for elf18 was always longer than that for flg22, and cotreatment with both peptides never led to a significant increase above the response obtained with the stronger stimulus alone (data not shown). In contrast, an additive effect of the two PAMPs was observed in combined application at nonsaturating doses of the elicitors (Figures 2A and 2B).

Activation of MAP kinases is an early signaling event in plants treated with different pathogen- and wound-related signals (Nakagami et al., 2005). In-gel assays with myelin basic protein (MBP) as a substrate showed rapid, strong, but transient activation of two kinases of ~48 kDa and ~45 kDa in cells treated with 100 nM elf18 or flg22 (Figure 2C). As observed above for medium alkalinization, the overall kinetics of induction was similar for both stimuli, but the onset of the response occurred slightly faster with flg22 than with elf18. In further experiments, kinase activity was compared after treatment of cells for 10 min with flg22, elf18, or a combination of both PAMPs (Figure 2D). No additive effect was detectable for cotreatment with both PAMPs, suggesting that the kinases activated by both PAMPs represent enzymes belonging to a common pool within the cells.

Treatment of *Arabidopsis* leaves with either elf18 or flg22 restricts subsequent growth of virulent *Pst* DC3000 (Kunze et al., 2004; Zipfel et al., 2004). To test for an additive effect of flagellin and EF-Tu, leaves were pressure infiltrated with 100 nM of either one or both peptides (Figure 2E). Bacterial

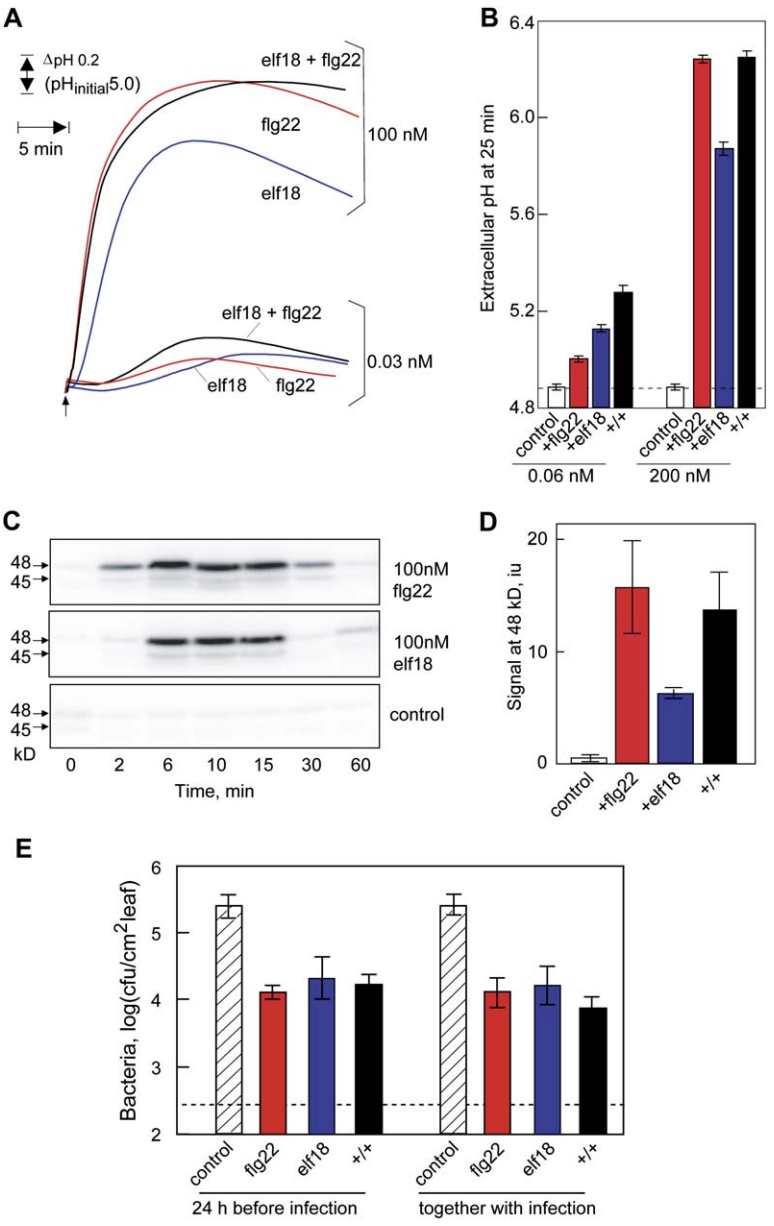

Figure 2. EF-Tu and Flagellin Induce Common Responses in *Arabidopsis*

(A) Extracellular pH in cells treated with flg22 or elf18 as indicated.

(B) Extracellular pH in cells treated for 25 min with flg22, elf18, or both peptides as indicated. Results show means and standard deviations of n = 4 replicates and dashed line indicates pH at t = 0 min.

(C) Time course of MBP kinase activity in mock-treated cells (control) or in cells treated with 100 nM flg22 or 100 nM elf18. Gels were loaded with equal amounts of protein and no additional radiolabeled bands were observed in the parts of the gel clipped from the figure.

(D) MBP kinase activity after 10 min of treatment with 100 nM elf18, 100 nM flg22, or a combination of both peptides. Results show means and standard deviations of the radioactivity in the 48 kDa band from extracts of n = 3 replicate treatments. Linearity of the assay was verified by loading gels with double amounts of extracts (data not shown).

(E) EF-Tu and flagellin limit growth of *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 in *Arabidopsis*. Leaves were pressure infiltrated with 100 nM flg22, 100 nM elf26, or both peptides either 24 hr before or concomitantly with the *Pst* DC3000 (10^5 cfu/ml). Results show bacteria (cfu) in leaves 4 days post-infection as means and standard errors from n = 5 leaves. The dashed line indicates cfu extractable 1 hr after inoculation (0 dpi).

growth restriction observed after a combined treatment with both peptides was not more efficient than pre- or co-treatment with one of the peptides alone (Figure 2E).

Gene Expression and Number of Receptor Sites after Treatment with EF-Tu and Flagellin

Using the whole genome array (ATH1, Affymetrix) we have previously described changes in mRNA levels of ~1000 genes in response to flg22 (Zipfel et al., 2004). Using the same experimental setup, *Arabidopsis* seedlings were treated with EF-Tu-derived peptides for 30 or 60 min. Applying a threshold-filter of 2-fold, 427 genes were upregulated after 30 min treatment with 1 μM elf26, and this number further increased to 866 after 60 min (Table S1 and Figure 3A). A decrease in mRNA levels was observed for 7 genes after 30 min and 83 genes after 60 min. In a further series of experiments, elf18 was found to cause changes in the same set of genes in *fls2* seedlings defective in the flagellin receptor FLS2 (Table S1). In contrast, these genes showed no significant changes after treatment with the inactive peptide elf12 (Table S1). Interestingly, the genes induced or repressed by EF-Tu and flagellin clearly correlate (Figure 3A, Zipfel et al. 2004, and Table S1). There was no evidence for subsets of genes with flagellin- or EF-Tu-specific regulation.

Among the genes that are rapidly induced by flagellin and EF-Tu are >100 of the 610 RLKs present in the genome (Shiu et al., 2004) (Table S2). Interestingly, the flagellin receptor *FLS2* (At5g46330) is one of them, and we wondered whether the increased level of *FLS2*-mRNA

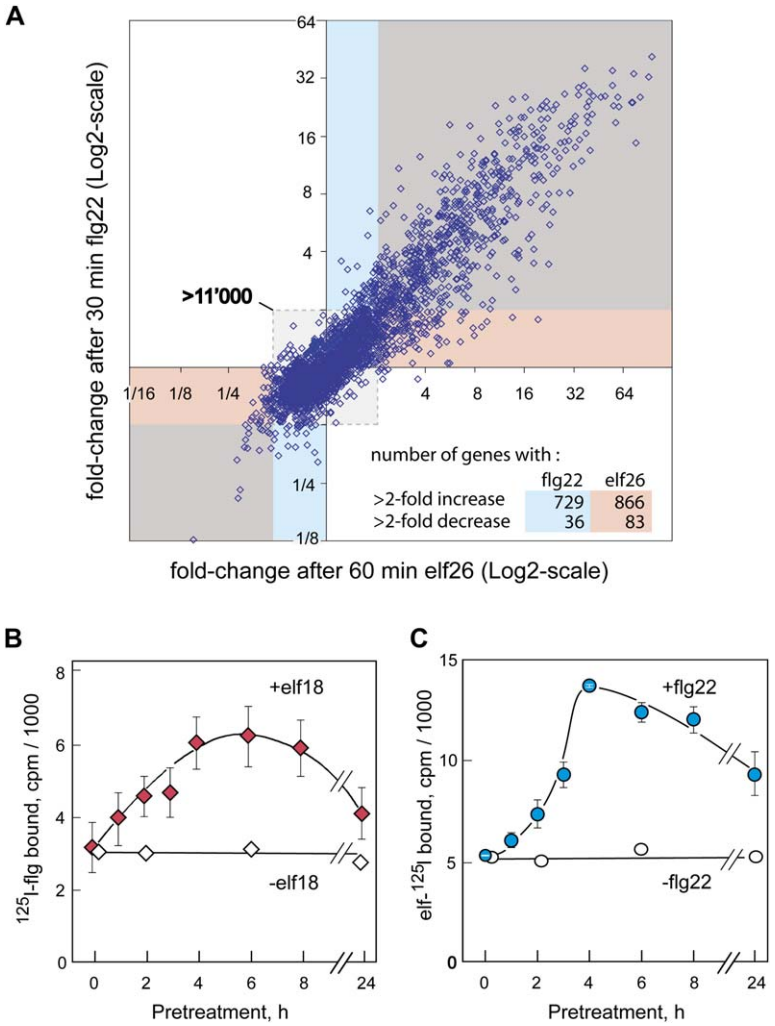

Figure 3. Changes in Transcriptome and in Number of Receptor Binding Sites in Plants Treated with Flagellin and EF-Tu

(A) Whole genome transcriptome analysis with *Arabidopsis* seedlings treated with flg22 and elf26. Fold-changes (log_2) over control levels after treatment with elf26 for 60 min (*x* axis) are plotted against changes previously described for flg22 after 30 min (*y* axis, values from Zipfel et al., 2004).

(B) Receptor sites for flagellin after treatment of seedlings with elf18. Binding was assayed with 10 nM ^{125}I-flg (66 Ci/mmol) in extracts of Col-0 seedlings after mock treatment (−elf18) or treatment with 1 μM elf18 (+elf18). Radiolabel bound in the presence of 10 μM nonlabeled flg22 (nonspecific binding) was ~800 ± 50 cpm for all samples and was subtracted from total binding. Values represent means and standard deviations of n = 3 plant extracts.

(C) Receptor sites for EF-Tu after treatment with flg22. Binding was assayed with 10 nM elf-^{125}I (66 Ci/mmol) in extracts of seedlings after mock treatment (−flg22) or treatment with 1 μM flg22 (+flg22). Nonspecific binding was at ~3000 cpm in all samples and was subtracted from total binding. Values represent mean and standard deviation of n = 3 plant extracts.

results in an increase of flagellin receptor sites and, in turn, whether the number of receptor sites for EF-Tu would change in parallel. Flg22 and elf18 interact with their receptor sites in a nonreversible manner, (Bauer et al., 2001 and Figure 1A), which impedes accurate analysis of binding sites after autostimulation. Thus, we tested the number of binding sites for flagellin after pretreatment with elf18 (Figure 3B) and, vice versa, the number of EF-Tu binding sites after pretreatment with flagellin (Figure 3C). Indeed, in both cases, the number of specific binding sites significantly increased after 1 to 2 hr of pretreatment and reached a level >2-fold higher than controls within 4 to 6 hr of treatment. Similarly, >2-fold increases in specific receptor sites were also observed in cultured *Arabidopsis* cells treated with flg22 or elf18 (data not shown).

Identification of a Mutant Insensitive to EF-Tu

The above results suggest that the EF-Tu receptor is encoded by one of the genes induced by both PAMPs. Since perception of EF-Tu and flagellin share many features, we further speculated that the EF-Tu receptor might involve a RLK related to *FLS2*. Starting with close relatives of FLS2 we established a collection of homozygous T-DNA-tagged mutants for induced leucine-rich repeat (LRR)-RLKs. Similar to flg22 (Gómez-Gómez and Boller, 2000), elf18 induces seedling growth inhibition (Figure 4A). Growth inhibition was observed for wild-type Col-0, the flagellin receptor mutant *fls2*, and all of the T-DNA insertion lines tested with the exception of the line *SALK_044334*, termed *efr-1*, which proved completely insensitive to elf18 (Figure 4A and Figure S1A). In contrast to wild-type and the *fls2 mutant*, *efr-1* plants also did not respond to EF-Tu-derived elicitors with an oxidative burst (Figure 4B), increased ethylene biosynthesis (Figure S1B), or induced resistance to *Pst* DC3000 (Figure S1C). However, *efr-1* seedlings were as sensitive to flg22 as wild-type, suggesting that this line is specifically affected in EF-Tu perception (Figure 4B; Figure S1). Apart from insensitivity to EF-Tu, *efr-1* plants were indistinguishable from wild-type, and no other phenotype became apparent over several generations (data not shown).

When assayed for the presence of receptor sites, specific binding of elf-^{125}I was detectable in extracts from wild-type but not from *efr-1* plants (Figure 4C). Similarly,

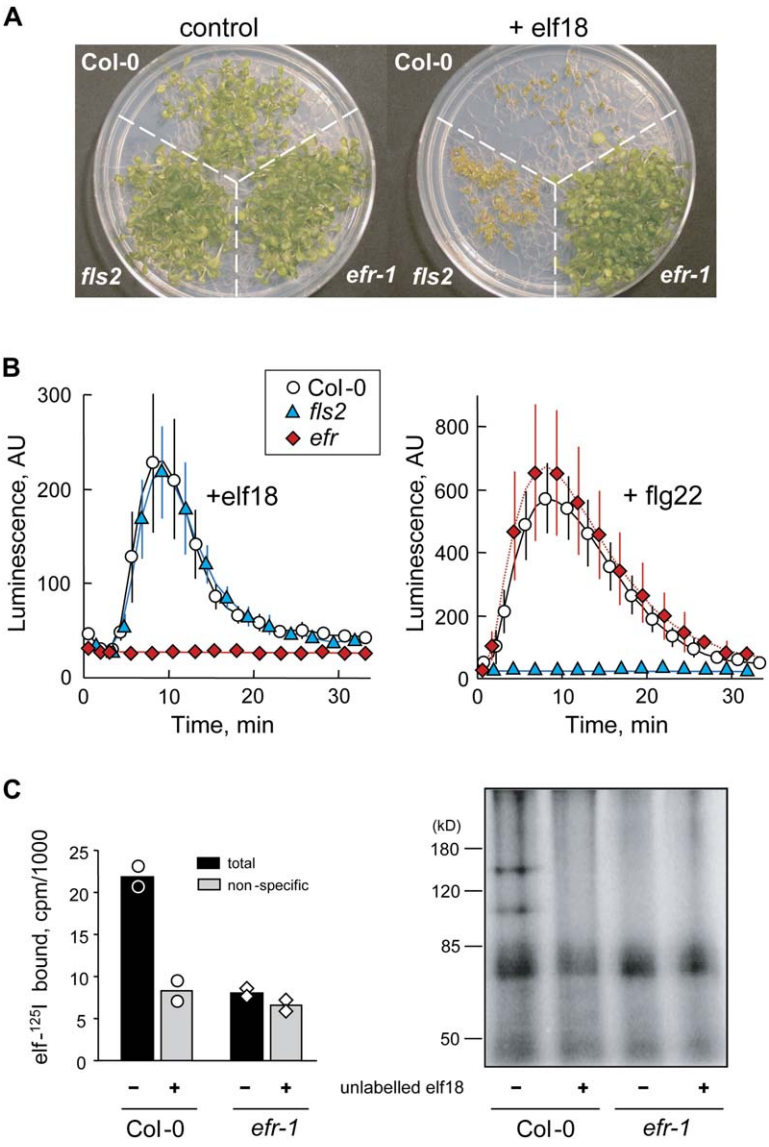

Figure 4. The Mutant *efr-1* Is Insensitive to elf18

(A) Effect of elf18 on seedlings from wild-type Col-0, *efr-1* (SALK_044334), and *fls2*. Five-day-old seedlings were treated with MS medium without (left panel) or with 1 μM elf18 (right panel) and photographed after one week of treatment.

(B) Oxidative burst in leaves of wild-type Col-0, *efr-1*, and *fls2* plants after addition of 1 μM elf18 (left panel) or flg22 (right panel). Results are averages ± standard errors (n = 8).

(C) EF-Tu receptor binding sites are absent in *efr-1* plants. Binding activity (left panel) and chemical crosslinking (right panel) with extracts from wild-type and *efr-1* plants in the absence or presence of 10 μM unlabeled elf18 as competitor. Bars represent means of two replicate measurements (symbols).

crosslinking experiments with *efr-1* extracts did not label the 150 kDa and 100 kDa polypeptides that were specifically labeled in extracts from wild-type (Figure 4C).

EFR Is the EF-Tu Receptor

The line *SALK_044334* (*efr-1*) carries a T-DNA insert in *At5g20480*, which was tentatively termed *EFR* for *EF*-Tu *r*eceptor (Figure 5A). A second insertion line, *efr-2* (*SALK_068675*, Figure 5A), proved insensitive to EF-Tu as well (data not shown). No *EFR* mRNA was detectable in *efr-1* by RT-PCR, and complementation of *efr-1* with wild-type *EFR* restored responsiveness to elf18 (data not shown).

The *EFR* gene codes for a predicted protein with 1031 amino-acid residues (113 kDa) with all the characteristics of a LRR-RLK (Figure 5B). It has a hydrophobic N terminus predicted to act as a signal peptide for secretion, an extracellular domain with 21 tandem copies of a 24-residue LRR (residues 96 to 606). The LRR domain is flanked by two pairs of cysteins with the characteristic spacing observed in several plant LRR-RLKs (Dievart and Clark, 2003). A single trans-membrane domain (residues 650 to 673) is predicted to separate the extracellular from the intracellular domain, which shows all the signatures of a serine-threonine protein kinase (residues 712 to 1000) (Hanks and Quinn, 1991). Like FLS2 (Chinchilla et al., 2006), EFR might be glycosylated at some of its 21 potential N-glycosylation sites (N-X-S/T) in the LRR domain. Thus, the 150 kDa band detected by crosslinking with elf-^{125}I might correspond to the glycosylated EFR protein (Figures 1C and 4C).

Nicotiana benthamiana plants, like all plants outside the *Brassicaceae* tested so far, are nonresponsive to EF-Tu (Kunze et al., 2004). To examine whether this is due to

Figure 5. *EFR* Encodes a LRR Receptor Kinase

(A) Schematic representation of the *EFR* gene (*At5g20480*) with exons as black boxes and the sites of T-DNA insertions in the *efr-1* and *efr-2* mutants indicated by open triangles.

(B) Primary structure of the EFR protein divided into the predicted signal peptide; the N-terminal domain containing paired Cys residues (black boxes connected with a line); the LRR domain with residues identical to the LRR consensus marked in gray; the extracellular juxta-membrane domain with paired Cys residues (black boxes connected with a line); the transmembrane domain with hydrophobic residues (shaded) flanked by basic residues (bold); the intracellular juxta-membrane domain with the putative endocytosis motif YXXØ (underlined); the Ser/Thr kinase domain; and the C-terminal tail.

a lack of functional EFR, we transiently expressed *EFR* in *N. benthamiana* leaves. Transformation with *EFR* resulted in specific binding of elf-^{125}I peptide, while plants agroinfiltrated with a control construct (*FLS2)* showed no binding of EF-Tu (Figure 6A). Importantly, the leaves expressing *EFR* gained responsiveness to EF-Tu, as illustrated by enhanced biosynthesis of ethylene (Figure 6B) and induction of an oxidative burst (Figure 6C). No responses were observed after treatment with elf12 used as a control (Figures 6B and 6C). Also, no response to elf18 was observed in nontransformed leaves (data not shown) or in leaves transformed with the control construct encoding *FLS2* (Figures 6B and 6C).

In summary, *EFR* expressed in *N. benthamiana* plants codes for a functional receptor binding site that can induce PAMP responses when triggered with EF-Tu.

EF-Tu Perception Limits *Agrobacterium tumefaciens* Infection

PAMP perception by plants plays a role in induction of basal resistance, and *fls2* mutants lacking a functional flagellin receptor are more susceptible to spray inoculation with the bacterium *Pst* DC3000 (Zipfel et al., 2004). However, whereas *Pst* DC3000 has a flagellin that is fully active as PAMP in *Arabidopsis*, its EF-Tu exhibits a strongly reduced elicitor activity (Kunze et al., 2004 and Figure 1C). Therefore this pathogen is not suitable for testing the role of EF-Tu perception in activation of basal resistance. In contrast, the plant pathogen *Agrobacterium tumefaciens* has an EF-Tu that is fully active as an elicitor in *Arabidopsis* (Kunze et al., 2004). The infection process of *Agrobacterium* critically depends on the transfer of a part of the bacterial DNA, termed T-DNA, into the genome of the plant hosts (Gelvin, 2005; Escobar and Dandekar, 2003). The T-DNA transfer to plant cells can serve as a convenient readout for the success of *Agrobacterium* to infect its host. To test if EF-Tu perception plays a role in restricting *Agrobacterium* transformation, wild-type (Col-0) and *efr-1* leaves were injected with the hyper-virulent nontumorigenic *Agrobacterium* strain GV3101 containing a binary plasmid with a β-glucuronidase (GUS)-intron construct that allows expression in plants but not in bacteria. In Col-0 leaves only weak GUS activity was detectable at 2 or 4 days post-infection (dpi) while *efr-1* leaves exhibited intense GUS staining (Figure 7A). In several independent repetitions of this experiment, Col-0 showed considerable leaf-to-leaf variation, and transformation in the *efr-1* mutants was always higher and more uniform (Figure 7A). The allelic mutant line *efr-2* exhibited enhancement of transformation as *efr-1*, and *efr-1* plants complemented with a functional *EFR* gene showed the low level of transformation characteristic for wild-type (data not shown). In experiments using spray inoculation with *Agrobacterium*, only very few transformation events, visible as single cells with GUS staining, could be detected in wild-type leaves whereas ~50-fold more events were detectable in *efr* mutants (Figure 7B). This result indicates that EF-Tu recognition lowers frequency of transformation events. To further

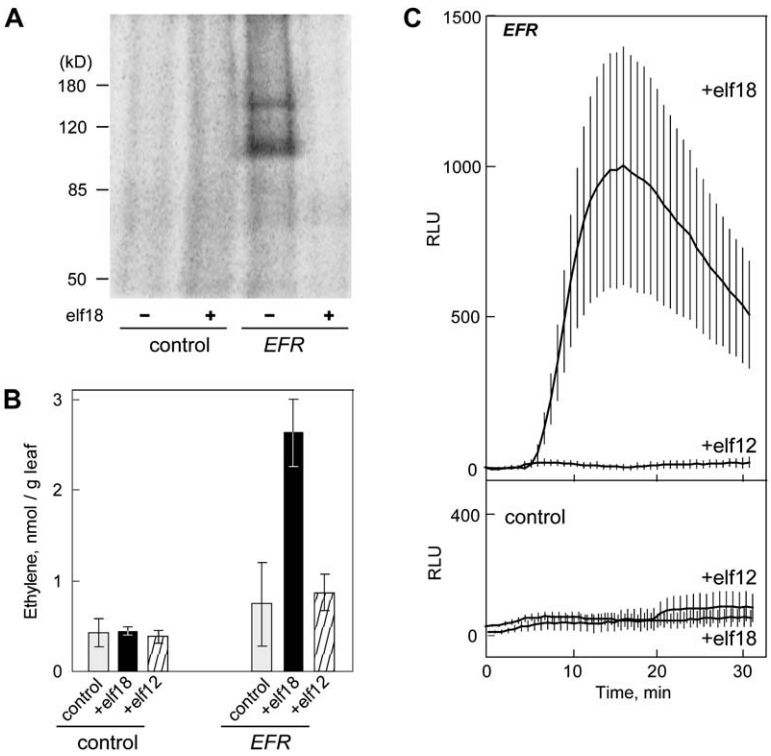

Figure 6. *N. benthamiana* Plants Transformed with *EFR* Show Responsiveness to EF-Tu

(A) EF-Tu receptor binding sites detected by chemical crosslinking in extracts from *N. benthamiana* leaves transiently transformed with EFR or FLS2 (control). Crosslinking with elf-^{125}I was performed in the absence (–) or presence (+) of 10 μM unlabeled elf18 as competitor.

(B) Induction of ethylene biosynthesis in leaves transiently transformed with *EFR* or *FLS2* (control). Leaf pieces were treated with 10 μM elf18 or of the inactive analog elf12 as indicated. Results of ethylene accumulating over 3 hr of treatment are averages ± standard deviations (n = 3).

(C) Oxidative burst in leaf tissues transiently transformed with *EFR* or *FLS2* (control). Luminescence of leaf slices in a solution with peroxidase and luminol was measured after addition of 100 nM el18 or elf12. Results are averages ± standard error (n = 6).

test the effect of PAMP-induced plant defenses on transformation, we injected *Agrobacteria* in combination with saturating doses of flg22 or elf18 and quantified GUS activity in extracts of these leaves at 4 dpi (Figure 7C). Compared to wild-type, *efr-1* mutants showed an 8- to 24-fold increase in GUS activity in four independent experiments (14-fold in the example shown in Figure 7C). Coinjection of flg22 nearly abolished *GUS* expression in leaves of wild-type and *efr-1* plants (Figure 7C). While not affecting transformation in *efr-1* plants, coinjection with elf18 similarly reduced transformation in wild-type plants (Figure 7C).

Interestingly, the enhanced transformation in *efr* mutants did not correlate with a significant increase in number of *Agrobacteria* in these leaves (data not shown). However, about half of the infiltrated *efr-1* leaves developed chlorotic symptoms within one week after inoculation whereas all wild-type leaves remained visually healthy (Figure 7D).

These results show that defense responses activated by PAMPs restrict plant transformation by *Agrobacterium*. In particular, they also clearly indicate EFR-dependent perception of bacterial EF-Tu to occur during *Agrobacterium* infection in planta.

DISCUSSION

EFR Is the *Arabidopsis* Receptor for EF-Tu

In this report we identify the receptor kinase EFR as the EF-Tu receptor based on loss-of-function experiments in *Arabidopsis* and gain-of-function experiments in *N. benthamiana* plants. The presence of a specific, high-affinity binding site for EF-Tu detectable by binding assays and chemical crosslinking correlates with the presence of an intact *EFR* gene in *Arabidopsis* and *N. benthamiana*, supporting the hypothesis that EFR is the receptor site for EF-Tu. Perception of EF-Tu and flagellin exhibit many common characteristics of ligand-receptor interaction and mode of response induction. Consistent with these similarities, EFR and FLS2 are closely related LRR receptor kinases belonging to the subfamily LRR-XII of RLKs (Shiu et al., 2004). Although a number of other PAMPs are perceived by plants (Nürnberger et al., 2004), the corresponding PRRs have been reported only for *Phytophtora* β-glucan in soybean (Umemoto et al., 1997) and for the fungal elicitor EIX (ethylene-inducing xylanase) in tomato (Ron and Avni, 2004). Whereas the glucan binding protein is a soluble, extracellular protein with an intrinsic β-1,3-glucanase activity (Fliegmann et al., 2004), the EIX binding sites are receptor-like proteins with extracellular LRR domains and transmembrane domains but no intracellular kinase domains. The large protein families of RLKs and RLPs probably comprise receptors for further PAMPs. A reverse-genetic approach, as used in this report, might help to identify them among the subgroup induced in response to treatment with PAMPs. A search in the gene expression database Genevestigator (Zimmermann et al., 2004) revealed that *FLS2* and *EFR* are induced also by bacterial LPS, fungal chitin, and the oomycete-derived NPP1. Overall, these different PAMPs seem to trigger changes in a common set of genes, indicating that plants do not distinguish bacteria, fungi, and oomycetes

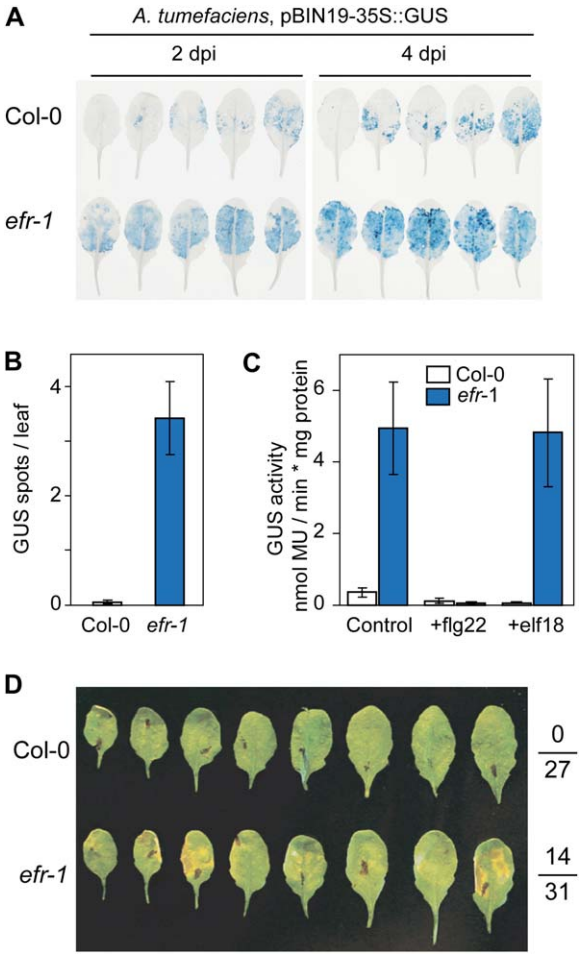

Figure 7. The *efr-1* Mutant Is More Susceptible to *Agrobacterium tumefaciens*

(A) Staining for GUS activity in leaves of wild-type and *efr-1* after infiltration with 2 × 10^8 cfu/ml *Agrobacterium* carrying *pBIN19-35S::GUS*.

(B) Number of cells with GUS staining 4 days after spray-inoculation with 5 × 10^8 cfu/ml *Agrobacterium pBIN19-35S::GUS*. Result show means and standard errors of GUS spots in n = 44 Col-0 leaves and n = 48 *efr-1* leaves.

(C) Quantitative GUS assays with extracts from leaves of wild-type and *efr-1* mutants 4 days after infiltration with 2 × 10^8 *Agrobacterium* carrying *pBIN19-35S::GUS*. Leaves were injected with bacterial suspensions supplemented with 1 μM flg22 or elf18 as indicated. Results represent means and standard errors of independent extracts from n = 6 leaves.

(D) Symptoms of wild-type and *efr-1* leaves 7 days after pressure infiltration with 2 × 10^8 cfu/ml *Agrobacterium*. Values on the right indicate numbers of leaves with chlorotic symptoms versus total numbers of leaves infiltrated with *Agrobacterium*.

on the basis of the signaling signature of their PAMPs. Rather, presence of one type of PAMP seems to serve as an indicator of injury or danger in general.

In plants, the repertoire of PAMPs perceived appears to depend on the species, indicating divergence and rapid evolution of PRRs specificities even between closely related species. In this work we made use of absence of EF-Tu perception in plants outside the *Brassicaceae* to demonstrate the function of the *Arabidopsis EFR* gene when expressed in *N. benthamiana* leaves. Apparently, EFR is the only component missing in these plants for EF-Tu perception. This also indicates feasibility of strategies to introduce additional PAMP recognition systems to crop plants in order to increase their capacity to detect and ward off microbial pathogens.

EFR Perceives a Cytoplasmic Bacterial Protein

EF-Tu is one of the most conserved and abundant proteins in bacteria (Jeppesen et al., 2005), exerting its fundamental role in protein translation in the bacterial cytoplasm. However, our finding that the *efr* mutants of *Arabidopsis* have an enhanced susceptibility to *Agrobacterium* gives strong functional evidence that the EFR receptor, which presumably resides in the plasma membrane of the plant cells, is exposed to bacterial EF-Tu during infection. How and why EF-Tu gets released from the bacterial cells is still unclear, but EF-Tu has been detected in the secretome of *Xanthomonas campestris*, *Pseudomonas fluorescens*, and *Erwinia chrysanthemi* (Watt et al., 2005; Singh et al., 2004; Kazemi-Pour et al., 2004), or in membrane "blebs" constitutively released from *Neisseria meningitidis* (Post et al., 2005). It has also been found associated with the surface of *Mycoplasma pneumoniae* and *Lactobacillus johnsonii* where it appears to play a role in adhesion to mammalian host cells and, notably, as an activator of pro-inflammatory responses (Dallo et al., 2002; Granato et al., 2004).

Convergence of EF-Tu and Flagellin Signaling

Previous studies have shown that flg22 activates a MAP kinase cascade, including MEKK1, MKK4/5, and MPK3/6 (Asai et al., 2002; Nühse et al., 2000). These MPKs are probably part of general stress signaling since they have been found activated by other PAMPs such as chitin and by other biotic or abiotic stress conditions (Nühse et al., 2000; Nakagami et al., 2005). Although not allowing unequivocal attribution to MPK3 and MPK6, the in-gel assays to detect kinase activation indicate that elf18 and flg22 induce the same activities (Figures 2C and 2D). Moreover, combined treatment with both PAMPs induces the same kinases without an additive effect, indicating that these kinases belong to the same pool of enzymes in the cell. Hence, signaling induced by the two PAMPs appears to converge at a step upstream of these kinases. The cytoplasmic domain of EFR is only ~30 % similar to FLS2. Further studies will be required to test whether signaling occurs via common adaptors or by convergence at a later step in the pathway.

Perception of Multiple PAMPs Ensures Efficient Recognition of Pathogens

The apparent redundancy of the chemosensory systems to detect bacteria opens questions on the interplay and functional integration of the different signals. In our experiments with concomitant application of flagellin and

EF-Tu, we could observe additive effects only when both PAMPs were applied at low, nonsaturating doses. A strongly cooperative or synergistic interaction would correspond to regulatory systems obeying the logic of a Boolean "and", with induction of responses only in the presence of both stimuli. This type of signal integration might increase safety for severe decisions such as turning on programmed cell death or a hypersensitivity response. PAMP induced responses, as described here, rather indicate signal integration corresponding to a Boolean "or," which might increase sensitivity and ensure detection of pathogens that manage to evade a single recognition system of the host. Indications for strategies to evade the recognition of flagellin has been obtained for several plant pathogens carrying flg22 domains nondetectable by FLS2 in *Arabidopsis* (Felix et al., 1999; Pfund et al., 2004) and for several human pathogenic bacteria with flagellins that cannot be recognized by TLR5 (Ramos et al., 2004; Andersen-Nissen et al., 2005). Similarly, the N terminus of EF-Tu from some plant pathogens like *Pst* DC3000 and *Xylella fastidiosa* show strongly reduced eliciting activity in *Arabidopsis* (Kunze et al., 2004). Although correlative, these peculiar alterations in this otherwise highly conserved protein might hint at an evolutionary pressure to avoid recognition by the defense system of the plants.

The Plant Basal Defense Restricts *Agrobacterium*-Mediated Transformation

Agrobacterium causes crown gall on many plant species by an infection process relying on transfer and integration of part of its DNA into genome of its host (Escobar and Dandekar, 2003). This property has been widely used to introduce transgenes into plants, fungi, and also human cells (Gelvin, 2005) and has prompted extensive research on the processes by which *Agrobacterium* transfers its T-DNA and manipulates gene expression of its host cells. Recent studies show that *Agrobacterium* interferes with plant defense gene expression (Veena et al., 2003; Ditt et al., 2005) and that the *Arabidopsis cep1* mutant, which constitutively expresses defense-related genes, is more resistant to *Agrobacterium* infection (Zhu et al., 2003). In this work we demonstrate a role of PAMP-induced defense in restricting *Agrobacterium* infection. On the one side, *efr* mutants are clearly more susceptible to transformation than wild-type. On the other side, application of flagellin or EF-Tu almost completely suppresses plant transformation in plants with functional perception for these PAMPs (Figure 6).

N. benthamiana, in contrast to *Arabidopsis*, is highly sensitive to *Agrobacterium* transformation (Wroblewski et al., 2005). Whether part or all of this difference is due to distinct capacities of these plants to detect *Agrobacterium* remains to be tested. On the one side, it will be interesting to measure transformation efficiency in *N. benthamiana* plants stably expressing *EFR*. On the other side, transformation efficiency in *efr* mutants of *Arabidopsis* seems still lower than that observed in *N. benthamiana*, and one might think of further increasing this efficiency by further lowering defense against *Agrobacterium.* Interestingly, *efr* mutants were still responsive to *Agrobacterium* extracts (G.F., unpublished data), suggesting that *Arabidopsis* can detect *Agrobacterium* via PAMPs other than EF-Tu.

Plant defense could interfere at any step of *Agrobacterium*-mediated transformation, starting with attachment of bacteria to the plant cell and ending with gene expression and stable integration of T-DNA into the plant genome. Our studies do not indicate which steps get blocked by the plant defense response, and transient expression of GUS does not necessarily depend on stable integration of the T-DNA into the plant genome. Nevertheless, we can anticipate that plant defense might also affect the frequency of stable transformation. Future studies will address this question that is also of practical interest for genetic engineering. Our results raise the possibility that lowering plant defenses might provide a way to more efficiently transform crop plants recalcitrant to *Agrobacterium* transformation. In turn, our findings also suggest that enhancing the repertoire of PAMP perception might help to engineer crops more resistant to diseases such as the ones caused by *Agrobacterium* species.

EXPERIMENTAL PROCEDURES

Materials

Peptides were synthesized by F. Fischer (Friedrich Miescher-Institute, Basel, Switzerland) or obtained from Peptron (Daejeon, South-Korea). Elf18, elf26 (Kunze et al., 2004), and the C-terminally extended elf26-Tyr-Cys were used as fully active EF-Tu-derivatives, while elf12 was used as an inactive control peptide. Tyr-flg22 and elf26-Tyr-Cys were labeled with [^{125}I] iodine at their Tyrosine residues to yield ^{125}I-Tyr-flg22 (^{125}I-flg) and elf26-^{125}I-Tyr-Cys (elf-^{125}I) with specific radioactivity of 2000 Ci/mmol by Anawa Trading SA (Wangen, Switzerland).

Plant Material

A. thaliana and *N. benthamiana* plants were grown in single pots at 20°C–21°C and 8 hr photoperiod, or on plates containing MS medium (Duchefa), 1% sucrose, and 0.8% agar under continuous light. The *Arabidopsis* cell culture was maintained and used for experiments 4–8 days after subculture as described (Felix et al., 1999).

Bioassays with Plant Tissue and Cell Cultures

Seedling growth inhibition was assessed as described (Gómez-Gómez and Boller, 2000), or seedlings were treated directly on the agar plates by adding liquid MS containing the peptides to be assayed. Oxidative burst, ethylene production, and induced resistance were assayed as described (Felix et al., 1999; Zipfel et al., 2004).

For in-gel MBP protein kinase assays, proteins were extracted as described (Meindl et al., 1998) and kinase activity was determined after re-naturation of proteins separated by SDS-PAGE containing 0.2 % (w/v) MBP (Sigma) (Suzuki and Shinshi, 1995).

Binding Assays and Chemical Crosslinking

Binding assays with intact cells or plant extracts were done as described (Bauer et al., 2001) except that the binding buffer was 25 mM MES (pH 6.0), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM KI, 2 mM KCl, and 1 mM DTT for assays with elf-^{125}I. Radiolabeled peptides were added at concentrations of 0.3 nM in standard assays (specific activity of 2000 Ci/mmol), 10 nM to determine the number of binding sites (66 Ci/mmol), or specified concentrations in saturation studies (200 Ci/mmol). After incubation for 25 min at 4°C, unbound ligands

were removed by filtration (Bauer et al., 2001) except that paper filters were used in binding assays with EF-Tu.

Crosslinking experiments were performed according to Chinchilla et al. (2006) using elf-^{125}I and ^{125}I-flg as radioligands and EGS (ethylene glycol bis(succinimidylsuccinate), Pierce) as crosslinker.

Affymetrix ATH1 Array

Experimental conditions for treatment of *Arabidopsis* seedlings, RNA extraction, microarray hybridizations, and statistical analyses were performed as in Zipfel et al. (2004). The microarray experiment accession number at the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) is E-MEXP-547.

Isolation of T-DNA Insertion Mutants

The *EFR* 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). *EFR*- and T-DNA-specific primers were used to select plants homozygous for the inserts.

EFR Cloning

A 4.1 kb fragment including *EFR* (At5g20480) and 1080 bp of upstream sequence was amplified from Col-0 genomic DNA using the Expand High Fidelity System (Roche) and placed upstream to a GFP coding sequence in a pGEM-T Easy plasmid (Promega). After digestion with NotI, a *EFRp::EFR* fragment was cloned into the binary vector pGREENII/T-0229 (Hellens et al., 2000). The final construct was verified by sequencing and electroporated into *Agrobacterium* EHA101 containing the helper plasmid *pSOUP*.

Agrobacterium-Mediated Transient Expression

For transient expression in *N. benthamiana*, *Agrobacteria* harboring pGREENII-*EFRp::EFR* or pCAMBIA2300-*FLS2p::FLS2* (Zipfel et al., 2004) were grown overnight in YEB medium and transferred to induction medium (Nimchuk et al., 2000) with 50 μM acetosyringone for 4 hr until OD_{600} reached 0.4 to 0.5. Bacteria were diluted with one volume of 10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 μM acetosyringone, and pressure infiltrated into leaves of 4- to 5-week-old *N. benthamiana* plants.

Agrobacterium-mediated expression in *Arabidopsis* was performed as described in Wroblewski et al. (2005) with the *Agrobacterium* GV3101 strain carrying the GUS-intron transgene in pBIN19g. Bacteria were resuspended in water at an OD_{600} of 0.4 for injection into leaves of 3- to 4-week-old plants, or they were resuspended in water with 0.04% Silwett at an OD_{600} of 1 for spray infection. At least 6 plants/genotype and 4 leaves/plant were used per experiment. Qualitative and quantitative measurements for GUS activity were performed according to Jefferson et al. (1987).

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/125/4/749/DC1/.

ACKNOWLEDGMENTS

The authors would like to thank J. Leung (CNRS, Gif-sur-Yvette) for the gift of the *pSOUP* and *pGreen* plasmids, the Salk Institute Genomic Analysis Laboratory (SIGnAL) and the NASC stock center for the T-DNA insertion lines, and D. Hann and J. Rathjen for critical reading of the manuscript. This work was supported by the Swiss National Foundation and the European Molecular Biology Organization.

Received: December 12, 2005
Revised: February 11, 2006
Accepted: March 16, 2006
Published: May 18, 2006

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