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

Gernot Kunze,[a] Cyril Zipfel,[a] Silke Robatzek,[a] Karsten Niehaus,[b] Thomas Boller,[a] and Georg Felix[a,1]

[a] Zürich-Basel Plant Science Center, Botanisches Institut der Universität Basel, CH-4056 Basel, Switzerland
[b] Fakultät für Biologie, Universität Bielefeld, D-33501 Bielefeld, Germany

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 lipopolysaccharides. 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

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
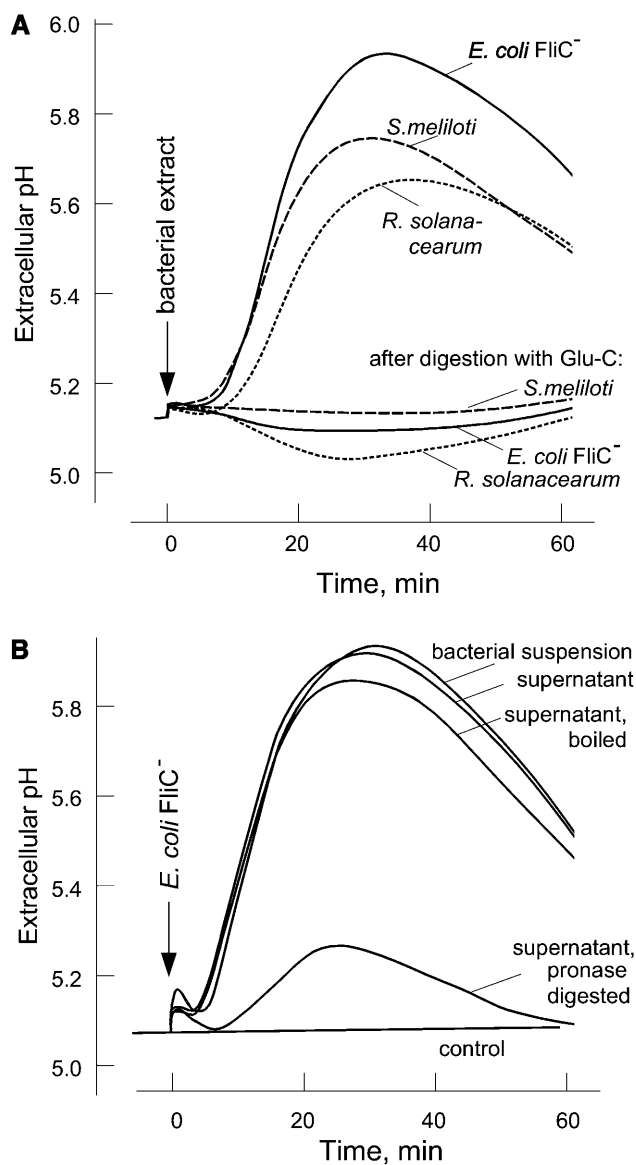

Figure 1. Induction of Extracellular Alkalinization by Bacteria and Bacterial Extracts.

(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
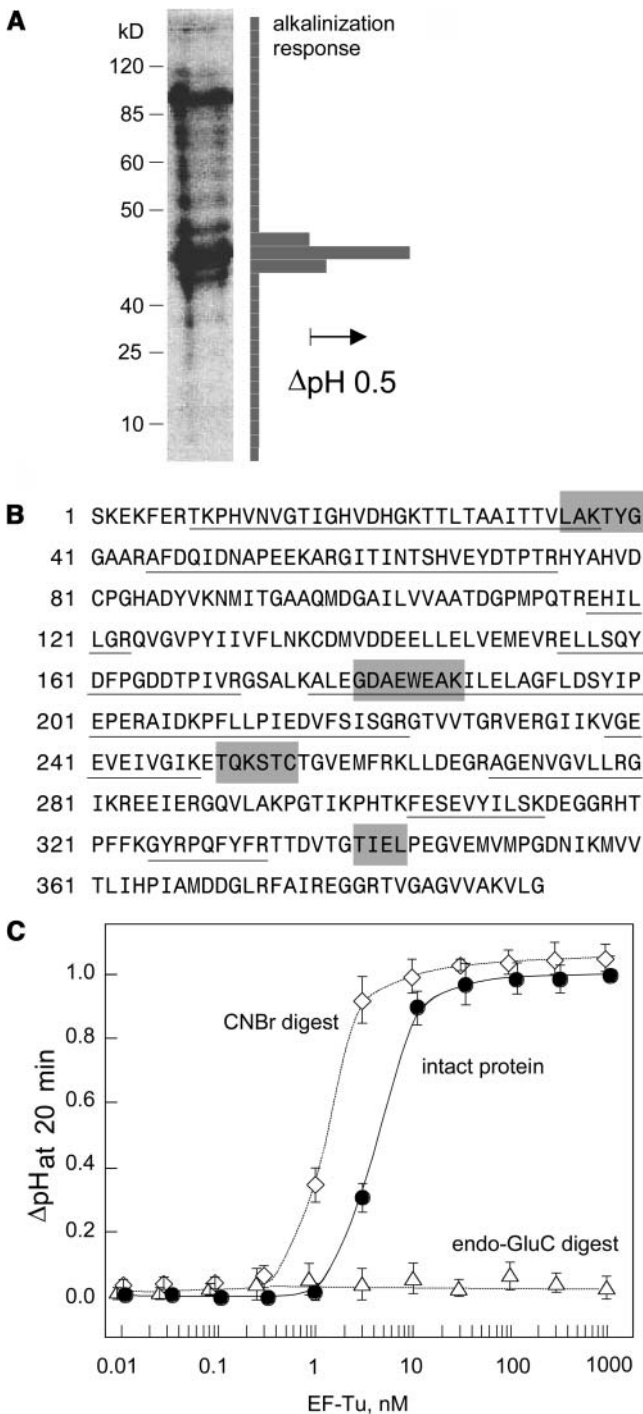
A

B

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  1 SKEKFERTKPHVNVGTIGHVDHGKTTLTAAITTVLAKTYG
 41 GAARAFDQIDNAPEEKARGITINTSHVEYDTPTRHYAHVD
 81 CPGHADYVKNMITGAAQMDGAILVVAATDGPMPQTREHIL
121 LGRQVGVPYIIVFLNKCDMVDDEELLELVEMEVRELLSQY
161 DFPGDDTPIVRGSALKALEGDAEWEAKILELAGFLDSYIP
201 EPERAIDKPFLLPIEDVFSISGRGTVVTGRVERGIIKVGE
241 EVEIVGIKETQKSTCTGVEMFRKLLDEGRAGENVGVLLRG
281 IKREEIERGQVLAKPGTIKPHTKFESEVYILSKDEGGRHT
321 PFFKGYRPQFYFRTTDVTGTIELPEGVEMVMPGDNIKMVV
361 TLIHPIAMDDGLRFAIREGGRTVGAGVVAKVLG
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C

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,

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

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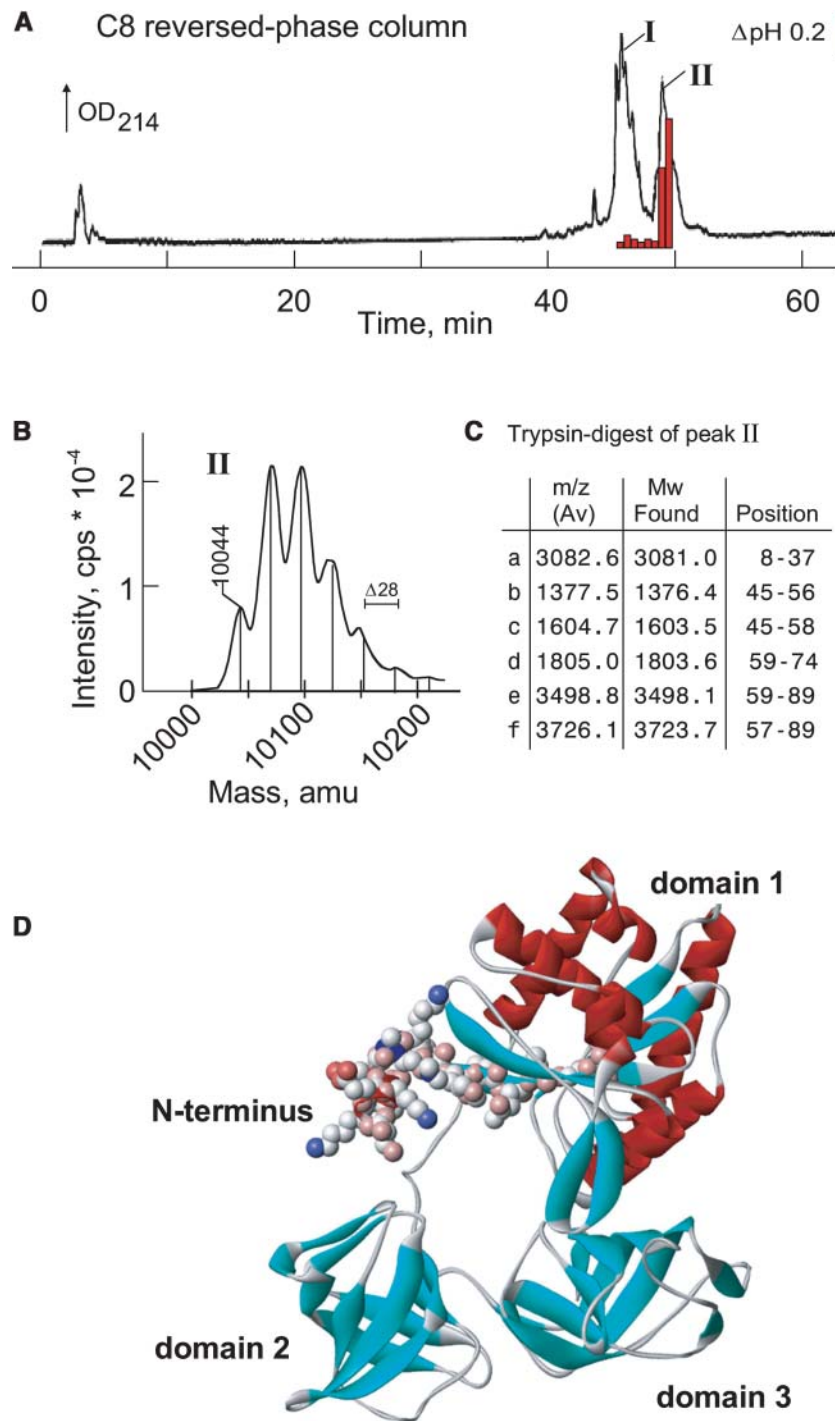
Figure 3. Identification of the CNBr Fragment Carrying Elicitor Activity.

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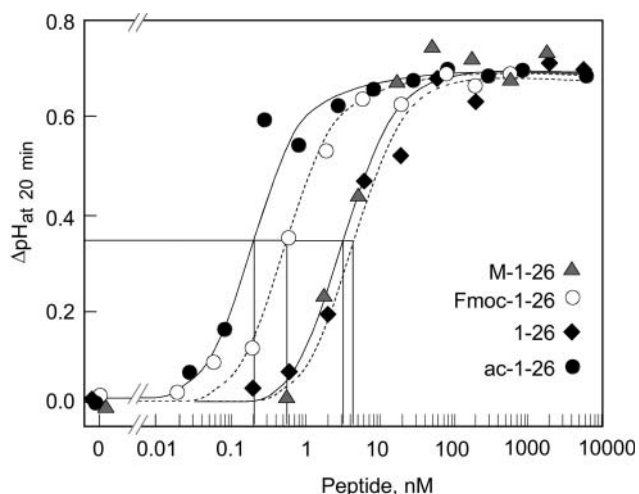
Figure 4. Elicitor Activity of Peptides Representing the N Terminus of EF-Tu.

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* Gl826 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.
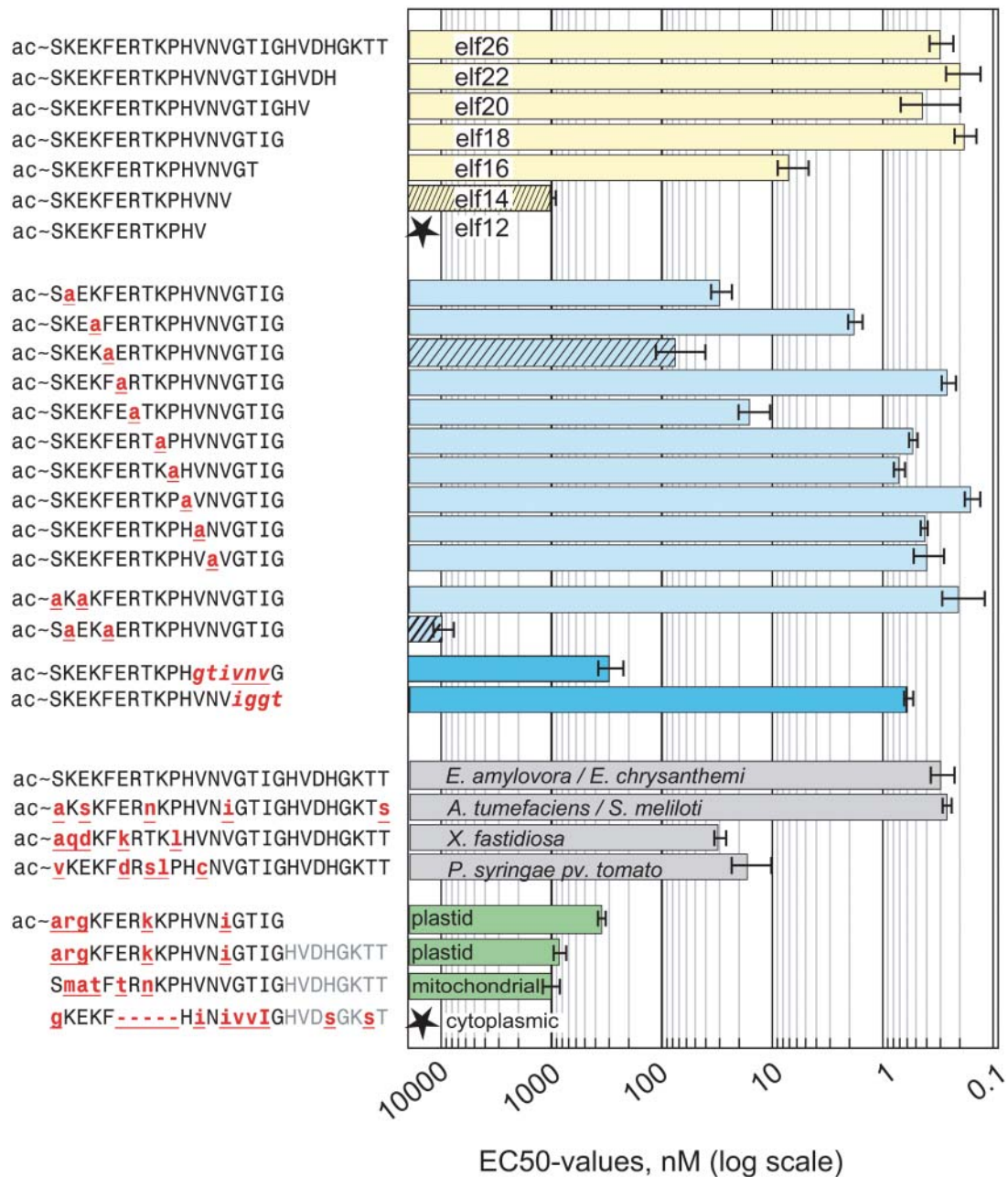
Figure 5. Alkalinization-Inducing Activity of EF-Tu N-Terminal Peptides.

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
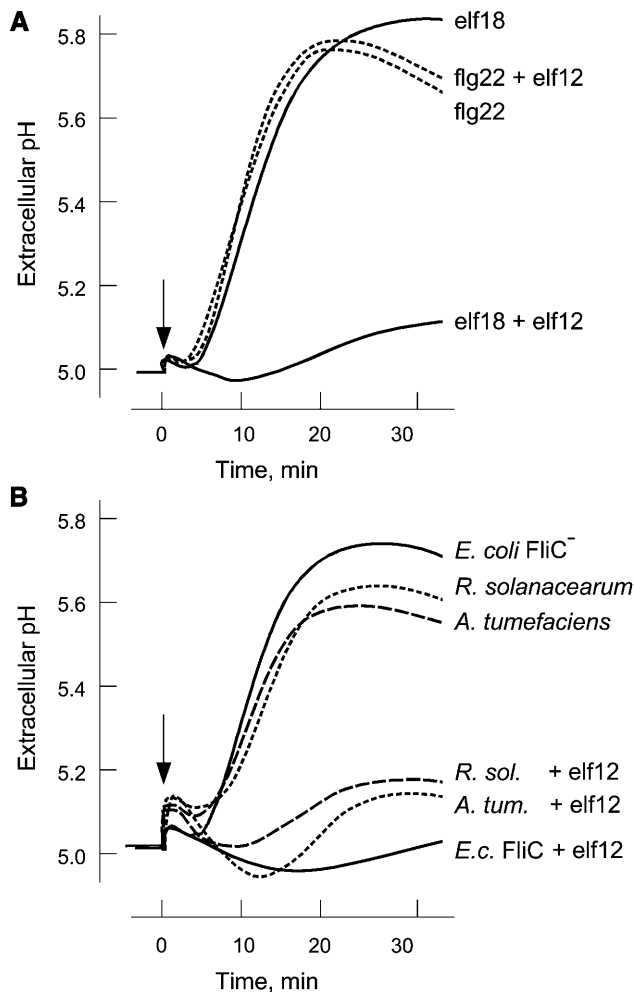
A

B

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
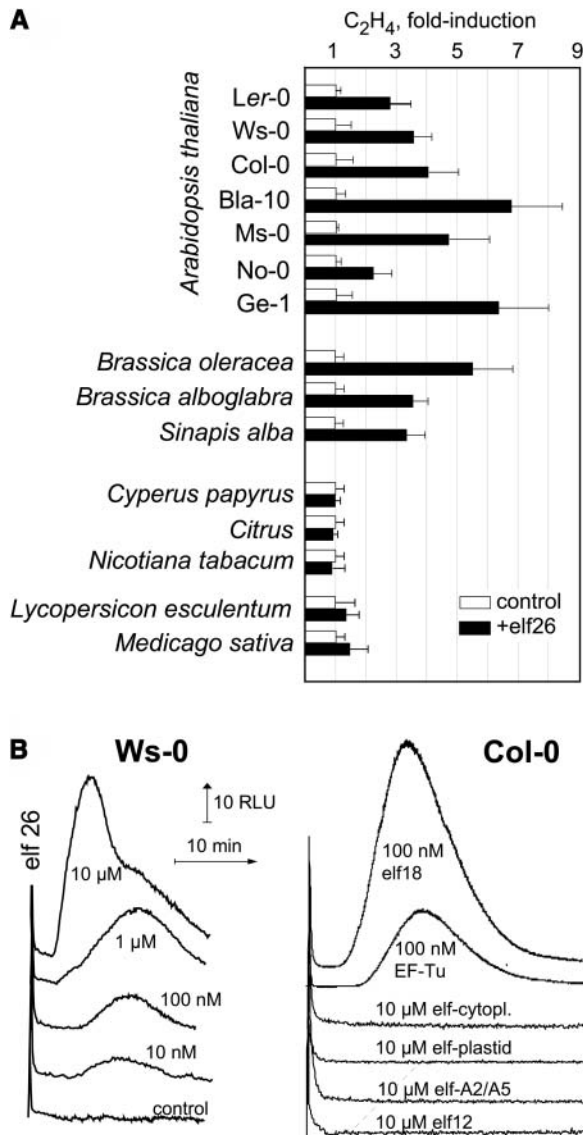
A

B

Ws-0   **Col-0**

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
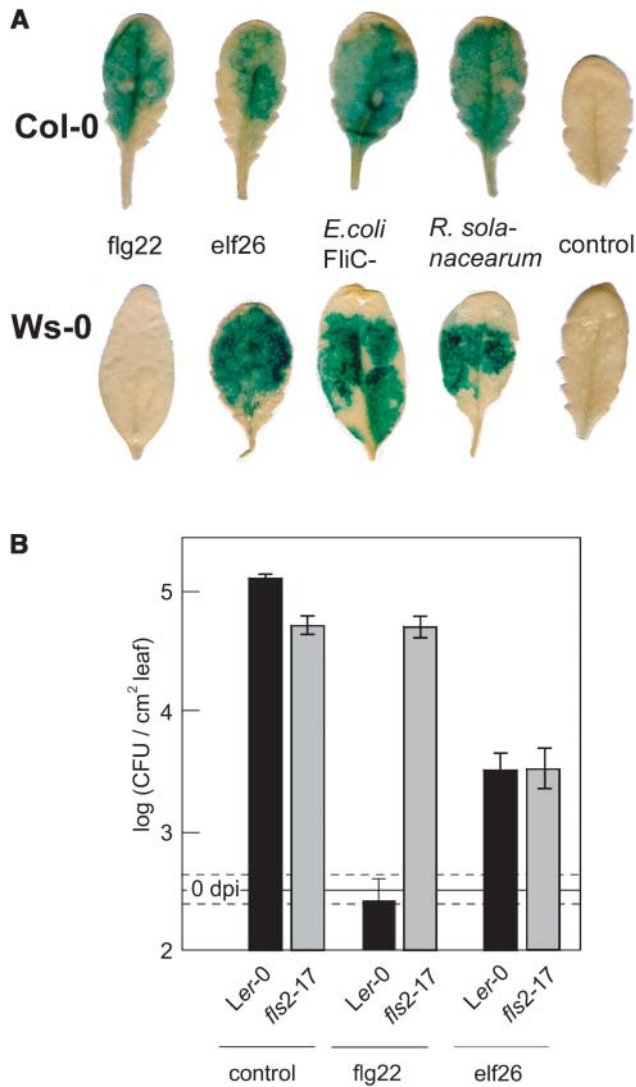
A

Col-0

flg22   elf26   *E.coli* FliC-   *R. sola-nacearum*   control

Ws-0

B

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 pro-karyotes.

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 cyto-plasm, 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. Re-cently, 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 in-duced 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, endopro-tease Arg-C, endoprotease Asp-N, endoprotease Lys-C, and endopro-tease 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 solana-cearum* (from DSM, Braunschweig, Germany) were grown in King's B broth at 26°C on a rotary shaker. Bacteria were harvested by centrifuga-tion 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 frac-tionated 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 seg-ments. 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 Biosys-tems/MDS Sciex, Thornhill, Ontario, Canada). Peptides further digested with trypsin were analyzed by matrix-assisted laser desorption/ioniza-tion–time of flight analysis on a TofSpec 2E.

Plant Cell Cultures and Alkalinization Response

The *Arabidopsis thaliana* cell culture (May and Leaver, 1993) was main-tained 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 (needle-less 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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