

Flagellin Is Not a Major Defense Elicitor in *Ralstonia solanacearum* Cells or Extracts Applied to *Arabidopsis thaliana*

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The phytopathogenic bacterium *Ralstonia solanacearum* requires motility for full virulence, and its flagellin is a candidate pathogen-associated molecular pattern that may elicit plant defenses. Boiled extracts from *R. solanacearum* contained a strong elicitor of defense-associated responses. However, *R. solanacearum* flagellin is not this elicitor, because extracts from wild-type bacteria and *fliC* or *flhDC* mutants defective in flagellin production all elicited similar plant responses. Equally important, live *R. solanacearum* caused similar disease on *Arabidopsis* ecotype Col-0, regardless of the presence of flagellin in the bacterium or the FLS2-mediated flagellin recognition system in the plant. Unlike the previously studied flg22 flagellin peptide, a peptide based on the corresponding conserved N-terminal segment of *R. solanacearum*, flagellin did not elicit any response from *Arabidopsis* seedlings. Thus recognition of flagellin plays no readily apparent role in this pathosystem. Flagellin also was not the primary elicitor of responses in tobacco. The primary eliciting activity in boiled *R. solanacearum* extracts applied to *Arabidopsis* was attributable to one or more proteins other than flagellin, including species purifying at approximately 5 to 10 kDa and also at larger molecular masses, possibly due to aggregation. Production of this eliciting activity did not require *hrpB* (positive regulator of type III secretion), *pehR* (positive regulator of polygalacturonase production and motility), *gspM* (general secretion pathway), or *phcA* (LysR-type global virulence regulator). Wild-type *R. solanacearum* was virulent on *Arabidopsis* despite the presence of this elicitor in pathogen extracts.

Swimming motility, mediated by extracellular flagella, allows bacteria to move in response to their environment (Moens and Vanderleyden 1996). The structural components of flagella include a basal body capable of rotary motion, a hook apparatus, and thousands of flagellin monomers that polymerize to form the flagellar filament (Schuster and Khan

1994). Polymerization is facilitated by conserved domains near both the N- and C-termini of each flagellin protein, while the central domain is hypervariable (Wilson and Beveridge 1993). In addition to its role in motility, flagellin is a known elicitor of animal innate immune responses mediated by toll-like receptors (Aderem 2001; Imler and Hoffmann 2001; Jacchieri 2003; Khush and Lemaitre 2000; Kopp and Ghosh 1994; Sieling and Modlin 2002). Flagellin can also elicit defense responses in plants. A series of studies with plant pathogenic and nonpathogenic bacteria have demonstrated that flagellin contains pathogen-associated molecular patterns that can be recognized by some plants, leading to activation of defense responses to counter pathogen attack (Che et al. 2000; Felix et al. 1999; Gomez-Gomez and Boller 2002). However, host plant perception of flagellin has not been shown to successfully limit bacterial growth or disease damage in plants.

We have investigated the role of motility in *Ralstonia solanacearum*. *R. solanacearum* is the causal agent of bacterial wilt, a vascular disease that affects over 50 families of plants worldwide, including tomato, potato, tobacco, banana, and plantain. This soil-borne disease causes substantial crop losses in the tropics and subtropics, resulting in great economic and human hardship (Hayward 1991). Nonmotile mutants of *R. solanacearum* are significantly reduced in virulence on tomato plants, indicating that motility plays an important role (Tans-Kersten et al. 2001). *R. solanacearum* mutants lacking flagella caused reduced disease in a naturalistic soil soak inoculation assay that requires the bacteria to find and invade plant roots from the soil but caused wild-type levels of disease when introduced directly into the plant vasculature (Tans-Kersten et al. 2001). This suggests that motility plays a role early in infection and colonization. In several animal host systems, bacterial motility has been shown to be an important virulence factor mediating adhesion, invasion, and colonization (Giron et al. 2002; Ottemann and Lowenthal 2002; Tomich et al. 2002; Young et al. 2000).

As motility contributes to virulence and flagella are readily apparent on the bacterial cell surface, it is reasonable that plants might have evolved means to perceive flagella and subsequently activate defense mechanisms. However, this type of activity would have to be carefully balanced, because unnecessary defense activation in response to benign or beneficial bacteria would be likely to carry selective penalties. The discovery of flagellin perception by plants originated from the observation that many phytopathogenic bacteria can trigger an oxidative burst in the host when added to suspension-cultured plant cells

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(Felix et al. 1999; Gomez-Gomez and Boller 2002). The oxidative burst is an indicator of defense activation in plants (Baker and Orlandi 1995; Doke 1983). Bacterial extracts from *Pseudomonas syringae* pv. *tabaci* were most effective at eliciting an oxidative burst when they were heat-denatured by boiling (Felix et al. 1999). Biochemical analysis of these boiled extracts demonstrated that flagellin was the major protein responsible for eliciting an oxidative burst in tomato suspension culture cells (Felix et al. 1999). Flg22, a 22-amino acid peptide derived from a conserved domain in the N-terminus of many flagellins, is sufficient for elicitation of the oxidative burst in tomato, tobacco, potato, and *Arabidopsis* suspension-culture cells and may serve as a pathogen-associated molecular pattern for certain plants (Felix et al. 1999). Similar flg22 peptides based on flagellins from a wide range of gram-negative bacteria, including some phytopathogens, elicited a response. However, *Agrobacterium*- and *Rhizobium*-based peptides were inactive (Felix et al. 1999). *R. solanacearum* sequences apparently were not tested.

In *Arabidopsis* seedlings, flg22 peptide can elicit rapid defense-associated responses such as callose deposition, an oxidative burst, and induction of pathogenesis-related (PR) gene expression (Felix et al. 1999; Gomez-Gomez et al. 1999). These defense responses are associated with a measurable inhibition of seedling growth upon prolonged treatment with flg22 (Gomez-Gomez et al. 1999). Importantly, recognition of flg22 has been attributed to the leucine-rich repeat (LRR) receptor-like kinase FLS2, as *Arabidopsis* lines carrying a mutated version of this receptor fail to respond to flg22 (Gomez-Gomez and Boller 2000). Additional studies of FLS2 have suggested that flg22 may bind directly to FLS2 and have identified a kinase cascade that functions downstream of FLS2 (Asai et al. 2002; Bauer et al. 2001; Gomez-Gomez and Boller 2002; Gomez-Gomez et al. 2001).

We sought to investigate the role of *R. solanacearum* flagellin in the elicitation of defense in *Arabidopsis*. We hypothesized that boiled extracts from *R. solanacearum* would elicit defense-associated responses in plants and that *R. solanacearum* strains lacking flagellin would not induce these responses. We further hypothesized that bacterial strains able to elude detection by the plant's flagellin-recognition system would have enhanced virulence. To test these hypotheses, we measured *Arabidopsis* seedling responses to boiled extracts from flagellate and aflagellate *R. solanacearum* strains and to a flg22 peptide based upon *R. solanacearum* flagellin. We detected one or more defense-eliciting proteins in boiled extracts

of *R. solanacearum*. However, flagellin and the host FLS2 receptor were neither associated with response elicitation nor with overall reduction of virulence.

RESULTS

Extracts from aflagellate *R. solanacearum* strains elicit a defense response in *Arabidopsis* seedlings.

Previous studies using *Arabidopsis* seedlings demonstrated activation of defense responses upon exposure to the consensus flagellin-derived peptide flg22 (Felix et al. 1999). Seedlings exhibited induction of several classic defense response marker genes, including *PR-1*, within 24 h of treatment with flg22 (Asai et al. 2002; Gomez-Gomez et al. 1999). Because wild-type *R. solanacearum* K60 synthesizes flagellin, we hypothesized that exposure of *Arabidopsis* seedlings to boiled extracts of K60 should result in increased expression of defense marker genes. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to test for induction of the classic defense marker *PR-1*. Seedlings treated with either flg22 or with *R. solanacearum* K60 boiled extracts showed increased expression of *PR-1* after 24 h (Fig. 1).

If flagellin is the major elicitor of *PIZ* gene induction in boiled extracts from K60, then boiled extracts from strains lacking flagellin should not induce *PR-1* gene expression. To test this hypothesis, *Arabidopsis* seedlings were treated with boiled extracts from two mutant strains of *R. solanacearum*. Strain K701 carries an insertion in the *fliC* gene encoding flagellin and K702 carries an insertion in the *flhDC* locus, which encodes the master regulator of flagellin biosynthesis (Table 1) (Tans-Kersten et al. 2001; Tans-Kersten et al. 2004). Both of these strains are completely nonmotile in culture and in planta

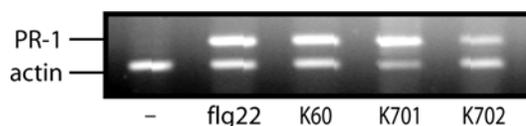


Fig. 1. Boiled extracts of *Ralstonia solanacearum* lacking flagellin induce defense gene expression. *PR-1* RNA levels as detected by reverse transcription-polymerase chain reaction in whole Col-0 seedlings 24 h after treatment with water, 11 μ g flg22, or 45 μ g (total protein) of the indicated boiled bacterial extracts from K60 (wild-type), K701 (*fliC*), or K702 (*flhDC*). The top band was amplified using primers for *PR-1*, the bottom band was amplified using primers for actin (*ACT2*).

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
DH5 α	F ⁻ endA1 relA ϕ 80 <i>lacZAM15 hsdR17 supE44 thi-1 recA1 gyrA96</i>	Hanahan 1983
<i>Ralstonia solanacearum</i>		
K60	Wild-type, race 1, biovar 1, tomato isolate	Kelman 1954
K71	Lacks the regulator <i>pehSR</i> , causing decreased polygalacturonase production and non-motile phenotype K60 <i>pehR::Tn3-uidA</i> , Km	Allen et al. 1997
K701	Disrupted gene for flagellin K60 <i>fliC::Gm^r</i>	Tans-Kersten et al. 2001
K702	Disrupted master regulator of flagellin biosynthesis K60 <i>flhDC::Gm^r</i>	J. Tans-Kersten, unpublished data
K100	Disrupted general secretion pathway K60 <i>gspM::Km^r</i>	This study
K200	Disrupted <i>hrpB</i> regulatory locus K60 <i>hrpB::Gm^r</i>	This study
AW-D	Wild-type race 1 biovar 1 tomato isolate AW-1 <i>gspM::Tn5, Km^r</i>	Kang et al. 1994
K60- <i>phcA</i>	Disrupted LysR-type global regulator of virulence factors K60 <i>phcA::strept</i>	Allen et al. 1997
Plasmids		
pSTBlue-1	A-T cloning vector, Km ^r	Novagen, Madison, WI, U.S.A.
pUCGM	Contains gentamycin resistance cassette, Gm ^r	Novagen
pHRPB1	1,400-bp <i>hrpB</i> fragment in <i>EcoRV</i> site of pSTBlue-1	This study
pHRPB2	PhrpB1 with 850-bp Gm ^r cassette in <i>BglII</i> site of <i>hrpB</i>	This study

^a Km = kanamycin, Gm = gentamycin, and ^r = resistance.

(Tans-Kersten et al. 2001; Tans-Kersten et al. 2004). Furthermore, boiled extracts prepared from strains K701 and K702 grown in culture lacked an approximately 30-kDa band presumed to be flagellin, when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). *Arabidopsis* seedlings treated with boiled extracts from strains K701 and K702 for 24 h showed increased PR-1 expression, similar to the response to boiled extracts from the wild-type strain K60 (Fig. 1). This suggests that flagellin is not necessary for the observed increase in PR-1 gene expression in *Arabidopsis* seedlings in response to these boiled extracts.

Extracts of aflagellate *R. solanacearum* strains elicit growth inhibition in *Arabidopsis* seedlings.

Previous results using *Arabidopsis* seedlings showed that prolonged exposure to the flagellin-derived peptide flg22 resulted in marked growth inhibition, correlating with the activation of defense responses (Gomez-Gomez et al. 1999). Growth inhibition is common in *Arabidopsis* constitutive disease-resistance mutants (Bowling et al. 1994; Clarke et al. 1998; Greenberg and

Ausubel 1993; Maleck et al. 2002; Petersen et al. 2000; Yu et al. 1998). Therefore, we used growth inhibition as a facile, indirect measure of defense gene activation (Fig. 1) (Gomez-Gomez et al. 1999). *Arabidopsis* seedlings grown for 14 days in the presence of boiled extracts of *R. solanacearum* K60 were significantly reduced in weight compared with water-treated controls (Fig. 2A). The level of growth inhibition caused by 45 μg (total protein) of boiled extract per well was comparable to the growth inhibition caused by 11 μg (7.5 μM) flg22 peptide, which was previously characterized as a strong growth inhibitor (Gomez-Gomez et al. 1999). This effect was detectable within 7 days of treatment (data not shown). This growth inhibitory response was not due to a general inhibition caused by the presence of bacterial proteins at this concentration, as similarly prepared extracts from another phytopathogenic bacterium, *Xanthomonas campestris* pv. *campestris*, applied at this same concentration did not inhibit growth (C. Pfund and A. F. Bent, unpublished data).

Arabidopsis seedlings treated with boiled extracts from aflagellate *R. solanacearum* K701 and K702 demonstrated significant growth inhibition that was indistinguishable from the response to extracts from the wild-type parent strain K60 (Fig. 2A). Moreover, the dose-response curve was the same for seedlings treated with boiled extracts from all three strains of *R. solanacearum*, suggesting that growth inhibition by these strains is due to one or more similar elicitors (Fig. 2B). Together, the above results (Figs. 1 and 2) suggest that flagellin is not necessary for defense gene activation or the correlative growth inhibition of *Arabidopsis* seedlings caused by boiled extracts of *R. solanacearum*.

Elicitation does not occur through the *Arabidopsis* FLS2 receptor.

We tested the ability of the *R. solanacearum* extracts to elicit a growth response in *Arabidopsis* seedlings lacking functional FLS2. FLS2 is a transmembrane LRR receptor kinase that has been shown to bind flg22 and activate defense gene transcription in *Arabidopsis*; mutations in *FLS2* render *Arabidopsis* seedlings unresponsive to the flg22 peptide (Gomez-Gomez and Boller 2000). If flagellin is the primary elicitor in boiled extracts of K60 and if FLS2 is the primary flagellin receptor, then plants lacking FLS2 would be expected to show less of a response to boiled extracts. In addition, if the *R. solanacearum* nonmotile mutants K701 and K702 retain some elicitation capacity because of an undetected ability to synthesize low levels of flagellin (K702) or an N-terminal fragment of flagellin

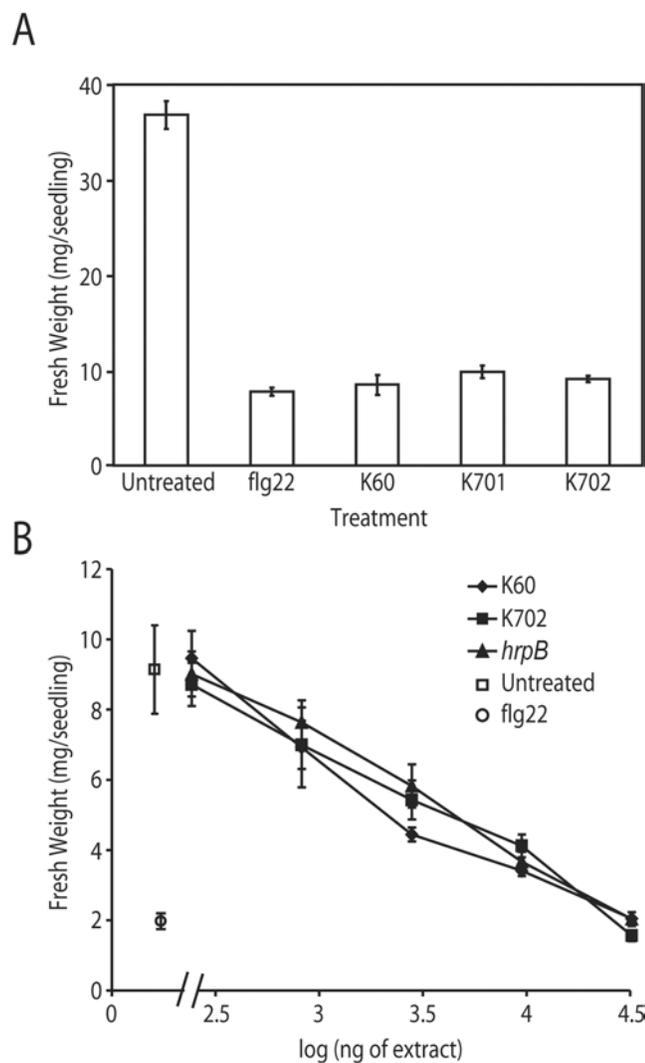


Fig. 2. Boiled extracts of *Ralstonia solanacearum* lacking flagellin elicit a growth inhibition in *Arabidopsis* seedlings. Average fresh weight measurements of nine *Arabidopsis* Col-0 seedlings. Seedlings **A**, 14 days after treatment with water, 11 μg flg22, or 45 μg (total protein) of the indicated boiled bacterial extracts and **B**, 7 days after treatment with water, 11 μg of flg22 (open symbols), or varying levels of the indicated extracts (closed symbols) ranging from 0.35 μg to 35 μg total protein. Bars represent standard error of the mean.

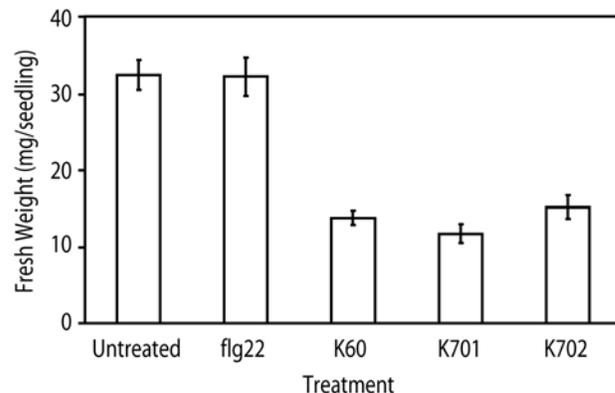


Fig. 3. Elicitation of growth inhibition does not require FLS2 receptor. Average relative fresh weight measurements of nine *Arabidopsis fls2-101* seedlings treated with water, 11 μg flg22, or 45 μg of the indicated boiled bacterial extract. Fresh weights of seedlings were determined 14 days after treatment. Bars represent standard error of the mean.

(K701), one could hypothesize that disruption of the putative flagellin receptor would detectably reduce the responsiveness of seedlings to boiled extracts from these strains.

We identified an *Arabidopsis* line homozygous for a T-DNA insertion in FLS2 (*fls2-101*). As expected, *flsr2-101* seedlings were unresponsive to flg22 (Fig. 3). However, *fls2-101* seedlings did demonstrate a marked response when treated with boiled extracts from K60, K701, and K702 (Fig. 3). In replicated experiments, there were no reproducible differences between the weights of Col-0 and *fls2-101* seedlings in response to a given extract (Figs. 2A and 3). These data suggest that the primary elicitor in *R. solanacearum* is not flagellin and demonstrate that this elicitation does not require the flagellin receptor FLS2.

Tobacco responds to nonmotile bacterial cells and extracts.

We also wanted to determine if cell-associated proteins from wild-type and aflagellate *R. solanacearum* strains could elicit a response in tobacco (*Nicotiana tabacum*), a natural host of strain K60. We infused the apoplastic space of tobacco leaves with live bacteria or semipurified cell-associated proteins from cells grown in minimal medium. When live cultures of K60, K701, and K702 were infused into tobacco leaves at 1×10^7 CFU/ml, the infused leaf tissue became chlorotic after 18 h and was completely collapsed and necrotic 40 h after infusion, with no difference between strains (data not shown). There was a minor response to bacterial protein extracts. At 500 μ g/ml boiled extracts from K701, K702, and wild-type K60 all caused slight chlorosis (leaf yellowing) after 24 h and showed marked chlorosis after 76 h, with no distinguishable difference between treatments. There was no detectable plant response from extracts applied to leaves at 50 μ g/ml, possibly because the relevant protein concentrations were too low. These results suggest that cell-associated proteins from live *R. solanacearum* cells or crude bacterial preparations can elicit a response in tobacco leaves, even in the absence of the putative flagellin elicitor.

The flg22 region of K60 does not elicit a response in *Arabidopsis*.

Our results suggest that flagellin is not the major elicitor in *R. solanacearum*. However, we wanted to further investigate the eliciting capacity of *R. solanacearum* flagellin by focusing on its flg22 region. This region is more conserved across diverse bacterial species than are most other regions of bacterial flagellin. Peptides based on the flg22 sequence from *Pseudomonas aeruginosa*, *Escherichia coli*, and other species have strong eliciting activity (Felix et al. 1999). We compared the amino acid sequence of the consensus flg22 oligopeptide to that of the same region in the flagellin of *R. solanacearum* K60 (flg22-K60). The flg22-K60 sequence differs from the previously studied flg22 sequence at several positions, some of which have previously been shown to be critical for full elicitation in tomato cells (Fig. 4A). In particular, the Gly to Ala change at position 18 has previously been shown to reduce the alkalization-inducing activity of flg22 in tomato cells by 96% (Felix et al. 1999).

To test the elicitation ability of the flg22-K60, *Arabidopsis* seedlings were treated with 10 μ M of either flg22 or flg22-K60 for seven days. Seedlings heated with the flg22-K60 did not show a significant growth inhibition compared with untreated controls, while the original flg22 peptide did (Fig. 4B). This absence of flg22-K60 perception by the plant may be coincidental or may have arisen due to selective pressures. For example, the flg22 region of *R. solanacearum* flagellin may have evolved to escape detection by the plant FLS2-flagellin detection system.

The boil-resistant elicitor from *R. solanacearum* is protein.

We pursued initial structural characterization of the boil-resistant, nonflagellin elicitor. Extracts of *R. solanacearum* K60 treated with proteinase K no longer elicited significant growth inhibition in *Arabidopsis* seedlings (Fig. 5A). Treatment of K60 extracts with a generic protease gave similar results (data not shown). These data suggest that the elicitor is a protein. Gel filtration over Sephadex G-50 separated elicitation activity into both higher and lower molecular weight (MW) fractions (Fig. 5D and E). Elicitation from the later fractions was assignable to low-MW proteins, as opposed to the tail of a broad peak of higher-MW proteins because, in separate experiments, elicitation activity was present in extracts that had been centrifuged through filters with a stated molecular mass cut-off range centered at 5 kDa (Fig. 5C).

The possibility that the elicitor is a small peptide was also investigated, as some well-known microbially derived plant defense elicitors and toxins are small peptides (Hahn 1996; Scheel and Parker 1990). K60 extracts reproducibly showed similar elicitation activities before and after extensive dialysis, using dialysis membrane with a stated molecular mass cut-off range centered at 7 kDa (Fig. 5B). Extracts dialyzed using membranes with a molecular mass cut-off of 10 kDa in four independent experiments showed either no detectable reduction in elicitation or some reduction of elicitation (data not

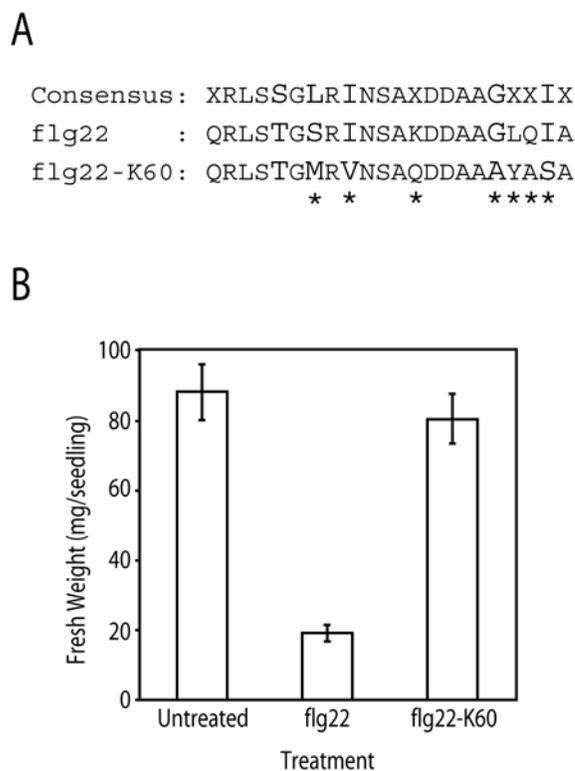


Fig. 4. A peptide derived from the flg22-region of K60 flagellin does not elicit a growth response in *Arabidopsis* seedlings. **A**, Amino acid alignment of the N-terminal flg22 region of flagellin derived from a consensus sequence (Felix et al. 1999), the standard flg22 peptide used in many studies, including the present study (Felix et al. 1999), or the sequence of the flg22-K60 peptide designed based on the corresponding region of flagellin from *Ralstonia solanacearum* K60. Amino acid differences between the original flg22 and the *R. solanacearum*-derived peptide are noted with an asterisk (*). Amino acids in the *R. solanacearum*-derived peptide that differ from the consensus are shown in larger font. **B**, Average fresh weight measurements of nine *Arabidopsis* (Col-0) seedlings treated with 10 μ M flg22 or 10 μ M flg22-K60. Weights of seedlings were taken 7 days after treatment. Bars represent standard error of the mean.

shown). Collectively, the above experiments suggest that the eliciting activity is not caused primarily by a small peptide but is, instead, caused by one or more proteins with a molecular mass of roughly 5 to 10 kDa.

Additional eliciting activity was evident in larger MW fractions collected after gel filtration (Fig. 5D and E). We were unable to determine if the larger elicitors were single large proteins or aggregates of the smaller elicitor. SDS treatment to dissociate possible aggregates followed by SDS removal eliminated elicitation activity (Fig. 5C). This may have been due to denaturation of one or more boil-resistant eliciting proteins by SDS or due to loss of small elicitor proteins during the post-SDS dialysis, which was required to remove SDS so that extracts could be assayed on *Arabidopsis* seedlings. Note that fractions from gel filtration were re-boiled immediately prior to application to seedlings for elicitation experiments, possibly re-denaturing or redissociating proteins, or both, that

had previously been boiled during the initial preparation of all bacterial extracts.

Elicitor production is not controlled by known virulence regulators.

To further investigate the nature of this elicitor, we examined the elicitation activity of boiled extracts derived from *R. solanacearum* strains carrying mutations in secretion pathways, regulators of known virulence factors, or both (Denny 2000). Strain K60-*phcA* no longer expresses PhcA, the LysR-type global regulator of virulence factors. This strain is motile but has severely reduced exopolysaccharide and endoglucanase production, has increased extracellular polygalacturonase production, and is essentially nonvirulent (Brumbley et al. 1993). Strain K71 lacks the two-component regulator product of *pehS*R, causing decreased polygalacturonase production and a nonmotile phenotype (Allen et al. 1997); K71 exhibits sig-

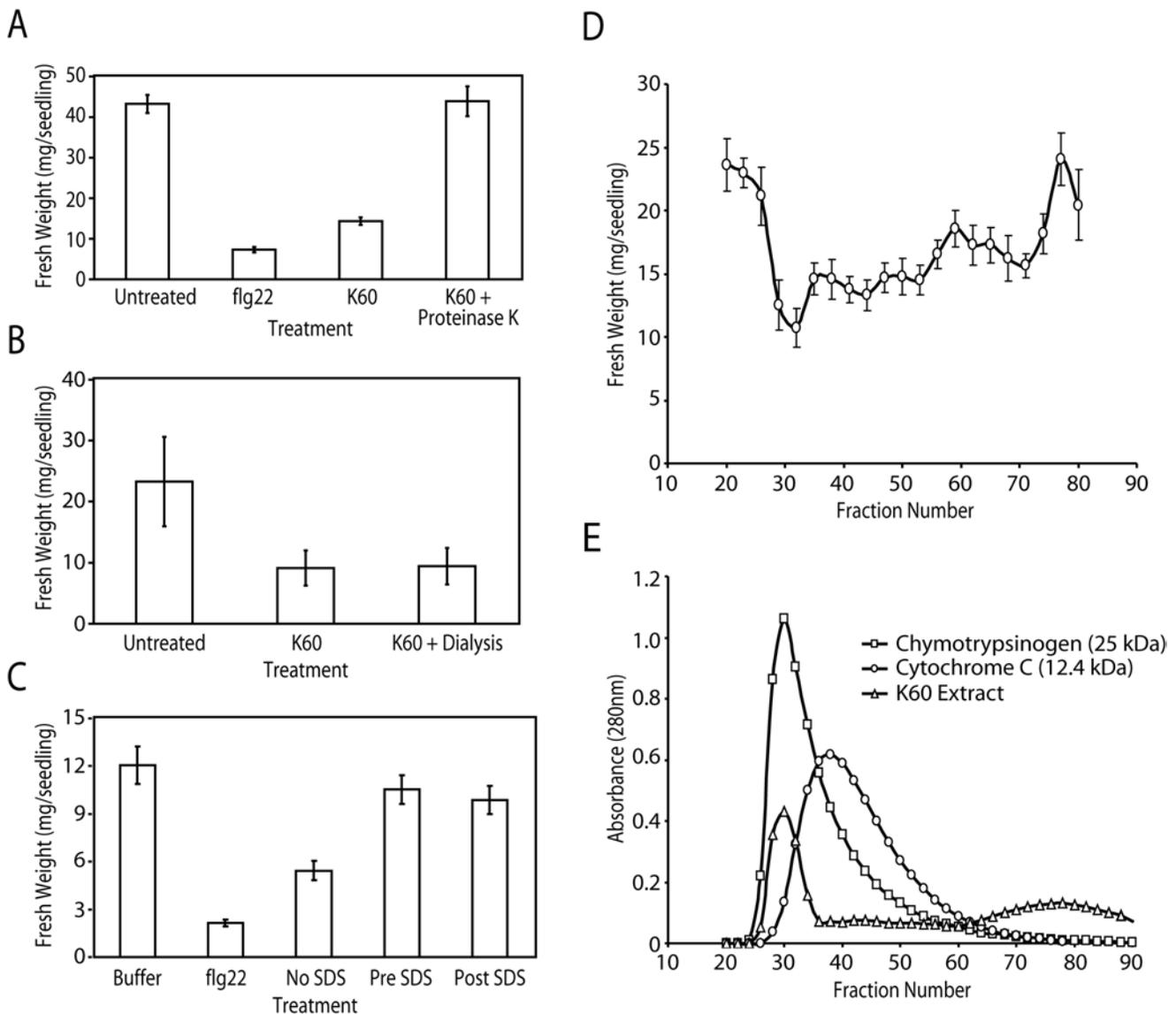


Fig. 5. Seedling growth inhibition is caused by one or more proteins purifying between 5 to 10 kDa as well as by proteins that purify at a larger apparent size. Average fresh weight measurements are for nine *Arabidopsis* (Col-0) seedlings weighed after 14 days of treatment. Bars represent standard error of the mean. **A**, Seedlings treated with water, 10 μ M flg22, or 45 μ g of boiled bacterial extracts from K60 either with or without proteinase K treatment. **B**, Seedlings treated with approximately 30 μ g K60 extract that was or was not dialyzed in 7-kDa molecular mass cut-off dialysis tubing. **C**, Seedlings treated with water, 10 μ M flg22, K60 extract passed through a 5-kDa molecular mass cut-off ultrafiltration unit, or K60 extract treated with 0.4% sodium dodecyl sulfate (SDS) before (pre) or after (post) passage through a 5-kDa molecular mass cut-off ultrafiltration unit and then dialyzed in 3.5-kDa molecular mass cut-off dialysis tubing to remove SDS. **D**, Seedlings treated with K60 extract fractions from a Sephadex G-50 gel filtration column (top panel). **E**, Elution profile for the K60 extract fractions used in panel D and for two protein size standards previously run on the same Sephadex G-50 column.

nificantly reduced virulence. The 'out' mutant K100 has a Tn5 insertion in the *gspM* locus that disrupts the general secretion pathway and leads to dramatically reduced virulence (Kang et al. 1994). K200 has a gentamycin resistance cassette insertion in the *hrpB* regulatory locus. HrpB is a positive regulator of the *R. solanacearum* type III secretion system, which transfers effector proteins into host plant cells. HrpB is also required for expression of a suite of putative effector proteins. *hrpB* mutants can no longer cause disease on host plants or induce a hypersensitive response in nonhost plants (Genin 1992).

Boiled extracts from these strains were tested for their ability to elicit defense-associated responses in *Arabidopsis* seedlings. Extracts from K100 (*gspM*), K200 (*hrpB*), K71 (*pehR*), and K60-*phcA* all elicited a seedling growth-inhibition response indistinguishable from that elicited by the wild-type K60 extract (Figs. 1B and 6A and B). Thus, mutations disrupting these known regulators and secretory pathways do not affect expression of one or more elicitors detected in *R. solanacearum* boiled extracts.

The *R. solanacearum* elicitor is not an avirulence factor.

It can be hypothesized that the elicitor in *R. solanacearum* K60 boiled extracts is an avirulence gene product. Pathogen avirulence (*avr*) genes are defined by their functional interaction with the plant host through the action of corresponding plant resistance (*R*) genes, which typically activate rapid and robust defense responses that restrict pathogen invasion

(Dangl and Jones 2001). If K60 contains an *avr* gene product that is recognized by *Arabidopsis* Col-0, then K60 should be largely unable to cause disease on *Arabidopsis* Col-0 and might elicit a hypersensitive response. We tested the virulence of K60 on *Arabidopsis* plants using a soil soak assay following root wounding, which has previously been used to demonstrate virulence of another *R. solanacearum* strain, GMI1000 (Deslandes et al. 2002). *R. solanacearum* K60 was virulent on *Arabidopsis* Col-0 (Fig. 7), the same *Arabidopsis* genotype used in our earlier experiments, indicating that the defense-eliciting activity present in boiled extracts of K60 is not causing an *avr* phenotype, in that it does not correlate with effective whole plant disease resistance in the same plant genotype.

We also tested the ability of wild-type strain K60 and nonmotile strain K701 to cause disease in wild-type Col-0 *Arabidopsis* and in Col-0 plants lacking the FLS2 receptor. If flagellin or another elicitor is recognized by this receptor, it should activate defense induction and cause a corresponding decrease in disease. However, K60 caused statistically indistinguishable levels of disease on wild-type Col-0 and *fls2-101* (Fig. 7). The loss of flagellin also had little or no impact on pathogen virulence (Fig. 7). These data indicate that the presence of FLS2 in the plant or flagellin in the pathogen does not significantly affect the whole-plant response to live *R. solanacearum* bacteria in our assays.

Separate from the main focus of this study, the data presented in Figure 7 also support the previous finding that flagella-mediated motility is not necessary for virulence if bacteria are delivered to wounded plant surfaces at a sufficiently high concentration (Tans-Kristen et al. 2001).

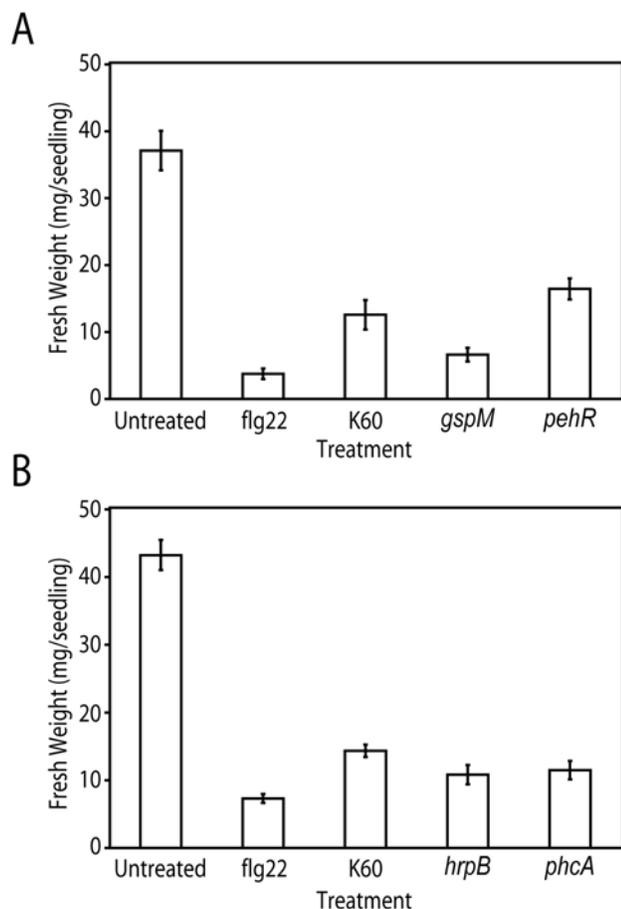


Fig. 6. Extracts from diverse *Ralstonia solanacearum* virulence mutants elicit a response in *Arabidopsis*. Average relative fresh weight measurements of nine *Arabidopsis* (Col-0) seedlings treated with water, 11 μ g flg22, or 45 μ g of the indicated boiled bacterial extract. Fresh weights of seedlings were determined 14 days after treatment. Panels A and B depict independent experiments. Bars represent standard error of the mean.

DISCUSSION

A critical component of immunity is the ability of an organism to recognize nonself invaders. Conserved pathogen-associated molecular patterns displayed on the surface of microbes

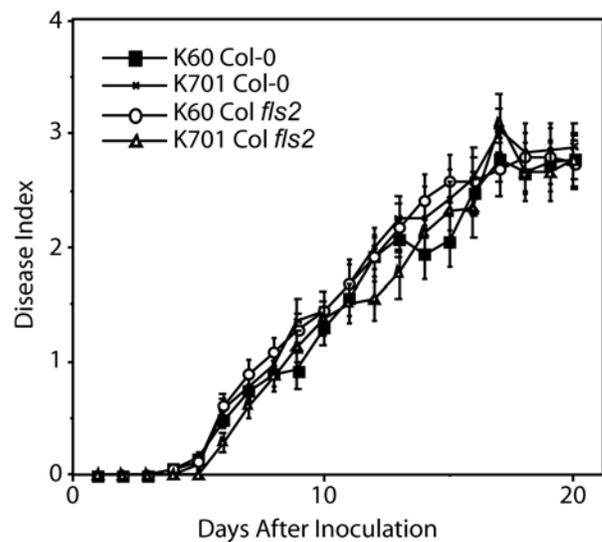


Fig. 7. Wild-type and aflagellate *Ralstonia solanacearum* strains cause similar disease levels on *Arabidopsis* with and without the FLS2 receptor. Soil-soak virulence assay on *Arabidopsis* Columbia (Col-0) and *Arabidopsis* Col *fls2* (*fls2-101*). In three separate experiments that each included all treatments, four-week-old *Arabidopsis* plants were inoculated by soil drench with 10 ml of a bacterial suspension immediately after roots had been injured by a serrated knife passing through the soil. Each datapoint is the mean \pm standard error of the mean for 45 to 46 plants total for each treatment, which incorporates data from all three replicate experiments. Solid squares represent K60 (wild-type) on Col-0; crosses represent K701(*flhC*) on Col-0; open circles represent K60 (wild-type) on Col-0 *fls2*; and open triangles represent K701(*flhC*) on Col-0 *fls2*.

are thought to serve as recognition cues that the host uses to trigger defenses (Darvill et al. 1984; Ebel and Scheel 1997; Medzhitov et al. 2002; Nurnberger and Brunner 2002). In animal innate immune systems, toll-like transmembrane receptors are involved in recognition of pathogen-associated molecular patterns, including bacterial flagellins and in the subsequent activation of immune responses (Aderem 2001; Hayashi et al. 2001; Imler and Hoffmann 2001; Khush and Lemaitre 2000; Smith and Ozinsky 2002). Recent studies have focused on the recognition of bacterial flagellin by several plants (Donnelly and Steiner 2002; Felix et al. 1999; Gomez-Gomez and Boller 2002; Taguchi et al. 2003; Tananka et al. 2003). Recognition of flagellin by *Arabidopsis* has been attributed to FLS2, a leucine-rich receptor-like kinase that activates plant defense at least in part via a MAP kinase pathway (Asai et al. 2002; Gomez-Gomez and Boller 2000, 2002). However, the impact of flagellin expression on bacterial virulence and the efficacy of the plant defenses activated following flagellin recognition remain unclear.

Phytopathogenic *R. solanacearum* bacteria require motility for full virulence (Tans-Kersten et al. 2001). Previous work using cultured tomato cells implicated flagellin as the primary elicitor of plant defense responses in boiled extracts from many phytopathogenic bacteria (Felix et al. 1999). Furthermore, exposure of *Arabidopsis* seedlings to a peptide derived from a conserved N-terminal flagellin domain (flg22) leads to induction of classic defense marker genes and a correlative seedling growth inhibition (Gomez-Gomez et al. 1999). We considered that flagellin of *R. solanacearum* may serve as a pathogen-associated molecular pattern recognizable by host plants and capable of activating plant immune responses. We found that boiled extracts of *R. solanacearum* K60 did inhibit seedling growth and induce defense gene expression (Figs. 1 and 2). However, flagellin from *R. solanacearum* was not the primary elicitor in boiled extracts (Figs. 1 and 2), and the virulence of *R. solanacearum* on *Arabidopsis* plants did not differ between flagellate and aflagellate strains (Fig. 7). Moreover, the absence of the FLS2 receptor in *Arabidopsis* did not significantly affect the ability of *Arabidopsis* to respond to this elicitor (Fig. 3) or to defend itself against *R. solanacearum* infection (Fig. 7). As an aside, our data also demonstrated that *R. solanacearum* does not require motility for virulence in *Arabidopsis* following a soil-soak method of inoculation that uses wounded plants and a high titer of bacteria (Fig. 7).

The above findings were somewhat unexpected, as earlier work had implicated flagellin as the major elicitor of defense responses in boiled extracts from many phytopathogenic and nonpathogenic bacteria (Felix et al. 1999). However, those experiments were done in tomato and not *Arabidopsis*, and were done in suspension culture cells rather than whole plants. It is possible that *Arabidopsis* seedlings possess a functional receptor for one or more additional elicitors that are lacking in tomato suspension culture cells. However, in a very recent study of boiled extracts from more than 20 different bacteria, all of which elicited defense-associated responses, elicitation activity in some cases was attributed to a cold-shock protein rather than flagellin (Felix and Boller 2003). Note also that not every boiled bacterial extract can elicit plant responses. We have observed that extracts from some strains of the phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris* do not elicit growth inhibition in *Arabidopsis* seedlings (C. Pfund, unpublished data).

A preliminary analysis of the elicitor or elicitors present in *R. solanacearum* extracts indicated that it is proteinaceous in nature and resistant to boiling. At least one primary fraction of elicitor has an apparent molecular mass of 5 to 10 kDa; dialysis experiments suggested that elicitation is not substantially

due to a small peptide, but gel filtration work did indicate that elicitation is also caused by larger proteins, aggregates, or both (Fig. 5). Previous work on elicitors from phytopathogenic bacteria suggested that this elicitor might be a harpin, which are small, heat-resistant proteins that are secreted via the type III secretion apparatus and can activate plant defenses (He et al. 1993; Wei et al. 1992). Our results indicated that this is not the case, as extracts from a *R. solanacearum* *hrpB* mutant retain the ability to elicit plant response (Figs. 2B and 6B). *HrpB* is a positive regulator of expression, not only of the type III secretion apparatus, but also of many putative effector proteins, such as the PopA harpin (Denny 2000; Genin 1992). Extracts from *R. solanacearum* strains harboring mutations in *phcA*, *pehR*, or *gspM* also retained wild-type ability to elicit responses in *Arabidopsis* seedlings, indicating that expression of elicitor also does not require the *PhcA* or *PehR* regulons or the general secretory pathway. Other potential elicitors, such as lipopolysaccharides, pectic fragments, and glycoproteins (Nuhse et al. 2000) would likely be sensitive to boiling or insensitive to proteinase K treatment, and therefore, do not fit our profile.

Use of seedling growth as an assay that correlates with defense activation has been extensively documented (Figs. 1 and 2) (Gomez-Gomez and Boller 2000; Gomez-Gomez et al. 1999; C. Pfund and A. F. Bent, unpublished data), and many laboratories have observed that constitutive activation of defenses causes *Arabidopsis* plants to be dwarfed (Bowling et al. 1994; Clarke et al. 1998; Greenberg and Ausubel 1993; Maleck et al. 2002; Petersen et al. 2000; Yu et al. 1998). The flg22 peptide causes both PR gene expression and seedling growth inhibition, and these two traits correlated in all cases that we tested with *R. solanacearum* extracts. However, if the eliciting activity in *R. solanacearum* boiled extracts is, in fact, attributable to more than one different protein, the possibility exists that elicitation of defense and of seedling growth inhibition may be separable.

Recent work has revealed the presence of another major boil-resistant elicitor of plant defense responses found in extracts of *Micrococcus lysodeikticus* (Felix and Boller 2003). This elicitor is a member of the cold-shock protein (CSP) family. While the *R. solanacearum* genome does encode proteins annotated as CSPs, two pieces of data suggest that our elicitor may not be a CSP. First, a peptide derived from a conserved domain found in CSPs did not elicit a response in *Arabidopsis* culture cells (Felix and Boller 2003). Secondly, treatment of extracts of *M. lysodeikticus*, using molecular mass cut-off 10 kDa, completely eliminated the CSP elicitor (Felix and Boller 2003), whereas we retained elicitation activity following dialysis in membrane with a stated cut-off of 10 kDa. Further biochemical analysis of extracts from *R. solanacearum* should help identify one or more elicitors that are capable of causing seedling growth inhibition and the corresponding induction of defense gene in *Arabidopsis* and, presumably, other plants.

In tobacco, a natural host of *R. solanacearum*, live cells of the *flhC* mutant K701 elicited a rapid tissue chlorosis and necrosis that was indistinguishable from the response induced by wild-type strain K60. Furthermore, crude boiled extracts derived from nonmotile strains K701 (*flhC*) and K702 (*flhDC*) caused mild chlorosis in tobacco, just like extracts from the wild-type strain K60. This preliminary result extends our findings beyond *Arabidopsis* and suggests that *R. solanacearum* produces nonflagellin elicitors that are detected by a natural host species.

While our data indicate that flagellin is not the major elicitor of plant responses in boiled extracts of *R. solanacearum*, our data also indicated that the *R. solanacearum* flagellin may not be recognized by the *Arabidopsis* flagellin-recognition

system (Fig. 4). Previous data indicated that the conserved f1g22 region of bacterial flagellin is sufficient to elicit response in *Arabidopsis* (Gomez-Gomez et al. 1999). We found that a peptide derived from the *R. solanacearum* version of this same region did not elicit a response in *Arabidopsis* (Fig. 4B). The sequence of this *R. solanacearum* f1g22 region contains at least one significant change from a consensus sequence derived from the flagellin of many bacteria (Fig. 4A) (Felix et al. 1999). Felix and associates (1999) demonstrated that peptides derived from the flagellins of *Agrobacterium* and *Rhizobium* spp. had no elicitation activity and suggested that some plant-associated bacteria may have altered flagellins that elude flagellin-dependent detection. *R. solanacearum* flagellin may also fall into this group. Interestingly, *FLS2* was found to be highly expressed in the plant vasculature (Gomez-Gomez and Boller 2002). As *R. solanacearum* is a vascular pathogen, these data are consistent with a strong selection pressure for vascular pathogens that can elude detection via *FLS2*.

It is unclear at this point whether plants that do detect flagellin recognize assembled flagella shed from the bacterium, free flagella, or fragments of degraded flagellin. Alternative epitopes of flagellin may be displayed by shed flagella compared with intact flagellin. The f1g22 region of flagellin is normally buried in the assembled polymer's tertiary structure (MacNab 1996), suggesting that the flagellin molecule recognized by *FLS2* may be unfolded or partially degraded. Recent evidence suggests that the N-terminal f1g22 region may not be the sole determinant of flagellin recognition by plants (Che et al. 2000; Taguchi et al. 2003; Tananka et al. 2003). Our results with aflagellate *R. solanacearum* strains demonstrated that any elicitation caused by other flagellin domains was insufficient to be detectable over and above the other elicitors present in the strains or extracts used.

Our investigation was initially focused on flagellin elicitation, but it raised general questions as to whether elicitors identified in pathogen extracts offer a productive route to identification of biologically relevant elicitors. Despite the presence of an extractable defense elicitor, *R. solanacearum* K60 is highly virulent on *Arabidopsis*. It is possible that the defenses activated in response to this elicitor have only minor effects on pathogen virulence that were undetectable in our assays. Alternatively, *R. solanacearum* may be effective in overcoming or suppressing the modest host resistance activated by this elicitor. Other host or nonhost species may be more responsive to the presence of such elicitors. However, the possibility must also be considered that these elicitors are not biologically relevant.

We demonstrate that flagellin is not the major elicitor of *Arabidopsis* defense gene induction in boiled extracts of *R. solanacearum*, although these *R. solanacearum* extracts do contain defense-eliciting activity. There may be many molecules capable of eliciting plant defenses (Hahn 1996; Scheel and Parker 1990). However, the relevance of such putative pathogen-associated molecular patterns in plant disease is unclear. Identification of these elicitors, isolation of mutant strains that are disrupted in elicitor production, and subsequent analysis of the virulence of these live pathogens will help us to better understand the role of these elicitors in plant disease and disease resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *R. solanacearum* strains were grown at room temperature in CPG broth or CPG agar amended with 0.05% triphenyltetrazolium chloride (Hendrick and Sequeira 1984). Boucher's minimal media broth (BMM) (Boucher et al. 1985)

with 0.2% glucose was used when minimal media was required. Antibiotics (Sigma Chemical, St. Louis) were added as necessary in the following concentrations (mg per liter): tetracycline, 15; gentamycin, 25; kanamycin, 25; and streptomycin, 30.

Cloning and mutagenesis of *gspM* and *hrpB* genes.

Isolation of plasmid and chromosomal DNA, restriction mapping, subcloning, and PCR were performed using standard methods (Ausubel et al. 1995). *Escherichia coli* was transformed by electroporation as previously described (Ausubel et al. 1995). *R. solanacearum* was transformed either by electroporation (Allen et al. 1991) or by natural transformation (T. Denny, personal communication).

To create the *gspM* (out) mutant K100, AW-D (*gspM*::Tn5; Table 1) genomic DNA was naturally transformed into *R. solanacearum* K60. Briefly, recipient strain K60 was grown overnight in CPG broth to an optical density at 600 nm (OD_{600}) of 0.5. Cells were centrifuged at $7,000 \times g$ for 3 min and the supernatant was discarded. Cells were mixed with 3 μ g of genomic DNA, were spotted on a CPG plate, and were incubated overnight at 28°C. Cells were subsequently scraped and re-plated on selective media. Kanamycin-resistant transformants were screened for lack of extracellular cellulase and polygalacturonase activities by the cellulase plate assay (Chatterjee et al. 1995) and thin-layer chromatography, respectively (Huang and Allen 1997).

The *hrpB* locus was cloned, using PCR primers designed from the *R. solanacearum* GMI1000 genomic sequence available on INRA's *Ralstonia solanacearum* webpage. The primers were 5' ATGCTGGGAAACATCTAC 3' and 5' GCTTCG TTGAACTGCTTG 3'. A 1,400-bp product containing *hrpB* was cloned into pSTBlue-1 to create pHRPB1, and the correct sequence was confirmed by sequencing. A single *Bgl*III site within *hrpB* was used to insert an 850-bp gentamycin resistance cassette (pHRPB2). This mutagenesis construct was marker-exchanged into the *R. solanacearum* K60 genome by homologous recombination. Gentamycin-resistant transformants were screened by PCR for an 850-bp cassette insertion in the *hrpB* gene. Mutants were also screened for inability to cause a rapid necrosis in tobacco leaves (Boucher et al. 1985) and for dramatically reduced virulence by petiole inoculation in tomato plants (discussed below) (Tans-Kersten et al. 2001). The *R. solanacearum* K60 *hrpB* mutant strain was named K200 (Table 1).

Arabidopsis lines and seedling growth conditions.

Arabidopsis thaliana ecotype Col-0 plants were used as wild-type plants. The *fls2-101* mutant allele was generated at the Salk Institute *Arabidopsis* Knock-Out Facility (JP71.4 G09; La Jolla, CA, U.S.A.) (Alonso et al. 2003). The homozygous presence of an insertion in the *hLS2* locus was subsequently confirmed by Southern blot analysis, PCR, and insensitivity to the f1g22 peptide by growth-inhibition analysis. For seedling growth assays, all seeds were sterilized by standard bleach sterilization (Clough and Bent 1998), were stratified for 2 days, were germinated on $1/2$ Murashige-Skoog (MS) plates containing 0.8% plant tissue culture agar (Sigma) containing 2% sucrose and vitamins (1 ml/liter) (Sigma), and were grown for 5 days at room temperature (22°C) under 18 h of light. Seedlings were then transferred to 24-well culture dishes containing 400 μ l $1/2$ MS media. Total protein (45 μ g of each boiled extract unless otherwise indicated) or 11 μ g (7.5 to 9.0 μ M) f1g22 peptides were added to each well (1.5-cm diameter), and the seedlings were incubated for the indicated time at 22°C under 8 h of light. Seedling tissue was either frozen in liquid N₂ for RNA blot analysis or was weighed and photographed after 14 days.

Preparation of boiled extracts.

Crude boiled extracts to be tested for elicitation in the *Arabidopsis* seedling assay were prepared in the following manner: 500 ml of BMM + 0.2% glucose broth was inoculated with 1 ml of bacterial culture ($OD_{600} = 0.7$) and was incubated for 18 h (to mid-log phase) at 28°C in a shaker. Cultures were centrifuged at $7,000 \times g$ for 30 min, were washed with 200 ml of sterile distilled water, and were recentrifuged at $7,500 \times g$ for 30 min. The cell pellet was resuspended in 1 to 2 ml of sterile distilled water or 50 mM Tris, 1 nM EDTA, pH 7.5, for centrifugal ultrafiltration experiments or in 4.3 mM MES, pH 5.8, for gel filtration experiments, and then, in all cases, was boiled for 10 min. Samples were centrifuged for 10 min at $10,000 \times g$, and the supernatant was collected. Protein concentrations were determined using the BCA assay (Sigma) and were visualized using SDS-PAGE stained with Coomassie blue. All extracts were again boiled for 10 min immediately before use in seedling assays.

Treatment of extracts.

For elicitor fractionation experiments, boiled extracts were dialyzed to remove low molecular mass components using 7- or 10-kDa molecular mass cut-off dialysis tubing (Pierce, Rockford, IL, U.S.A.). Extracts were dialyzed for 18 h in $2,000 \times$ volume of ddH₂O with three changes of ddH₂O. For centrifugal ultrafiltration experiments, extracts were spun through a 5,000 molecular mass cutoff polyethersulfone membrane (Vivascience, Hanover, Germany) and, in some cases, were treated with 0.4% SDS before or after passage through the membrane, and then, dialyzed afterward using 3.5-kDa molecular mass cut-off dialysis tubing (Pierce). These extracts were dialyzed for 24 h in $5,000 \times$ volume of buffer with three buffer changes. In gel filtration experiments, K60 boiled extract was separated on a calibrated Sephadex G-50 column (Amersham Biosciences, Piscataway, NJ, U.S.A.), and 1-ml fractions were collected. To remove proteins from crude boiled extracts, extracts were incubated at 37°C for 30 min with proteinase K (Sigma P-2308) at 0.006 units per μg of extract protein or a Type IX protease preparation from *Bacillus polymyxa* (Sigma P-6141) at 0.005 units per μg of extract protein. In all seedling treatments, except as noted, 45 μg of crude boiled extract or an aliquot of a fractionated extract representing approximately 45 μg of original extract were placed in each well.

Tobacco response to crude cellular extracts and live bacteria.

The response of tobacco to crude boiled extracts or to live bacteria was determined by infusing the apoplastic space of 8-week-old leaves (*N. tabacum* cv. Bottom Special) with full-strength crude boiled extracts (500 μg of protein per ml), 10-fold dilutions of extracts (50 $\mu\text{g}/\text{ml}$), or washed live bacterial suspensions (1×10^7 CFU/ml). The infused leaf areas were observed for development of chlorosis and necrosis over a period of 4 to 5 days.

Oligopeptides.

The flg-22 oligopeptide (QRLSTGSRINSKVDAAAGLQIA) (Felix et al. 1999) was synthesized at Commonwealth Biotechnology (Richmond, VA, U.S.A.). Sequence of the *R. solanacearum* K60 *fliC* (flagellin) is available through GenBank accession number AF283285 (Tans-Kersten et al. 2001). *R. solanacearum* flg-22 oligopeptide (QRLSTGMRVNSAQDDAAAYASA) was synthesized at PeptideXpress (Dublin, OH, U.S.A.).

RT-PCR analysis.

Whole seedlings were harvested and frozen in liquid N₂. Total RNA was extracted from seedlings using TRIzol Reagent

(Invitrogen, Carlsbad, CA, U.S.A.). Briefly, seedlings were ground in liquid N₂, and tissue (<100 mg) was resuspended in 1 ml TRIzol and was extracted using published protocol. cDNA was synthesized using the First-Strand cDNA synthesis kit (Invitrogen). A $1/10$ fraction of the RT reaction was used as template for PCR reactions performed with *Taq* polymerase (Promega, Madison, WI, U.S.A.) for 40 cycles at 51°C. PCR amplifications were performed using the following primers: PR-1 primers, 5'GTAGGTGCTCTTGTCTTCCC3' and 5'CA CATAATCCCACGAGGATC3'; ACT2 primers, 5'AGGTTCTGTTCCAGCCATC and 5'TTAGAAGCAATTCCTGTGAAC. The PCR products were separated by gel electrophoresis and were stained with ethidium bromide.

Virulence assays.

To measure the virulence of *R. solanacearum* strains on *Arabidopsis* Col-0 or *fls2-101* plants, bacteria were grown overnight in CPG broth with appropriate antibiotics to mid-log phase, were centrifuged at $6,000 \times g$ for 20 min, were washed with distilled water, and then, the cells were resuspended in distilled water and were diluted to an OD_{600} approximately 1.0, corresponding to approximately 1×10^9 CFU/ml. Actual concentrations applied to plants in the three experiments were 2.4×10^8 to 9.6×10^8 CPU/ml. Three- to four-week-old *Arabidopsis* plants were grown in Sunshine Mix #1 (SunGro Horticulture, Bellevue, WA, U.S.A.) in approximately 5-cm square pots at 22°C with an 8-h day. Plants were inoculated by wounding the roots with a small serrated knife without uprooting them, and then, 10 ml of bacterial suspension (1×10^{10} total cells) were poured on the soil surface. Plants were coded and rated daily for 20 days by a rater blind to treatment identity, using a disease index scale of 0 to 4 (0 = no symptoms; 1 = one to three inverted leaves or visible anthocyanin production, or both; 2 = more than three inverted leaves and anthocyanin production; 3 = plant wilting over less than 50% of leaf surface; and 4 = more than 50% of leaf surfaces wilted or dead).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

INRA Bioinformatique *Ralstonia solanacearum* webpage:
sequence.toulouse.inra.fr/ralsto/Complete/doc/Complete.html