The *Ralstonia solanacearum* csp22 peptide, but not flagellin-derived peptides, is perceived by plants from the Solanaceae family

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**Summary**

*Ralstonia solanacearum*, the causal agent of bacterial wilt disease, is considered one of the most destructive bacterial pathogens due to its lethality, unusually wide host range, persistence and broad geographical distribution. In spite of the extensive research on plant immunity over the last years, the perception of molecular patterns from *R. solanacearum* that activate immunity in plants is still poorly understood, which hinders the development of strategies to generate resistance against bacterial wilt disease. The perception of a conserved peptide of bacterial flagellin, fig22, is regarded as paradigm of plant perception of invading bacteria; however, no elicitor activity has been detected for *R. solanacearum* fig22. Recent reports have shown that other epitopes from flagellin are able to elicit immune responses in specific species from the Solanaceae family, yet our results show that these plants do not perceive any epitope from *R. solanacearum* flagellin. Searching for elicitor peptides from *R. solanacearum*, we found several protein sequences similar to the consensus of the elicitor peptide csp22, reported to elicit immunity in specific Solanaceae plants. A *R. solanacearum* csp22 peptide (csp22<sup>2nd</sup>) was indeed able to trigger immune responses in *Nicotiana benthamiana* and tomato, but not in *Arabidopsis thaliana*. Additionally, csp22<sup>2nd</sup> treatment conferred increased resistance to *R. solanacearum* in tomato. Transgenic *A. thaliana* plants expressing the tomato csp22 receptor (SICORE) gained the ability to respond to csp22<sup>2nd</sup> and became more resistant to *R. solanacearum* infection. Our results shed light on the mechanisms for perception of *R. solanacearum* by plants, paving the way for improving current approaches to generate resistance against *R. solanacearum*.

**Keywords**: ralstonia, elicitor, flagellin, fig22, csp22, bacterial wilt.

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**Introduction**

*Ralstonia solanacearum* is a soil-borne bacterial pathogen able to cause disease in more than 250 plant species (Jiang et al., 2017; Mansfield et al., 2012). It has an extremely versatile lifestyle, surviving in water, soil and plant debris. *R. solanacearum* perceives root exudates and employs its flagellum for movement and efficient root invasion (Tans-Kersten et al., 2001; Yao and Allen, 2006). After invading plant tissues through wounds, root tips or cracks at the sites of lateral root emergence, *R. solanacearum* colonizes the root cortex, reaches the vasculature and spreads through xylem vessels, colonizing the plant systemically (Mansfield et al., 2012). Subsequent massive bacterial replication leads to a quorum sensing-dependent metabolic switch in bacterial cells (Khokhani et al., 2017; Perrier et al., 2016; Peyraud et al., 2016), triggering the production of virulence factors and exopolysaccharide (EPS; McGarvey et al., 1999), which causes the disruption of the plant vascular system and eventual plant wilting (Digonnet et al., 2012; Turner et al., 2009). Given its lethality, unusually wide host range, persistence and broad geographical distribution, *R. solanacearum* is currently considered one of the most destructive bacterial pathogens in agricultural systems.

To fend off pathogens, plants have evolved to perceive different microbial molecules. Conserved pathogen-associated molecular patterns (PAMPs) can be perceived at the plant cell surface by receptors localized at the plasma membrane, termed pattern-recognition receptors (PRRs (Zipfel, 2014)). Moreover, proteins injected by pathogens inside plant cells can also be perceived, in a direct or indirect manner, by intracellular receptors containing nucleotide-binding and leucine-rich repeat domains (NLRs; Khan et al., 2016). Applying a broad view of the plant immune system, bacterial elicitors perceived either extracellularly and intracellularly can be generally regarded as invasion patterns (Cook et al., 2015), and their perception leads to the activation of immune responses aimed at restricting bacterial infection (Bigeard et al., 2015; Boller and Felix, 2009; Tsuda and Katagiri, 2010). Despite the extensive research on plant immunity over the last years, little is known about the perception of immune elicitors from *R. solanacearum*, and our understanding of *R. solanacearum* perception by plants comprises almost exclusively the recognition of effector proteins delivered inside plant cells through the type III secretion system (Deslandes and Genin, 2014; Huet, 2014; Jayaraman et al., 2016). As a consequence, current agricultural approaches to control bacterial wilt disease caused by *R. solanacearum* are limited (Huet, 2014; Jiang et al., 2017), suggesting a need for identification of additional immune elicitors present in *R. solanacearum* that could be perceived by different plant species, and a characterization of the plant immune components that mediate this perception.
Currently, different species from the Solanaceae family show varying degrees of resistance to *R. solanacearum*, mostly based on the recognition of specific bacterial type III effectors (Huet, 2014; Poueymirou et al., 2009). Similarly, specific tomato cultivars also display some degree of disease resistance dependent on the putative recognition of bacterial exopolysaccharide, which otherwise acts as a virulence factor (Milling et al., 2011).

The bacterial flagellum is formed by polymerized flagellin protein (encoded by the FlIC gene). The perception of a conserved peptide of bacterial flagellin, flg22, is possibly the best studied example of plant recognition of invading bacteria, and occurs in plants harbouring the PRR FLS2 (Felix et al., 1999; Gómez-Gómez and Boller, 2000). However, no elicitor activity has been detected so far for *R. solanacearum* flg22 (flg22Rsol) in Arabidopsis, tomato or tobacco plants (Mueller et al., 2012a; Pfund et al., 2004), probably due to the presence of several polymorphisms in the amino acid sequence of this peptide compared to flg22 peptides from other bacteria (Mueller et al., 2012a; Sun et al., 2013). This observation has generated the original assumption that *R. solanacearum* has evolved to evade plant recognition of its flagellin by altering this flg22 sequence.

In recent years, it has been reported that specific plants have evolved to perceive peptides within bacterial flagellin other than flg22 (Cai et al., 2011; Clarke et al., 2013; Katsuragi et al., 2015). Several plants from the Solanaceae family are able to perceive a distinct 28 amino acid peptide within flagellin from several *Pseudomonas syringae* strains, named flgll-28 (Cai et al., 2011; Clarke et al., 2013). This perception is mediated by the PRR FLS3 (Hind et al., 2016) and is restricted to specific species within the Solanaceae family, including tomato, potato and pepper (Clarke et al., 2013). Other Solanaceae plants, such as tobacco, eggplant or *Nicotiana benthamiana*, or non-Solanaceae plants, including Arabidopsis, cannot perceive flgll-28 (Clarke et al., 2013). Whether FLS3 can recognize flgll-28 from *R. solanacearum* is currently unknown. In the light of these reports, and considering the current limitations in our understanding of plant perception of *R. solanacearum* immune elicitors, we designed a systematic approach with the aim of determining whether other peptides from *R. solanacearum* flagellin could be perceived by plants from the Solanaceae family. This work was based on the following reasoning:

1. Tomato is a host for *P. syringae* species harbouring immunogenic flgll-28. Similarly, specific Solanaceae plants may have co-evolved with *R. solanacearum* and developed specific mechanisms for the perception of *R. solanacearum* flgll-28 (flgll-28Rsol) or other conserved elicitors.

2. There is no report on the ability of tomato plants (or other non-Nicotiana solanaceous plants) to perceive flgll-28Rsol or other peptides of *R. solanacearum* flagellin. It is known that *R. solanacearum* flagellin is not an elicitor in Arabidopsis and tobacco (Pfund et al., 2004), but these species do not respond to flgll-28 (Clarke et al., 2013). Therefore, a potential elicitor activity of flgll-28Rsol may have been overlooked in previous studies.

3. There is an example of another bacterial species, *P. cannabina pv. alisalensis* ES4326 (Pcal ES4326; formerly known as *P. sulcicola*), whose flgll-28, but not flg22, is recognized by tomato (Clarke et al., 2013; Hind et al., 2016).

4. Most elicitation assays published to date have been performed in leaf tissue or cultured cells, without taking into consideration root responses. Given that root is the most common entry point into plant tissues for *R. solanacearum*, the ability of this organ to perceive and respond to invading bacteria is of special relevance.

In this work, we tested a synthetic flgll-28Rsol peptide and full *R. solanacearum* flagellin for elicitation in different plant species, including tomato, pepper, eggplant, tobacco and *N. benthamiana*, using either leaf or root tissues. Our results show that none of these species is able to respond to *R. solanacearum* flagellin.

We next sought to identify other *R. solanacearum* elicitor peptides that could be perceived by plants. The csp22 peptide is present in the conserved COLD-SHOCK PROTEIN from several bacterial species (Felix and Boller, 2003) and is perceived by PRRs from several Solanaceae species (Saur et al., 2016; Wang et al., 2016). Our results show that specific Solanaceae species, including tomato, tobacco and *N. benthamiana*, can perceive the csp22 peptide derived from *R. solanacearum* cold-shock protein (csp22Rsol), and that the reported csp22 receptor CORE (Wang et al., 2016) confers responsiveness to csp22Rsol in Arabidopsis. Moreover, csp22Rsol induces immune responses that are able to restrict *R. solanacearum* growth in tomato. This work allowed us to uncover a *R. solanacearum* peptide perceived by a PRR present in specific Solanaceae species, paving the way for the potential design of novel strategies to confer or increase resistance to *R. solanacearum* in additional plant species through interfamily transfer of the associated PRR.

**Results**

**Solanaceae plants cannot perceive flgll-28Rsol or flgll-28Rsol in leaves or roots**

The amino acid sequence of the FlIC gene product in *R. solanacearum* GMI1000 contains both flg22 and flgll-28 sequences, showing several polymorphisms compared to flg22 and flgll-28 from *P. syringae* strains (Clarke et al., 2013; Mueller et al., 2012a; Figure S1). To test whether flgll-28Rsol could act as an elicitor of immune responses in Solanaceae plants, we monitored the elicitor-triggered burst of reactive oxygen species (ROS) in leaf tissues, which is a widely used readout to test elicitor activities in different plant species (Clarke et al., 2013; Hind et al., 2016). Our results show that none of the plant species tested (tomato, *N. benthamiana*, eggplant, tobacco, pepper, and Arabidopsis, the latter used as control) showed a detectable response to flgll-28Rsol (Figures 1a and S2). As previously reported, all these species responded to flg22 from *P. syringae pv tomato* DC3000 (flg22Rpto), while none of them responded to flgll-28Rsol (Figures 1a and S2). As reported before, only tomato and pepper responded to flgll-28 from *P. syringae pv tomato* T1 (flgll-28Rpto) (Figures 1a and S2; Clarke et al., 2013).

As a soil-borne pathogen, *R. solanacearum* mostly invades plant tissues through the roots. As most of the elicitation tests commonly used in plants are based on leaf tissue (or cultured cells), we sought to determine whether roots of tomato or *N. benthamiana* plants could respond to flg22Rsol or flgll-28Rsol, setting up an assay to measure ROS in roots of these plant species. However, elicitation patterns in roots mirrored those in leaves for these species and peptides: both species responded to flg22Rsol, only tomato responded to flgll-28Rsol, and none of them responded to flgll-28Rsol (Figures 1b and S3).

As an alternative method to test activation of PRR-dependent responses, we used a phosphorylation-dependent specific antibody to determine the phosphorylation of mitogen-activated protein kinases (MAPKs), which is a well-established elicitor-triggered response. The observed patterns of peptide-induced activation of immunity, measured as MAPK activation in tomato,
N. benthamiana, or Arabidopsis seedlings resembled those observed in ROS assays, with none of them responding to the flagellin-derived peptides from R. solanacearum (Figure S4).

Solanaceae plants cannot perceive other peptides from Ralstonia solanacearum flagellin in leaves or roots

To investigate whether Solanaceae plants can perceive other peptides from R. solanacearum flagellin different from flg22 and flgII-28, we purified the full-length flagellin protein (FliC) from R. solanacearum (FliC_Rs), fused to an N-terminal 6His-MSB tag and a C-terminal Strep tag (His-MSB-FliC_Rs-Strep; Figure S5). FliC from P. syringae pv tomato (His-MSB-FliC_Pto-Strep) was used as a positive control, and a GFP recombinant protein (His-MSB-GFP-Strep) as negative control. All proteins were expressed in Escherichia coli and purified as described in the methods section. The recombinant purified GFP did not trigger a

Figure 1 Flg22 or flgII-28 from Ralstonia solanacearum does not elicit responses in Solanaceae plants. Oxidative burst triggered by 100 nM of the indicated peptides or water (mock) in leaves (a) or roots (b) of plants from the indicated species, measured in a luminol- or L-012-based assay, respectively, as relative luminescence units (RLU) during 60 min. Values are average ± SE (n = 8). Asterisks indicate significant differences compared to the mock control according to a Student’s t-test (P < 0.001). The experiments were repeated at least three times with similar results.

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detectable response in leaves of any of the plants tested (Figure 2a), indicating that our purified recombinant proteins do not contain significant levels of contaminants from E. coli with eliciting activity, such as EF-Tu. All the plant species tested (tomato, N. benthamiana, eggplant, tobacco, pepper, and Arabidopsis) responded to His-MSB-FIC_Rs, while none of them showed a detectable response to His-MSB-FIC_Rs-Strep (Figures 2a, S6). The same pattern was observed in roots of tomato and N. benthamiana (Figures 2b, S7). To rule out a specific effect of the His-MSB-Strep tag or the purification process on the absence of elicitation by R. solanacearum flagellin, we expressed our recombinant proteins fused to a N-terminal 6His-maltose-binding protein (His-MBP; Figure S8a) tag in E. coli and purified them as described in the methods section (Figure S8b and C). Further analysis using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) confirmed that our recombinant protein contains the full sequence of R. solanacearum FIC (Figure S8b). However, recombinant His-MBP-FIC_Rs did not elicit a detectable response in tomato, N. benthamiana, or Arabidopsis (Figure S9). Of note, during the set-up of our assays, we noticed that gel filtration column buffer containing DTT caused a detectable luminescent signal in ROS assays (data not shown), and therefore, our recombinant proteins were eluted in buffer without DTT.

The perception of csp22Rsol leads to disease resistance to Ralstonia solanacearum in tomato plants

The effective activation of PRR-dependent immune responses leads to the strengthening of plant cells and protection against subsequent bacterial infections (Bigeard et al., 2015; Boller and Felix, 2009). To determine whether perception of csp22Rsol can trigger effective disease resistance in tomato plants, we inoculated R. solanacearum in tomato leaves 1 day after treatment with csp22Rsol or water (as mock control). Pretreatment with csp22Rsol was able to protect tomato leaves against a subsequent infection by R. solanacearum, as reflected by the significant reduction in bacterial growth in csp22Rsol compared to mock-treated leaves (Figure 4d). This result indicates that csp22Rsol is able to trigger the activation of effective immune responses against R. solanacearum in tomato cells.

The tomato SICORE receptor confers responsiveness to csp22Rsol

Several Solanaceae plants can perceive csp22, and this perception has been recently associated to specific PRRs in N. benthamiana and tomato (Saur et al., 2016; Wang et al., 2016). The N. benthamiana NbCSPR has been reported to confer responsiveness to csp22, but the presence of a tomato sequence homolog for this PRR is unclear (Saur et al., 2016). Another report associates this responsiveness in N. benthamiana and tomato to the presence of a different PRR, named NbCORE or SICORE, respectively (Wang et al., 2016). As we can detect a clear response to csp22Rsol in both N. benthamiana and tomato, we tested whether NbCORE and SICORE are associated with the perception of csp22Rsol. To this end, we first used Agrobacterium tumefaciens to transiently overexpress NbCORE or SICORE in 4-week-old N. benthamiana leaves. Control leaves overexpressing green-fluorescent protein (GFP) showed weak and nonconsistent response to csp22Rsol in keeping with our previous results using young N. benthamiana leaves (Figures 3b, S11). However, overexpression of NbCORE and, specially, SICORE led to an enhanced and robust responsiveness to csp22Rsol (Figures 5a–b, S12), suggesting that NbCORE/SICORE contributes to the plant response against csp22Rsol.

Arabidopsis does not perceive the consensus csp22 peptide or csp22Rsol (Felix and Boller, 2003; Figure 3b). To determine whether SICORE confers responsiveness to csp22Rsol in nonresponsive plants, we used Arabidopsis transgenic lines expressing SICORE, which have been previously shown to respond to the consensus csp22 peptide (Wang et al., 2016). As shown in Figure 5c, Arabidopsis transgenic lines expressing SICORE gained the ability to respond to csp22Rsol with a similar intensity to that displayed when using consensus csp22 peptide (Figures 5c, S12; Wang et al., 2016). Moreover, sustained exposure to csp22Rsol
caused a significant inhibition of growth in Arabidopsis seedlings expressing SlCORE, but not in wild-type plants (Figure 5d). These results indicate that heterologous expression of SlCORE is able to confer responsiveness to csp22Rsol in nonresponsive plants, and SlCORE-mediated perception of csp22Rsol triggers both early and late immune-related responses in Arabidopsis.

Expression of the csp22 receptor SICORE confers increased resistance to Ralstonia solanacearum in Arabidopsis

Given that the transgenic expression of SICORE conferred responsiveness to csp22Rsol, we sought to determine whether

Figure 2. Purified recombinant flagellin from Ralstonia solanacearum does not elicit responses Solanaceae plants. Oxidative burst triggered by 100 nM of the indicated recombinant proteins in leaves (a) or roots (b) of plants from the indicated species, measured in a luminol- or L-012-based assay, respectively, as relative luminescence units (RLU) during 60 min. Values are average ± SE (n = 8). Asterisks indicate significant differences compared to the GFP control according to a Student’s t-test (P < 0.001). The experiments were repeated at least three times with similar results.
this csp22$_{Rsol}$ responsiveness has an impact on Arabidopsis resistance to R. solanacearum. To this end, we inoculated roots of Arabidopsis seedlings grown in sterile MS medium with a suspension of R. solanacearum and recorded the presence of bacteria in shoots 2 days after inoculation. Most wild-type seedlings were colonized by R. solanacearum, showing detectable bacterial numbers in shoots (Figure 6a). However, a larger proportion of Arabidopsis seedlings expressing SlCORE did not contain detectable numbers of R. solanacearum colony-forming units in shoots 2 days after root inoculation, reflecting the inability of bacteria to colonize these plants at this time point (Figure 6a). Moreover, among those seedlings colonized by R. solanacearum, shoots of wild-type seedlings supported higher bacterial loads than seedlings expressing the SICORE receptor (Figures 6b, S13). Furthermore, mature transgenic plants expressing SICORE showed a small but reproducible delay in the development of disease symptoms, compared to wild-type plants, in soil-drenching infection assays (Figure 6c). These results suggest that transgenic expression of SICORE confers increased resistance to root infection by R. solanacearum in Arabidopsis.

**Discussion**

Although the perception of flg22 is a well-studied example of recognition of a bacterial elicitor by most plant species, certain bacterial strains, such as Pcal, show a small but reproducible delay in the development of disease symptoms, compared to wild-type plants, in soil-drenching infection assays (Figure 6c). These results suggest that transgenic expression of SICORE confers increased resistance to root infection by R. solanacearum in Arabidopsis.

**Figure 3** Csp22 from Ralstonia solanacearum is perceived in specific Solanaceae plants. (a) Sequence alignment of the csp22 sequences used in this study. Csp22 peptide (csp22$_{Rsol}$) corresponds to the csp22 sequence from R. solanacearum and recorded the presence of bacteria in shoots 2 days after inoculation. Most wild-type seedlings were colonized by R. solanacearum, showing detectable bacterial numbers in shoots (Figure 6a). However, a larger proportion of Arabidopsis seedlings expressing SlCORE did not contain detectable numbers of R. solanacearum colony-forming units in shoots 2 days after root inoculation, reflecting the inability of bacteria to colonize these plants at this time point (Figure 6a). Moreover, among those seedlings colonized by R. solanacearum, shoots of wild-type seedlings supported higher bacterial loads than seedlings expressing the SICORE receptor (Figures 6b, S13). Furthermore, mature transgenic plants expressing SICORE showed a small but reproducible delay in the development of disease symptoms, compared to wild-type plants, in soil-drenching infection assays (Figure 6c). These results suggest that transgenic expression of SICORE confers increased resistance to root infection by R. solanacearum in Arabidopsis.

**Figure 4** Csp22 from Ralstonia solanacearum triggers additional early and late PRR-dependent immune responses in Solanaceae plants. (a) MAPK activation assay in leaves of 3- or 5-week-old Nicotiana benthamiana plants after treatment with 1 μM csp22$_{Rsol}$ or water (mock) for 15 or 30 min as indicated. Immunoblots were analysed using antiphosphorylated MAPK antibody (α-pMAPK). Immunoblots were also analysed using α-actin antibody to verify protein accumulation. Molecular weight (kDa) marker bands are indicated for reference. (b) Growth inhibition of N. benthamiana seedlings treated with 100 nM flg22$_{Pto}$, csp22$_{Rsol}$ or water (mock) for 9 days. Values represent seedling weight as percentage of mock-treated seedlings (average ± SE; n = 12). (c) Photograph of seedlings after exposure to the different elicitors in (b). (d) Growth of R. solanacearum GMI1000 in tomato (cultivar M82) leaves pretreated with 1 μM csp22$_{Rsol}$ or water (mock) for 24 h and then syringe-infiltrated with a 10$^{6}$ cfu/mL bacterial inoculum. Bacterial growth was determined 3 days after inoculation. Values are mean ± SE (n = 3). Asterisks indicate significant differences compared to the mock control according to a Student’s t-test (P < 0.001). All experiments were performed three times with similar results.

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A. tumefaciens or R. solanacearum, have developed polymorphisms in the flg22 sequence, which abolish recognition by FLS2 (Clarke et al., 2013; Felix et al., 1999). This represents an additional level of the well-accepted arms race between pathogens and plants, and questions the previous idea that microbial elicitors constitute evolutionarily constrained PAMPs, therefore stable targets for recognition by plant cells. Interestingly, specific Solanaceae plants have an additional receptor, named FLS3, which can recognize a flagellin peptide different from flg22, named flgII-28 (Hind et al., 2016). This receptor can perceive the flgII-28 peptide from different Pseudomonas strains, including PcaL ES4326 (Hind et al., 2016), although it was unclear whether it can perceive flgII-28 from other polymorphic flagellin proteins. Our systematic approach

Figure 5 The SICORE receptor confers responsiveness to csp22 from Ralstonia solanacearum. (a and b) Oxidative burst triggered by 100 nM of csp22
Rsol in 5-week-old Nicotiana benthamiana leaves expressing GFP (control), NbCORE (a) or SICORE (b), measured in a L-012 (luminol)-based assay as relative luminescence units (RLU) during 60 min. (c) Dynamics of oxidative burst triggered by 1 µM csp22 consensus peptide (csp22Cons) or csp22Rsol in leaves of Col-0 wt Arabidopsis plants, or two independent Arabidopsis transgenic lines expressing SICORE, measured in a luminol-based assay as relative luminescence units (RLU) during 50 min. Values are average ± SE (n = 8). (d) Growth inhibition of seedlings of the Arabidopsis lines mentioned in (c) treated with 100 nM flg22Pto, 1 µM csp22Rsol or water (mock) for 9 days. Values represent seedling weight shown as percentage of mock-treated seedlings (average ± SE; n = 12). Asterisks indicate significant differences compared to the control according to a Student’s t-test (P < 0.001). All experiments were performed three times with similar results.

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Yali Wei et al.
shows that Solanaceae plants are unable to perceive synthetic flgII-28 or full flagellin from R. solanacearum. This lack of recognition suggests that flagellin from R. solanacearum has undergone a very efficient evolution process to avoid recognition by PRRs from Solanaceae plants, while keeping its ability to form a functional flagellum.

The conserved csp22 peptide from the CSP from several bacterial species has been found to elicit responses in specific plant species from the Solanaceae family (Felix and Boller, 2003). CSPs constitute a large family of abundant RNA-binding proteins in Gram-negative bacteria. Their name reflects the original observation that cold temperature leads to hyperaccumulation of these proteins. However, genes encoding CSP proteins are not only expressed in cold temperatures, but also constitutively and under other stresses (Bae et al., 2000). Besides their known roles in the regulation of cellular growth and adaptation to environmental conditions, recent studies have shown that specific CSPs are important for pathogenicity in the enterobacterial pathogen Salmonella enterica, where they are induced at higher temperatures, revealing a role for CSPs in bacteria–host interaction (Michaux et al., 2017). In R. solanacearum, genes encoding proteins that contain csp22-like sequences are expressed during the infection in tomato (Jacobs et al., 2012). According to our model, R. solanacearum encounters a stress-inducing environment when invading plant tissues, which may in turn lead to the hyperaccumulation of CSP proteins. As it has been proposed for other abundant PAMPs present inside bacterial cells (e.g. Ef-tu; Kunze et al., 2004), leakiness of damaged bacteria during the infection process or active export of CSPs may lead to the exposure of csp22. This elicitor could then be perceived at the plant plasma membrane by the extracellular domain of plant PRRs (Saur et al., 2016; Wang et al., 2016), triggering a signalling cascade that ultimately results in the onset of immunity.

Our work shows that csp22\textsubscript{Rsol} is able to trigger robust immune responses in tomato, N. benthamiana, and tobacco (Figures 3 and 4). Novel elicitation activities found using synthetic peptides may raise concerns regarding potential peptide contamination (Mueller et al., 2012b). During the course of this work, we have used other peptide preparations with broad elicitor activities, such as flg22\textsubscript{Pto} and flgII-28\textsubscript{Pto}. However, our csp22\textsubscript{Rsol} peptide solution does not trigger any detectable response in Arabidopsis, ruling out a contamination with flg22\textsubscript{Pto} (see Figures 1 and 3), while the absence of elicitation in pepper rules out contamination with flgII-28\textsubscript{Pto} (see Figures 1 and 3).

Figure 6  Expression of SICORE confers increased resistance to Ralstonia solanacearum in Arabidopsis. (a and b) A 10\textsuperscript{5} cfu/mL suspension of R. solanacearum was used to inoculate roots of 8-day-old Arabidopsis seedlings in MS plates. (a) Number of seedlings colonized by R. solanacearum, 2 days after root inoculation, in six independent biological replicates (average ± SE; n = 6). Eight seedlings were inoculated in each biological replicate. Each symbol represents the number of seedlings showing a detectable number of bacteria in the shoot in each biological replicate. The minimum number of detectable bacteria is 20 cfu. (b) Number of colony-forming units (cfu) of R. solanacearum detected in the shoot 2 days after root inoculation. N = 8 for Col-0; N = 3 for the CORE lines (the rest of the samples showed no detectable bacteria). A representation considering all the values (N = 8) is shown in the Figure S13. The result is representative of six independent biological replicates. Asterisk indicates significant differences compared to the control according to a Student’s t-test (P < 0.05). (c) A 10\textsuperscript{8} cfu/mL suspension of R. solanacearum was used to soak the pots of 4- to 5-week-old Arabidopsis plants. Twenty plants per genotype were used. The graph represents the progression of the average wilting symptoms over 14 days. The experiment was performed three times with similar results.

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Compared to flg22<sup>Rto</sup>, csp22<sup>Rsol</sup> triggers a weaker ROS burst or MAPK activation at early time points. Interestingly, however, both peptides show a comparable activity when a late response, such as the inhibition of seedling growth, is monitored (Figure 4b and c). Additionally, pretreatment of tomato tissues with csp22<sup>Rsol</sup> triggers a significant increase in resistance to subsequent <i>R. solanacearum</i> infection (Figure 4d), similar to what is usually reported for flg22<sup>TR</sup> treatment. This suggests that the outcome of the perception of both peptides is comparable at late time points or after sustained exposure to the elicitor.

Interestingly, responses to csp22 are reported to be age dependent and are only consistent in leaves from mature plants (older than 5 weeks old; Saur et al., 2016; Wang et al., 2016). We found a similar pattern for csp22<sup>Rsol</sup> (Figure 3b). This age dependency is counterintuitive, as it suggests that plants only mount this defence system after a certain age, when the developmental programme of plants is likely to prioritize reproduction rather than immune responses. However, our analysis showed that the plant response to csp22<sup>Rsol</sup> seems to be more consistent in root tissues. Contrary to the age-dependent response in leaves, roots of <i>N. benthamiana</i> seedlings (7 days old) consistently responded to csp22<sup>Rsol</sup> (Figure 3c). Furthermore, <i>N. benthamiana</i> seedlings displayed a robust reduction in growth after sustained exposure to csp22<sup>Rsol</sup>, showing a remarkable inhibition of root growth (Figure 4c). These results indicate that roots are better equipped for the perception of csp22 compared to leaves at early stages of plant development, and suggest that the biological relevance of CSP perception may be more significant for the perception of csp22 peptides from soil-borne pathogens, such as <i>R. solanacearum</i>. Interestingly, Arabidopsis transgenic plants expressing SICORE became more resistant to colonization by <i>R. solanacearum</i> after root inoculation (Figure 6), supporting the notion that csp22 recognition in roots is able to contribute to disease resistance.

A large number of effectors from different pathogens, including bacterial T3Es, have been found to suppress elicitor-dependent immune responses (Macho and Zipfel, 2015). Most experimental set-ups employed to activate elicitor-triggered immune responses in laboratory conditions involve the treatment with flg22 peptide from <i>Pseudomonas</i>, regardless of whether the studied immune-suppressing microbe (either bacterium, fungus or oomycete) contains an immunogenic flagellin or not. Although this approach has been very helpful in the identification of the immunosuppression capacity of many microbial molecules, it raises the question of whether such microbes are able to suppress immunity activated by their own effectors. The infection process by <i>R. solanacearum</i> most likely involves at least a quantitative suppression of elicitor-triggered responses, mediated either by T3Es (Mukaihara et al., 2016; Sang et al., 2018) or other molecules (Tran et al., 2016). From a practical point of view, our findings show that it is possible to use an actual <i>R. solanacearum</i> elicitor (csp22<sup>Rsol</sup>) to experimentally trigger immunity in <i>Solanaceae</i>, instead of employing a heterologous elicitor from <i>Pseudomonas</i>, such as flg22.

The csp22 peptide is not perceived outside of the <i>Solanaceae</i> family (Felix and Boller, 2003). Accordingly, Arabidopsis plants are unable to perceive csp22<sup>Rsol</sup>, although transgenic Arabidopsis plants expressing SICORE become responsive to csp22<sup>Rsol</sup>. Interestingly, when we inoculated roots of Arabidopsis seedlings with <i>R. solanacearum</i>, we noticed that a considerable proportion of transgenic seedlings expressing SICORE did not contain any detectable bacteria in the shoot (Figure 6a). Although this hinders the comparison of bacterial loads between wild-type and SICORE transgenic plants (creating a bias due to the presence of samples without actual numeric values; see Figures 6b and S13), it also suggests that expression of SICORE increases resistance to bacterial colonization in Arabidopsis seedlings. This is supported by the observation that transgenic plants expressing SICORE showed a delay in the development of disease symptoms in soil-drenching infection assays (Figure 6c). This observation indicates that, despite its ability to suppress immunity, <i>R. solanacearum</i> may not be able to cope as efficiently when facing the constitutive expression of additional PRRs components. Similarly, although <i>R. solanacearum</i> is able to cause infection in <i>Arabidopsis</i>, which contains the EF-Tu receptor (EFR), tomato transgenic plants expressing EFR have been reported to become more resistant to <i>R. solanacearum</i> (Lacombe et al., 2010). Our results indicate that the csp22<sup>Rsol</sup> peptide is not perceived by several important crops for which <i>R. solanacearum</i> is an agricultural threat, both within the <i>Solanaceae</i> family (e.g. pepper; Figure 3) and others (e.g. bean and peanut; data not shown). The fact that csp22<sup>Rsol</sup> is not perceived in these species suggests that transfer of SICORE, layered atop the pre-existing defences of a given crop plant or additional PRRs, could significantly contribute to the generation of <i>R. solanacearum</i>-resistant crops.

### Experimental procedures

#### Plant material and growth conditions

Except where indicated otherwise, plants used in this study were grown in an environmentally controlled growth room at 22 °C with a 16-h photoperiod, a 65% humidity and a light-intensity of 100–150 mE/m<sup>2</sup>/s<sup>1</sup>. Arabidopsis transgenic lines expressing SICORE (Wang et al., 2016) were kindly provided by Georg Felix.

#### Chemicals and peptides

All the chemicals used in this study were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated. Flg22, flgII-28 and csp22 peptides were purchased from ABClonal, using the following amino acid sequences: Flg22<sup>Rto</sup>: TRLSSGLKINSAKD-ESTNQRMLRELYSQSNDNSSTDRDA; flg22<sup>Rsol</sup>: QRLSTGLRVSQDSADAAAYAS; flgII-28<sup>Rto</sup>: QTLDGG; csp22<sup>Rsol</sup>: ATGTVKWFNETKFGF-FIPDGG; csp22 (consensus): AVGTVKWFNAEKGFGFITPDDG.

#### Agrobacterium-mediated transient expression

Agrobacterium-mediated transient expression in <i>N. benthamiana</i> was performed as described (Li, 2011). Before infiltration, the bacterial suspension was adjusted to a final OD<sub>600</sub> of 0.5. Samples were taken at 1–3 days postinoculation (dpi) for analysis based on experimental requirements, as indicated for each specific experiment.

#### Measurement of PAMP-triggered ROS burst

Reactive oxygen species (ROS) production in leaves upon PAMP treatment was measured following the protocol described by Sang and Macho (Sang and Macho, 2017). *Nicotiana benthamiana*, *Nicotiana tabacum* (cv. Petit Havana), eggplant (*Solanum*), pepper (California Wonder) and tomato (Moneymaker) plants were grown on soil. Leaf discs were taken from 3- to 6-week-old plants (as indicated for each specific experiment) for ROS measurement. Four- to five-week-old Arabidopsis plants grown in short day conditions were used.
The measurement of ROS production in roots was similar except for several minor modifications in sample collection. Tomato seeds were germinated on filter paper with water in a Petri dish, and 7-day-old seedlings were used for ROS assay. *Nicotiana benthamiana* and *Arabidopsis* seeds were first germinated on 1/2 MS (Murashige-Skoog) solid medium for 7 days and then transferred to 1/2 MS liquid culture for 4 days before ROS measurement. Roots of seedlings were cut into one-centimeter-long sections and allowed to recover for 5 h in 96-well plates with 100 µL H2O in each well. Eight root sections were analysed for each sample, using 100 nM of the corresponding peptide. For root assays, luminol was replaced by the more sensitive derivative L-012 (Wako Chemical, Japan), as stated in the figure legends. It is worth noticing that approximately 20% of tomato root samples showed very weak or no response to elicitor treatment, and this is most likely due to the sensitivity of tomato roots to the handling process. Although this leads to higher variation in ROS results, it did not affect the conclusions of the assays.

**MAPK activation assay**

To measure MAPK activation in leaves, intact leaves were immersed in 1 µM elicitor solution in a beaker and vacuum-infiltrated for 3 min. After 15 min, four leaf discs with 8 mm diameter were collected and frozen in liquid nitrogen for further analysis. Seedlings were immersed in 1 µM elicitor solution directly for 10/15 min and then were collected and frozen immediately in liquid nitrogen for further analysis. Western blots to determine MAPK activation were performed as described (Macho et al., 2012). Blots were incubated with anti-actin (Agrisera AS13264A0) to verify equal loading.

**Protein expression and purification**

The FliC gene from *Pseudomonas syringae* DC3000 or *R. solanacearum* GMI1000 and GFP gene were cloned into PET13-strep-msb-his vector and then transformed to Rosetta *E. coli* cells to express the His-MSB-strep tag fused protein. Three columns were sequentially used for protein purification: Ni column (Merck, Darmstadt, Germany), anion-exchange column (GE Healthcare, Pittsburgh, PA) and strep column (GE Healthcare, Chicago, IL), following the procedures described by the manufacturers. The genes were cloned into pDEST566 (Addgene, Cambridge, MA) to generate His-MBP tag fusion proteins. Ni column (Merck, Darmstadt, Germany), desalt column (GE Healthcare, Chicago, IL) and MBP column (NEB, Ipswich, MA) were used sequentially to purify the resulting proteins. Gel filtration (GE Healthcare, Chicago, IL) was used finally to remove unspecific proteins using Äktas purifier (GE Healthcare, Chicago, IL).

**Protein identification by LC-MS/MS**

*Escherichia coli* protein extracts were digested with trypsin and analysed by LC-MS/MS following the protocol described by Sang et al. (2018). The mass spectra were submitted to Mascot Server (version 2.5.1, Matrix Science, London, UK) for peptide identification and searched against the *E. coli* protein database supplemented with GFP protein sequence and flagellin protein sequences from P. syringae and *R. solanacearum*.

**Seedling growth inhibition**

*Arabidopsis* seeds were sterilized for 10 min in 75% ethanol and 0.1% Triton X-100, then washed two times in 75% ethanol and dried in 100% ethanol. Seed were germinated on 1.5% agar plates with MS medium for 48 h before transfer to 48-well plates containing 500 µL of liquid MS or liquid MS containing peptides (100 nM flg22 or 1 µM csp22). Seedlings grew at 21 °C with 12 h of light and 12 h of darkness. Fresh weight was recorded for each seedling 9 days after transfer to liquid media. *Nicotiana benthamiana* seeds were sterilized, sown and weighted as described above, but transferred to 24-well plates with 3 mL of liquid MS or liquid MS containing peptides.

**Ralstonia solanacearum infection assays**

*Ralstonia solanacearum* GMI1000 was grown overnight in B liquid medium (Plener et al., 2012). For soil inoculations, 20 four-week-old *A. thaliana* plants per genotype were grown in Jiffy pots (Jiffy International, Kristiansand, Norway) and inoculated by soil drenching with a 5 × 10⁷ colony-forming units/mL bacterial suspension. One litre of inoculum was used to soak 20 *A. thaliana*-containing pots. After 20-min incubation, plants were removed from the bacterial solution and placed on a bed of potting mixture soil in a new tray. Visual scoring of disease symptoms according to a scale ranging from ‘0’ (no symptoms) to ‘4’ (complete wilting) was performed as described previously (Vailleau et al., 2007). For internal growth curve assays (IGC), 8-day-old Arabidopsis seedlings were grown on MS plates and inoculated with 10⁵ cfu/mL GMI1000 bacterial suspension, as described in Lu et al. (2018), with minor modifications. IGC was performed by collecting aerial parts of Arabidopsis seedlings at 2 dpi, followed by washing in sterile water. Colony-forming units were counted by spreading serial dilutions on solid B medium. An unpaired Student’s t test was used to determine statistical differences between the samples. In cases where the data did not adjust to a normal distribution, a nonparametric Mann–Whitney U test was used.

For induced resistance assays in tomato, leaves of 4-week-old tomato plants (cultivar M82) were infiltrated with 1 µM csp22Rsol or water (mock). Twenty-four hours later, the same leaves were infiltrated with 10⁶ cfu/mL GMI1000 bacterial suspension. Three dpi, three 8-mm leaf discs were excised using a cork-borer, weighed and ground in 10 mM MgCl₂. Colony-forming units were counted by spreading serial dilutions on solid B medium. An unpaired Student’s t-test was used to determine statistical differences between the samples.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 Sequence alignment of the amino acid sequence of FliC from different bacterial species. The major elicitor peptides flg22 and flgll-28 are highlighted. Pto, Pseudomonas syringae; Rs, Ralstonia solanacearum.

Figure S2 Flg22 or flgll-28 from Ralstonia solanacearum do not elicit responses in leaves of Solanaceae plants. Dynamics of oxidative burst from the assays described in the Figure 1a, measured in a luminol-based assay as relative luminescence units (RLU) during 60 min. Values are average ± SE (n = 8).

Figure S3 Flg22 or flgll-28 from Ralstonia solanacearum do not elicit responses in roots of Solanaceae plants. Dynamics of oxidative burst from the assays described in the Figure 1b, measured in a luminol-based assay as relative luminescence units (RLU) during 60 min. Values are average ± SE (n = 8).

Figure S4 Flg22 or flgll-28 from Ralstonia solanacearum do not elicit MAPK activation in leaves of Solanaceae plants. MAPK activation assay in leaves of 5-week-old tomato (a), Nicotiana benthamiana (b) or Arabidopsis plants after treatment with 1 μM of the indicated peptides or water (mock) for 10, 15, or 20 min as indicated. Immunoblots were analysed using antiphosphorylated MAPK antibody (α-pMAPK). Immunoblots were also analysed using α-actin antibody to verify protein accumulation. Molecular weight (KDa) marker bands are indicated for reference.

Figure S5 Schematic representation of the 6His-MBP-FliC-Strep recombinant protein.

Figure S6 Purified recombinant flagellin from Ralstonia solanacearum does not elicit responses in leaves of Solanaceae plants. Dynamics of oxidative burst from the assays described in the Figure 2a, measured in a luminol-based assay as relative luminescence units (RLU) during 60 min. Values are average ± SE (n = 8).

Figure S7 Purified recombinant flagellin from Ralstonia solanacearum does not elicit responses in roots of Solanaceae plants. Dynamics of oxidative burst from the assays described in the Figure 2b, measured in a luminol-based assay as relative luminescence units (RLU) during 60 min. Values are average ± SE (n = 8).

Figure S8 His-MBP-FliC purification and quality control. (a) Schematic representation of the His-MBP-FliC recombinant protein. (b) Representative gel filtration analysis of the recombinant proteins. A red box indicates the fractions that were pulled together and used for elicitation assays. (c) SDS-PAGE analysis of the final purified protein used for elicitation assays. (d) LC-MS/MS analysis after tryptic digestion of the final purified FliC_Rs used for elicitation assays. Highlighted sequences represent the detected peptides.

Figure S9 Purified recombinant His-MBP-FliC from Ralstonia solanacearum does not elicit responses in leaves of Solanaceae plants. Oxidative burst triggered by 100 nM of the indicated recombinant proteins in tomato (a,b), Nicotiana benthamiana (c,d), and Arabidopsis (e,f), measured in a luminol-based assay as relative luminescence units (RLU) during 60 min. b,d and f show the dynamics of oxidative burst from the assays shown in a,c and e, respectively. Values are average ± SE (n = 8). The experiments were repeated at least three times with similar results.

Figure S10 Amino acid sequence alignment of csp22-like peptides from Ralstonia solanacearum GM10000 proteins. Locus identifiers for the genes encoding these proteins are shown as RpSPPP or RsSXXX, together with their annotation. Csp22_Rs6 indicates the peptide used in this work (sequence from Cspd3). Csp22_Consensus indicates the consensus peptide characterized in a previous report (Wang et al., 2016).

Figure S11 Csp22 from Ralstonia solanacearum is perceived in leaves of specific Solanaceae plants. Dynamics of oxidative burst from the assays described in the Figure 3a, measured in a luminol-based assay as relative luminescence units (RLU) during 60 min. Values are average ± SE (n = 8).

Figure S12 The CORE receptor confers responsiveness to csp22 from Ralstonia solanacearum. (a and b) Dynamics of oxidative burst from the assays described in the Figure 5a and b, measured in a luminol-based assay as relative luminescence units (RLU) during 60 min. (c) Accumulation of SICORE-GFP in Arabidopsis transgenic plants. The immunoblot was analysed using anti-GFP antibody (α-GFP). A molecular weight (KDa) marker band is indicated for reference, and a nonspecific band (marked with an asterisk) is used as control for equal loading. (d) Total RLU accumulated during 60 min from the assay described in the Figure 5c. Values are average ± SE (n = 8).

Figure S13 Expression of SICORE significantly reduce Ralstonia solanacearum infection in Arabidopsis. Number of colony-forming units (cfu) of R. solanacearum detected in the shoot 2 days after root inoculation. These data correspond to the experiment shown.
in the Figure 6b. In this representation, samples where no bacteria were detected are represented as 20 cfu, which corresponds to the minimum number of detectable bacteria. Therefore, \( N = 8 \) for all genotypes. The result is representative of six independent biological replicates. Asterisks indicate significant differences compared to the control according to a Student’s \( t \)-test (\( *P < 0.05; \quad ***P < 0.001 \)).