Induction of innate immune responses by flagellin from the intracellular bacterium, ‘Candidatus Liberibacter solanacearum’

Guixia Hao1, Marco Pitino1, Fang Ding1, Hong Lin2, Ed Stover1 and Yongping Duan1*

Abstract

Background: ‘Candidatus Liberibacter solanacearum’ (Lso) is a phloem-limited alphaproteobacterium associated with the devastating zebra chip disease of potato (Solanum tuberosum). Like other members of Liberibacter, Lso-ZC1 encodes a flagellin domain-containing protein (FlaLso) with a conserved 22 amino-acid peptide (flg22Lso). To understand the innate immune responses triggered by this unculturable intracellular bacterium, we studied the pathogen-associated molecular patterns (PAMPs) that triggered immunity in Nicotiana benthamiana, using the flg22Lso peptide and the full length flaLso gene.

Results: Our results showed that the expression of flaLso via Agrobacterium-mediated transient expression induced a slow necrotic cell death in the inoculated leaves of N. benthamiana, which was coupled with a burst of reactive oxygen species (ROS) production. Moreover, the expression of several representative genes involved in innate immunity was transiently up-regulated by the flg22Lso in N. benthamiana. The FlaLso, however, induced stronger up-regulation of these representative genes compared to the flg22Lso, especially that of flagellin receptor FLAGELLIN SENSING2 (FLS2) and respiratory burst oxidase (RbohB) in N. benthamiana. Although neither cell death nor ROS were induced by the synthetic flg22Lso, a weak callose deposition was observed in infiltrated leaves of tobacco, tomato, and potato plants.

Conclusion: The flagellin of Lso and its functional domain, flg22Lso, share characteristics of pathogen-associated molecular patterns, and trigger unique innate immune responses in N. benthamiana. Slow and weak activation of the innate immune response in host plants by the flagellin of Lso may reflect the nature of its intracellular life cycle. Our findings provide new insights into the role of the Lso flagellin in the development of potato zebra chip disease and potential application in breeding for resistance.

Keywords: Candidatus Liberibacter solanacearum, Flagellin, Flg22, Cell death, Reactive oxygen species, Gene expression

Background

Zebra chip (ZC) is an important potato disease causing millions of dollars in losses to both potato producers and processors in the United States [1]. The disease was first discovered in potato fields near Saltillo, Mexico in 1994, and was reported in Texas, USA in 2000 [2]. Since 2000, the disease has spread to several other states and has been accompanied by serious economic impacts [1]. The disease symptoms are characterized by necrotic flecking and medullary ray discolorations in tubers, leaf chlorosis, twisted stems, swollen nodes, vascular discoloration, leaf scorching, and wilting [3,4]. The putative causal agent of Zebra chip disease is ‘Candidatus Liberibacter solanacearum’ (Lso) (also known as Ca. Liberibacter psyllaurous), which is transmitted by the potato psyllid, Bactericera cockerelli [3,5]. Lso has a significantly reduced genome and shares a high degree of similarity with another important plant pathogen, ‘Ca. Liberibacter asiaticus’ (Las) [6,7]. Lso is also associated with diseases of other crops including tomato, carrot (Daucus carota L.) in Finland, and celery (Apium graveolens) in Spain [3]. Currently all commercial potato cultivars appear to be susceptible to Lso infection and the only strategy for controlling the spread of the disease is by managing the potato psyllid [1].
Plant immunity relies on two levels of defense response against pathogens: pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) that is a component of basal defense and effector-triggered immunity (ETI) that reflects immunity to specific strains [8,9]. PTI is associated with PAMP recognition and activation of plant plasma membrane receptors [8,10]. Typically, PTI induces several important signaling pathways including calcium ion (Ca2+) influx, reactive oxygen species (ROS) production, and mitogen-activated protein kinase (MAPK) pathway activation [10]. Interactions between the peptide flg22, which is located at the N-terminal domain of a flagellin, and host surface-localized pattern recognition receptors flagellin sensing 2 (FLS2) in Arabidopsis thaliana, are well studied. In A. thaliana, FLS2 interacts with the receptor kinase BAK1 by forming a functional FLS2/BAK1 complex, which is subsequently followed by a typical PTI response [8,10]. Orthologs of the Arabidopsis FLS2 have been identified in many plant species including N. benthamiana [11], Lycopersicum esculentum [12] and Oryza japonica [13]. Recently, it has been shown that silencing the expression of NbFLS2 compromised the expression of downstream genes induced by flg22 [14]. In Arabidopsis, the ROS burst induced by the flg22 is regulated by RbohD [15]. In N. benthamiana, RbohB, a homolog of RbohD, is essential for ROS production and silencing of RbohB completely abolishes the ROS burst [14,16]. In N. benthamiana, two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase (SIPK), and wound-induced protein kinase (WIPK) are activated quickly after elicitation [17]. Furthermore, both SIPK and WIPK are essential for bacterial immunity in N. benthamiana [14].

It has recently been shown that another member of the Liberibacter genus, Ca. Liberibacter asiaticus (Las), encodes a functional flagellin that partially restored the motility of Sinorhizobium meliloti fla mutant and shares characteristics of pathogen-associated molecular patterns [18]. Like Las, the Lso genome contains the same number of open reading frames (30 ORFs) that are essential for the structure and assembly of a flagellum [6]. In this study, due to the inability to grow Lso in culture, we investigated the PAMP activity of Lso flagellin (FlaLso) and its peptide flg22Lso in planta via infiltration or Agrobacterium-mediated transient expression. Our results demonstrate that transient expression of flaLso induces a delayed increase in ROS production and slow necrotic cell death in tobacco plants. Although the flg22 from P. aeruginosa (which was used as a control) induced the typical ROS bursts, the flg22Lso peptide did not induce ROS production in tobacco, tomato, or potato, but did induce callose deposition in these three species. We further demonstrated that the peptide flg22Lso, and the flagellin, FlaLso, induced expression of genes associated with PTI in N. benthamiana. These results provide new insights into the role of bacterial flagellin in the development of potato zebra chip disease.

Results

Lso encoding a flagellin with a conserved flg22Lso peptide

In the Lso-ZC1 genome, several clusters of flagellar biosynthesis related genes were identified by sequence analysis. CKC_02645 was characterized as encoding a flagellin domain-containing protein. This gene contains 1374 nucleotides and encodes a 457 amino-acid protein, designated as FlaLso. FlaLso shares 61% identity to the flagellin from Ca. Liberibacter asiaticus (Las), 59% identity to the flagellin from Ca. Liberibacter americanus (Lam) and 51% identity to the flagellin from L. crescens BTI. A conserved flagellin domain was identified consisting of 22 amino acids located at position 29 to 50 at the N terminus of FlaLso and was designated as flg22Lso. Flg22Lso shares high identity (86%) to the flg22 peptides from Las, Lam and L. crescens BTI and it shares 77% identity to the flg22 from the closely related species Agrobacterium tumefaciens and Sinorhizobium meliloti 1021, which do not induce plant immune responses [19,20]. Flg22Lso shares 41% identity with the flg22 from Pseudomonas aeruginosa and 55% identity with the flg22 from Pseudomonas syringae pv. tabaci, which trigger a strong nonhost innate immune response [21]. In flg22, the amino acid residue D42 is critical for its PAMP activity [22,23]. Although flg22Lso possesses amino acid D42, only two other amino acids are conserved in the central RIN-SAKDDA motif (Figure 1).
Slow necrotic cell death induced by transient expression of the fla₄₀ in N. benthamiana

For the transient expression assay of fla₄₀ in N. benthamiana, various concentrations of A. tumefaciens GV3101 with and without fla₄₀ were tested. At an OD₆₀₀ of 0.8 and 1.2, necrotic cell death was observed in the infiltration zone with pBin fla₄₀ at 8 days after inoculation; the control with pBin vector alone exhibited chlorosis but not necrosis, and GV3101 with no vector showed no visible response (Figure 2). In addition, cell death was measured by electrolyte leakage from leaf discs infiltrated with MgCl₂. GV3101 carrying pBin vector and pBin fla₄₀ constructs. Overexpression of the fla₄₀ in tobacco leaves increased electrolyte leakage on 4 and 5 dpi (Additional file 1: Figure S1). Taken together, this indicates that the expression of the flagellin gene, fla₄₀, causes necrotic cell death in the infiltrated zone of N. benthamiana. However, in tomato and potato inoculations, no obvious differences were observed after inoculation with various concentration of GV3101 containing the pBin vector alone or pBin fla₄₀ respectively (data not shown).

Callose deposition induced by flg22₄₀ and flg22₄₁

The conserved flagellin domain flg22₄₁ from Ca. Liberibacter asiaticus induced callose deposition when infiltrated into tobacco [18]. Compared to flg22₄₁, flg22₄₀ has three amino acid substitutions including a serine (S) to alanine (A) at position 38, and alanine (A) to serine (S) substitutions at positions 40 and 41 (Figure 1). Both synthetic peptides flg22₄₀ and flg22₄₁ at a concentration of 40 μM were infiltrated into leaves of N. benthamiana, tomato and potato. Neither peptide induced cell death in the infiltrated zone but callose deposition was observed in infiltrated N. benthamiana, tomato and potato, with more callose deposition from flg22₄₀ compared to flg22₄₁ in both tobacco and tomato (Figure 3). Compared to tomato and potato, a more robust callose deposition was observed in tomato after treatment with either one of the peptides (Figure 3E and F).

ROS production induced by the fla₄₀, but not by the flg22₄₀ in N. benthamiana

One of the important events of PTI response is a rapid and transient burst of ROS production. We examined whether ROS production is induced by flg22₄₀ or flg22₄₁. Neither flg22₄₀ nor flg22₄₁ produced an ROS burst when infiltrated at 0.1 μM, 1 μM, 10 μM or 40 μM in tobacco, tomato or potato; while the control flg22 from P. aeruginosa shows a typical ROS production at a 0.1 μM concentration in the three plant species (Figure 4). In contrast, ROS production was detected in transient expression of N. benthamiana leaves infiltrated with A. tumefaciens containing pBin fla₄₀ and pBin fla₄₁ clones respectively (Figure 5). The strongest ROS response was observed on the third and fourth day after infiltration. However, no ROS response was detected in tomato or potato infiltrated with these peptides and constructs (data not shown), which is consistent with the observation that no obvious cell death was induced in these plants.

PTI gene expression transiently up-regulated by flg22₄₀ in N. benthamiana

The expression of many plant genes is up-regulated in PAMP-triggered immunity [24,25]. To investigate expression levels of PAMP-associated genes in N. benthamiana after flg22₄₀ infiltration, reverse transcriptase quantitative PCR (RT-qPCR) was performed. After N. benthamiana was infiltrated with the flg22₄₀ peptide, the transcript abundance was measured at 0.5, 1.0, 3.0, and 6.0 hours post infiltration (hpi). The expression of NbFLS2 was up-regulated approximately 2 fold at 0.5 hpi, more than 3 fold at 1.0 hpi, and then decreased at 3.0 hpi with flg22₄₀ treatment (Figure 6). Previously, three marker genes in N. benthamiana, NbcYP71D20, NbACRE31 and NbACRE32, were found to rapidly increase following flg22₄₀ treatment [14,26]. These three PAMP-marker genes were transiently induced by flg22₄₀ at 1.0 hpi and then subsequently declined in our RT-qPCR assay, which indicates that flg22₄₀ induces a typical transient PAMP triggered immunity gene response. FLS2 directly binds bacterial flagellin and then interacts with BAK1 to form a recognition complex [8]. However, the expression level of NbBAK1 showed no obvious differences in our assay. Somatic embryogenesis receptor kinase 3 (NbSerk3)/BAK1 is required for PAMP-triggered immunity in N.
The expression pattern of NbSerk3/NbBAK1 did not show an obvious change upon flg22Lso treatment. Two MAPKs, NbWIPK and NbSIPK, were transiently increased at 1.0 hpi in our experiment. NbRbohB expression was up-regulated about 2 fold except at 3.0 hpi. Notably, most of these PTI related genes are up-regulated at 0.5 hpi or 1.0 hpi, and then diminish at 3.0 hpi. Plastocyanin, which plays a key role in photosynthesis, was reported to be induced in the PTI response to nonpathogenic P. fluorescens [11]. In our experiment, transcript abundance of NbPlastocyanin increased 5 fold at 1.0 hpi and almost 10 fold at 3.0 hpi (Figure 6). Taken together, our results showed that flg22Lso transiently induced PAMP-triggered gene expression in N. benthamiana.

Long lasting PTI gene expression induced by flaLso in N. benthamiana

The expression of flaLso, and its elicitation of PAMP-associated gene expression in N. benthamiana were investigated after infiltration with A. tumefasciens containing pBin:flaLso or the empty pBin vector plasmid. A high level of expression of flaLso was indicated by a strong band from 1 to 4 days after inoculation (dpi). A very faint band was observed on 8 dpi (Figure 7A). RT-qPCR data further showed that the transient abundance after inoculation increased to its highest level on 3 dpi and decreased to an undetectable level compared to the host reference gene NbEF1a (Figure 7B). Meanwhile, the gene expression levels of PAMP-associated genes were measured at 2, 3, 4, and 8 dpi (Figure 8). RT-qPCR data showed that the expression of NbFLS2 was up-regulated more than 9 fold at 4 dpi. The abundance of NbBAK1/NbSerk3 was increased about 2 fold on 4 dpi. The expression of three other PAMP-associated genes, NbCYP71D20, NbACRE31 and NbACRE32 were upregulated and varied only slightly at different time after infiltration. Two MAPKs, especially NbWIPK, were up-regulated more than 4 fold at 4 dpi. NbRbohB dramatically increased at 4 dpi, which agreed with our ROS assay results that the strongest ROS production was detected at 4 dpi. The expression level of plastocyanin gradually declined over the evaluation period, in contrast to the transient high induction at 1.0 and 3.0 hpi observed with flg22Lso. Collectively, the expression of PAMP-associated genes elicited by the flaLso was different from the transient up-regulation with peptide flg22Lso induction. Most of these genes were strongly induced at 4 dpi, and then decreased, which correlated with the expression pattern of the flaLso in N. benthamiana.

Discussion

Our results revealed that transient expression of the Lso flagellin gene (flaLso) induced a burst of ROS and slow necrotic cell death in N. benthamiana plants but not in the tomato or potato plants tested. Although the peptide flg22Lso did not induce cell death or a ROS reaction, it did induce callose deposition in all three plant species. We determined that the expression of PAMP-triggered
genes was transiently up-regulated with synthesized peptide flg22\textsubscript{Lso} treatment. We further showed that these genes were more strongly affected by \textit{Agrobacterium}-mediated transient expression of the full length gene, \textit{flaLso}. Our results demonstrate that Lso flagellin and its short peptide, flg22\textsubscript{Lso} have a PAMP activity, and both of them trigger PTI response.

Callose deposition induced by Lso and Las peptides

Many reports document that flg22\textsubscript{Lso} peptides can induce plant defense responses, including reactive oxygen species (ROS), pathogenesis related gene expression and callose deposition [27]. Amino acid D42 of flg22\textsubscript{Lso} has been demonstrated to be essential for the elicitor activity of \textit{Xanthomonas} and \textit{P. syringe pv. tabaci} in non-host species [22,23]. In our study, flg22\textsubscript{Lso}, which contains D42, did not induce visible cell death or ROS production in the plants tested, however, it was found to induce callose deposition in tobacco, tomato and potato plants. This suggests that flg22\textsubscript{Lso} may interact with the FLS2 receptors of all three plant species. In addition, we screened over one hundred potato genotypes using flg22\textsubscript{Lso} and flg22\textsubscript{Las}. We
found that flg22_Lso and flg22_Las induced ROS response in several potato cultivars and the full flagellin gene has the ability to interact with the potato FLS2 receptor in yeast two hybridization assay (Duan, unpublished data). The flg22 peptides from A. tumefaciens and S. meliloti possess the D42 amino acid but did not show PAMP activity [27]. This indicates that other amino acids are also important for flg22 elicitor activity in addition to D42. Amino acid changes made at S38 and A39 completely abolished the PAMP activity of flg22_Las, indicating that serine and aspartate at these positions are essential for flg22_Las recognition by tobacco plants [18]. Compared to flg22_Las, flg22_Lso has three amino acid substitutions and induces more callose deposition than flg22_Las in tobacco and tomato. It is worth noting that callose deposition was observed although no ROS production was detected in the infiltrated plants with either flg22_Lso or flg22_Las. However, callose deposition is sometimes seen in a host plant's response to pathogen infection as well as being a component of non-host resistance, and high levels of callose are formed in response to Las infection in citrus [28].

**Necrotic cell death induced by the fla_Lso in N. benthamiana**

Flagellin is a bacterial elicitor associated with the induction of plant and animal defense responses. In our study, we discovered that transient expression of the fla_Lso induced necrotic cell death in tobacco, which was much slower than that reported for a typical hypersensitive response (HR) [29]. Transient expression of P. syringae flagellin FlIC gene with or without signal peptide induced ROS and FLS2-dependent immunity in a non-host, but did not induce cell death within five days [30]. In our infiltration experiments, the expression of fla_Lso reached the highest level on 3 dpi and decreased to a very low level on 8 dpi, however necrotic cell death was not observed until 8–10

![Figure 6 Flg22_Lso-triggered changes in the expression of genes related to plant defense in N. benthamiana](image-url)
in plants [31]. Most studies on plant FLS2 have focused on interaction with flg22 of flagellin [27], as would occur with infection by *Pseudomonas* and many other bacterial pathogens. *Candidatus Liberibacter* species are strictly intracellular, and therefore, in order to function as a PAMP, their flagellins would require recognition within the plant cell. It has been demonstrated that transgenic expression of the flagellin from a rice-incompatible strain of *Acidovorax avenae* induced immune responses, expression of defense related genes, production of hydrogen peroxide and cell death in rice plants [33]. In another study, when FLS2-GFP was transgenically expressed, the FLS2 was primarily localized on the cell membrane before binding to flg22. After binding, the FLS2 accumulated into intracellular mobile vesicles and degraded upon flg22 activation [34]. In addition to being localized on cell membranes, FLS2 was also observed in intracellular vesicles of various sizes and shapes in a protoplast assay [35]. All of these together indicate that there is a unique pathway involving the interaction between FLS2 and Lso flagellin inside a plant cell. Further research is necessary to understand this pathway and downstream host response.

**PTI gene expression responses induced by Lso flagellin and peptide**

As a complement to the observations discussed above, we investigated flg22*Lso*-induced gene expression following infiltration into *N. benthamiana*. NbFLS2 and three other PAMP-marker genes, *NbCYP71D20*, *NbACRE31* and *NbACRE32*, were shown to be rapidly and transiently up-regulated (Figure 6) as was previously reported with the flg22 from *Pseudomonas* [14,26]. We also assessed several other genes which are reported to be associated with different host-defense systems. ACRE (Avr9/Cf9 rapidly elicited) genes associated with race-specific defense responses were found to be up-regulated by the infiltration of flg22*Lso* in *N. benthamiana*, similar to the result seen when resistant-race tobacco cells (Cf9 genotype) are treated with the fungal elicitor Avr9 [24].

When *P. fluorescens* was infiltrated into non-host *N. benthamiana*, the plastocyanin gene was markedly up-regulated, and silencing this gene compromised PTI [11]. Plastocyanin is a small Cu-containing protein which acts as an electron carrier between the cytochrome *b*₆*f* and photosystem 1 complexes in the photosynthetic electron-transfer chain [11]. Surprisingly, our results showed that the expression of *NbPlastocyanin* was the most highly induced gene in the PTI response by flg22*Lso*, treatment. However, the up-regulation of *NbPlastocyanin* expression gradually decreased over several days with *Agrobacterium*-mediated expression of full length *flaLso* which may have resulted from impaired chloroplast function as cell death was slowly induced. It has been reported that there is cross-talk between PAMP-triggered immunity and photosynthesis [32]. Though a typical pattern of PAMP-triggered gene expression was observed with flg22*Lso* infiltration, no ROS production was detected. This may be explained by the fact that two different
signaling pathways were separated after calcium influx initiation: one leading to the ROS burst and the other to MAPKs and other gene activation [14]. It was reported that domains other than flg22 contribute very little to the elicitation of the FLS2-mediated *Arabidopsis* defense response [22]. However, a second region designated as flgII-28 within flagellin apart from flg22 was recently identified in *P. syringae pv. tomato* and flgII-28 was shown to induce ROS in *N. benthamiana* not in *Arabidopsis* or bean [36]. Furthermore, the allelic variation of flg22 and flgII-28 were reported to affect the plant immune response significantly, and to have no effect on bacterial motility [36]. This may explain our findings that the full flagellin gene, flaLso, not the peptide flg22Lso, has the ability to induce ROS production and slow cell death in tobacco, which suggests other flagellin domains such as flgII-28 or post-translational modifications may also be associated with plant defense response. It is important to point out that transient expression of the flaLso only induced ROS production in tobacco but did not lead to ROS reaction in tested tomato and potato plants. This could be explained by the sequence variations within the FLS2 genes among species/varieties of host plants (Duan unpublished data).

It is established that FLS2 directly binds to flagellin in the *Pseudomonas* non-host system [27] and then interacts with BAK1 to form the FLS2/BAK1 complex, which activates the downstream signaling such as MAPK pathway. In our experiments, the expression of two MAPKs,NbWIPK and NbSIPK were indeed up-regulated in *N. benthamiana* by the flaLso (Figure 8). The prolonged activation of a MAPK pathway in cells may cause a redox imbalance and generate ROS, eventually leading to cell death [37]. Since MAP kinases are primarily regulated at protein levels, the levels of these proteins in *N. benthamiana* after inoculation with flg22Lso and or flaLso need a further investigation.

**Figure 8** Transient expression of the flaLso triggered changes in the expression of genes related to plant defense in *N. benthamiana*. *N. benthamiana* leaves were inoculated with *A. tumefaciens* GV3101 containing pBinflaLso and GV3101 containing empty vector pBin as a control. The infiltrated spots were harvested at 2, 3, 4 and 8 days post-infiltration (dpi) for RNA isolation and cDNA preparation. RT-qPCR was performed to check gene expression of NbFLS2, NbWIPK, NbSIPK, NbBAK1, NbRbohB, NbCYP71D20, NbACRE31, NbACRE32, NbPlastocyanin and NbSerk3. The samples were normalized against *NbEF1α*. Data were shown as average fold gene induction in response to vector infiltrated samples from three independent experiments.
PAMP-triggered immunity is important for plants to limit pathogen growth or generate signals for adaptation to secondary infections [38]. PAMPs, including flg22, activate components of the salicylic acid and jasmonic acid defense pathways, which protect against potential pathogenic bacteria [39]. The molecular events that occur during PTI and elicitor-triggered immunity (ETI) partially overlap including SA, ROS, and activation of MAPK cascades [40]. However, ETI elicits a much stronger response than PTI, indicating a quantitative difference between these two immunity responses [8]. The discovery that the FlaLso and flg22Lso have PAMP activity and trigger PTI reveals that the flagellin from intracellular bacteria can initiate plant defense responses. The identification of a compatible FLS2 is critical for the development of potato plants with increased resistance against Ca. L. solanacearum via marker-assisted conventional breeding and genetic engineering.

Conclusion
Zebra chip (ZC) is an important potato disease associated with the phloem-limited intracellular bacterium ‘Candidatus Liberibacter solanacearum’ (Lso). In this study, we examined the PAMP activity of the flagellin of Lso and its functional domain, flg22Lso in planta. We found that flg22Lso has the ability to induce callose deposition and it also triggers transient up-regulation of PTI associated genes in N. benthamiana. We determined that the expression of NbFLS2 and three marker genes, NbCYP71D20, NbACRE31 and NbACRE32, are rapidly upregulated. Surprisingly we found the expression of NbPlastocyanin increased dramatically, rising by 10 fold at 3 hpi. However, neither cell death nor ROS were induced by the flg22Lso. We also determined that expression of the full length flagellin gene induces a much stronger PTI response compared to peptide flg22Lso, especially upregulation of the PAMP-associated genes NbFLS2 and NbRbohB. In addition, the expression of flaLso induced ROS production and necrotic cell death in N. benthamiana via Agrobacterium-mediated transient expression. The discovery that the FlaLso and its short peptide have PAMP activity and the ability to trigger PTI provide new insights into the role of bacterial flagellin in the development of potato zebra chip disease and a potential application in breeding.

Methods
Plants and bacteria cultivation
Seeds of N. benthamiana and tomato were germinated in chambers with cycles of 16 h light and 8 h dark at 26°C. The seedlings were then transferred into Fafard® 4P Mix soil in plastic containers and grown in greenhouses. Potato cultivar (Atlantic) was planted directly in Fafard® 4P Mix soil in plastic containers and grown in greenhouses.

E. coli was grown at 37°C and A. tumefaciens was grown at 28°C in Luria-Bertani (LB) medium. Kanamycin (kan) was added to the medium at a concentration of 50 μg/mL.

flaLso construction for transient expression in plants
The full length gene of flaLso was amplified using genomic DNA as a template with primers flaLsoF and flaLsoR (5'-AAACC GGGAATTCGTTTCCAATTTTTAAGGAT A-3'; 5'- AAAAAAGGCTAACCACGGAAAAGAGAT AGAATT-3'). Italicized bases are SmaI restriction sites included for cloning. The PCR product was ligated into PCR2.1-TOPO vector and transformed chemically into E. coli TOPO10 cells following the manufacturer’s instruction (Invitrogen, Carlsbad, CA). The positive clones were used for plasmid isolation and sequence verification. The consensus clone was digested, gel purified and cloned into a binary vector pBINPLUS/ARS-2x35S (pBin) generating pBinflaLso. The recombinant vector was then transformed into A. tumefaciens GV3101 by a freeze-thaw method [41].

Peptide infiltration and callose deposition assay
The peptides of flg22Lso (DRVSSGLRVADSSDNAAYWS IA) and flg22Lso (DRVSSGLRVSDAADNAAYWSIA) were synthesized by Life Tein Company (South Plainfield, NJ). Synthetic peptides were diluted with double distilled water to a final concentration of 40 μM and infiltrated into plant leaves with a 1 mL needleless syringe. At the second day post-infiltration (dpi), callose deposition was detected with aniline staining as described previously [39]. Briefly, the tissue was cleared and dehydrated in 100% ethanol. At the second day post-infiltration (dpi), callose deposition was detected with aniline staining as described previously [39]. Briefly, the tissue was cleared and dehydrated in 100% ethanol. Cleared leaves were washed with distilled water and then stained overnight at room temperature in 0.01% aniline blue in 67 mM K2HPO4 (pH 12). Stained material was mounted in 50% glycerol and observed under ultraviolet of epifluorescence (Leitz DMR microscope, Leica Microsystems, Buffalo Grove, IL).

Plant infiltration and RNA isolation
A. tumefaciens GV3101 and A. tumefaciens GV3101 containing vector pBin or pBinflaLso were cultured overnight in 2 mL of LB medium with the addition of 50 μg/mL kan. Fifty microliter of the overnight cultures were inoculated into fresh 5 mL LB medium for another 24 hr with the addition of 50 μg/mL kan, 10 mM MES (2-(N-morpholino)-ethanesulfonic acid), and 100 μM acetylseringone. The overnight cultures were centrifuged, washed, and re-suspended in Agromix (10 mM MgCl2, 10 mM MES, and 100 μM acetylseringone). The suspension was adjusted to different OD600 values with Agromix and kept at room temperature for at least 3 hr. The final cell suspension was used to inoculate plant leaves with a 1 mL needleless syringe. N. benthamiana leaves were infiltrated with flg22Lso at 40 μM and water as a control. As flg22Lso induced rapid
and transient PAMP-triggered gene expression [14], the infiltrated zones were harvested at 0.5, 1, 3 and 6 h post-infiltration (hpi) for RNA isolation. For full flaLso transient expression, A. tumefaciens GV3101 containing vector pBin or pBin:flaLso was used to infiltrate N. benthamiana leaves at OD600 ~ 0.5. Similarly the infiltrated zones were harvested at 2, 3, 4 and 8 d post-infiltration (dpi) for RNA isolation because cell death was observed after 8 to 10 dpi. Trizol reagent was used for RNA extraction according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Total RNA was quantified using the Nanodrop and treated with RQ1 RNase-free DNase from Promega Corp (Madison, WI).

ROS assay and ion-leakage assay
For the flg22Lso and flg22Laso peptides, leaf discs of N. benthamiana, tomato and potato were floated on water overnight. Prior to ROS measurement, water was replaced with 100 μl of assay solution (17 μM lumino, 1 μM horse-radish peroxidase, flg22Lso or flg22Laso at concentrations of 0.1 μM, 1 μM, 10 μM and 40 μM). Luminescence was measured using the Perkin Elmer Victor3 V 1420 Multilabel Plate Counter (Waltham, Massachusetts). The flg22 peptide from P. aeruginosa was used at 0.1 μM as a positive control for the ROS assay.

For the FlaLso assay, A. tumefaciens GV3101 containing vector pBin or pBin:flaLso with an OD600 of 0.5 was used to infiltrate leaves of N. benthamiana, tomato and potato as described above. Leaf discs from infiltrated zones were taken on 2, 3, 4, and 5 dpi. ROS assay was performed as described above.

The ion-leakage assay was performed as described [42]. Briefly six leaf discs (5 mm in diameter) were collected at 2, 3, 4, 5 and 8 d with a sharp cork borer after infiltration with 10 mM MgCl2. A. tumefaciens containing pBin:flaLso or pBin vector alone as a control, and then washed with 10 mL distilled water for 30 min. Then they were transferred to fresh distilled water. Conductance was measured with an OAKTON electrical conductivity meter (Singapore).

Reverse transcription quantitative PCR (RT-qPCR)
DNase-treated RNA (~2 μg) was used to synthesize first-strand cDNA with 0.5 μg of oligo (dt) primer and 1 μL of SuperScript® III reverse transcriptase in a 20 μL reaction (Invitrogen). A negative control without the reverse transcriptase was performed to verify the absence of genomic DNA contamination. RT-qPCR was performed with SYBR in triplicate using an Eppendorf Mastercycler® Realplex thermal cycler. The 15 μL amplification reactions contained the following: 7.5 μL of SYBR® Green PCR Master Mix system (PERFECTA SYBR FASTMX LRX, VWR), 250 nM of each forward and reverse primer, and 2.0 μL of diluted cDNA template. The following protocol was used: 95°C for 5 min, 40 cycles of 30 s for denaturation at 95°C and 30 s for extension at 60°C. Primers for NbFLS2 were designed as 5’-TCAATGTGATGACTGGA-3’ and 5’-ATGATGTGCTGCTCCCCATCC-3’. The N. benthamiana elongation factor 1 alpha (NbEF1α) was amplified and used to normalize the values as an internal control with primers (5’-GACACCTGAAGTGGATCTGTT-3’; 5’-TAGCACCAGTGGTGCTCCTTCTT-3’). All other primers were used as previously reported (NbWPiK), NbSIPK, NbRbohB, NbACRE31, NbACRE32 and NbCYP71D20 as in publication [14]; NbPlastocyanin as in [11]; NbBAK1 as in [18] and NbSerk3 as in [26]). For evaluation of transient expression of the flaLso tissue collection, RNA isolation and cDNA amplification were carried out as described above. RT-PCR and RT-qPCR were performed to detect the flaLso expression levels at various times after infiltration. The primers for flaLso were designed as 5’-TTGCGTGTGTGCTGATCTTCTG-3’ and 5’-TCTG CCTGACAAATGGTGC-3’. The expression level was normalized against internal control NbEF1α. Meanwhile, the 2-△△CT method was used to calculate the changes in relative copy number of the target genes under treated conditions, 2-△△CT method was taken [43] where CT is the point at which the fluorescence signal crosses the threshold and △CT = CT (target gene) - CT (internal control) and △△CT = (CT, Target – CT, internal control) Time x - (CT, Target – CT, internal control) Time x.

Additional file

Additional file 1: Figure S1. Electrolyte leakage from leaf discs of N. benthamiana leaves inoculated with 10 mM MgCl2, Agrobacterium tumefaciens strain GV3101 containing the vector control pBin and the pBin: flaLso, constructs, respectively. * marked as significant change by student t-test.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
GH and YPD conceived and designed the experiments. GH and MP performed the experiments. GH, DF and MP analyzed the data. GH, MP and LH contributed reagents/materials/analysis tools. GH, ES and YPD wrote the manuscript. All authors read and approved the final manuscript.

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Author details
1S. Horticultural Research Laboratory, USDA-ARS, 2001 South Rock Rd, Fort Pierce, FL 34949, USA. 2San Joaquin Valley Agricultural Sciences Center, USDA-ARS, Parlier, CA 93648, USA.

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