Comparative Genomics Screen Identifies Microbe-Associated Molecular Patterns from ‘*Candidatus Liberibacter*’ spp. That Elicit Immune Responses in Plants

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Citrus huanglongbing (HLB), caused by phloem-limited ‘*Candidatus Liberibacter*’ bacteria, is a destructive disease threatening the worldwide citrus industry. The mechanisms of pathogenesis are poorly understood and no efficient strategy is available to control HLB. Here, we used a comparative genomics screen to identify candidate microbe-associated molecular patterns (MAMPs) from ‘*Ca. Liberibacter*’ spp. We identified the core genome from multiple ‘*Ca. Liberibacter*’ pathogens, and searched for core genes with signatures of positive selection. We hypothesized that genes encoding putative MAMPs would evolve to reduce recognition by the plant immune system, while retaining their essential functions. To efficiently screen candidate MAMP peptides, we established a high-throughput microtiter plate-based screening assay, particularly for citrus, that measured reactive oxygen species (ROS) production, which is a common immune response in plants. We found that two peptides could elicit ROS production in *Arabidopsis* and *Nicotiana benthamiana*. One of these peptides elicited ROS production and defense gene expression in HLB-tolerant citrus genotypes, and induced MAMP-triggered immunity against the bacterial pathogen *Pseudomonas syringae*. Our findings identify MAMPs that boost immunity in citrus and could help prevent or reduce HLB infection.

Keywords: ‘*Candidatus Liberibacter*’ species, citrus, citrus greening, defense signaling pathways, elicitors, genomics, huanglongbing, MAMPs, microbe-associated molecular patterns, PAMPs,

pattern-triggered immune response, plant responses to pathogens, reactive oxygen species

Citrus greening or huanglongbing (HLB) is a destructive disease that threatens the worldwide citrus industry. The disease is mainly caused by three gram-negative bacteria: ‘*Candidatus Liberibacter asiaticus*’, ‘*Ca. L. africanus*’, and ‘*Ca. L. americanus*’ (Gottwald 2010). The bacterium is transmitted by the citrus psyllid *Diaphorina citri* and colonizes the phloem of citrus (Bové 2006; Pelz-Stelinski et al. 2010). Infected citrus trees exhibit blotchy mottle and asymmetrical chlorosis on the leaves, discolored and bitter-tasting fruit, a short lifespan, and low productivity (da Graça et al. 2016). In the United States, the disease has already caused huge losses in Florida’s citrus industry and is spreading to other major areas of production in Texas and California (Wang and Trivedi 2013). Diverse methods have been applied to control HLB disease (Wang et al. 2017) such as the use of antimicrobial compounds (Zhang et al. 2011), plant hormones (Canales et al. 2016; Nehela et al. 2016), and thermotherapy (Hoffman et al. 2013). However, unfortunately, there is very limited means of controlling HLB in the field (Li et al. 2016). One of the major factors contributing to the rapid spread and devastation of HLB is the lack of natural resistance in hosts. Identifying infected trees is also challenging because HLB symptoms may not appear for more than 1 year after initial infection (Gottwald 2010; Manjunath et al. 2008). Bacterial colonization appears to be quite slow, perhaps due, in part, to its niche in the phloem (Bendix and Lewis 2018; Wang et al. 2017). In addition, ‘*Ca. L. asiaticus*’, ‘*Ca. L. africanus*’, and ‘*Ca. L. americanus*’ have been recalcitrant to culturing, which has limited further exploration of genetic factors contributing to virulence or recognition. As a result, there has been much interest in exploring plant innate immunity against ‘*Ca. Liberibacter*’ spp. (da Graça et al. 2016).

Plant immune response to biotrophic pathogens can be divided into microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI). MAMPs are recognized by membrane localized pattern recognition receptors (PRRs), which are commonly receptor-like kinases (RLKs) or receptor-like proteins (Segonzac and Zipfel 2011). The best characterized MAMP is flg22, from the highly conserved N-terminal domain of flagellin in *Pseudomonas aeruginosa*, which is recognized by the PRR FLAGELLIN-SENSING 2 (FLS2) in *Arabidopsis* (Boller and

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Felix 2009; Felix et al. 1999; Gómez-Gómez and Boller 2000). MTI induces a suite of immune responses, including reactive oxygen species (ROS) production, Ca²⁺ fluxes, stomatal closure, callose deposition, mitogen-activated protein kinase (MAPK)-dependent signal transduction, defense gene expression, and hormone biosynthesis (Boller and Felix 2009; Macho and Zipfel 2015). Successful pathogens attenuate MTI by delivering effectors to evade or suppress host immunity (Clark et al. 2018; Lewis et al. 2009; Toruño et al. 2016; Xin et al. 2018). These pathogenic proteins, when recognized in the cytoplasm by nucleotide-binding leucine-rich repeat proteins, trigger a second layer of plant defense, called ETI (Jones and Dangl 2006; Schreiber et al. 2016). ETI is an amplified MTI response (Jones and Dangl 2006; Tao et al. 2003; Tsuda and Katagiri 2010), resulting in rapid cell death at the infection site (Heath 2000; Wright and Beattie 2004).

Because many MAMPs are part of highly conserved molecules such as flagellin, elongation factor Tu (EF-Tu), and peptidoglycan, the MAMP-encoding gene is inferred to be essential for the pathogen (Gust et al. 2007; Kacar et al. 2017; Kunze et al. 2004; Zipfel et al. 2004). As a result, this means that a pathogen cannot easily lose a gene encoding a MAMP to avoid recognition. However, selection for variation within a MAMP might reduce its recognition by the plant immune system. Several comparative genomic studies identified core genes with signs of selection in *Pseudomonas syringae* or *Ralstonia solanacearum* (Eckshtain-Levi et al. 2018; McCann et al. 2012; Mott et al. 2016). Given the difficulties in manipulating HLB pathogens and the disease's long latency period, we pursued a bioinformatics-based comparative genomics screen to identify potential MAMPs from '*Ca. Liberibacter*' spp. We identified 13 candidate MAMPs from '*Ca. Liberibacter*' genomes, and screened for immune responses to candidate MAMPs in *Arabidopsis*, *Nicotiana benthamiana*, and citrus species. We identified two new MAMPs that elicit MTI. This study identifies new targets to boost the host immune system and potentially control HLB.

RESULTS

Computational identification of candidate MAMPs.

To identify putative MAMPs, we employed a computational comparative evolutionary genomics approach. MAMP-encoding genes are inferred to be essential for the pathogen because they are highly conserved. However, because MAMPs are recognized by the host, this creates strong evolutionary pressure on the pathogen to diversify parts of the protein that are not essential for its activity and that could lessen recognition by the plant immune system (McCann et al. 2012). Therefore, we sought to identify core genes (Supplementary

Table S1) with signatures of positive selection (Supplementary Fig. S1). We took advantage of publicly available genome sequences for '*Ca. L. asiaticus*' (seven strains), '*Ca. L. africanus*' (one strain), '*Ca. L. americanus*' (two strains), '*Ca. L. solanacearum*' (four strains), and *Liberibacter crescens* (two strains) to identify core and accessory genes using Roary (Page et al. 2015). We used codeml and Bayes empirical Bayes estimates to identify core genes under positive selection (Yang 2007; Yang et al. 2000, 2005).

We hypothesized that genes under positive selection would be shared by citrus-infecting strains ('*Ca. L. asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*') and possibly '*Ca. L. solanacearum*', which infects solanaceous plants, but not *L. crescens*, which is nonpathogenic (Fagen et al. 2014; Naranjo et al. 2019). Therefore, to refine our list of candidate MAMPs, we performed a second round of analysis to identify core genes that were shared between '*Ca. L. asiaticus*' and related strains, and were not under selection in *L. crescens*. We used codeml and Bayes estimates to identify core genes under positive selection in '*Ca. L. asiaticus*' strain psy62, because '*Ca. L. asiaticus*' had the most strains available (Yang 2007; Yang et al. 2000, 2005). We prioritized 13 candidate MAMPs (Table 1) for further study because they contained multiple sites of positive selection, and an additional candidate peptide because it was associated with the flagella. We designed and synthesized 20 to 26 amino acid peptides that encompassed the positively selected sites in the candidate proteins.

Candidate MAMPs elicit ROS production.

Apoplastic peroxidase (POX) is critical for generating ROS during pathogen invasion, as part of a concerted plant immune response (Daudi et al. 2012; O'Brien et al. 2012). To determine whether candidate MAMPs could elicit immune responses, we carried out a colorimetric assay for POX activity. The POX assay is highly sensitive, and allows for the detection of weak MAMPs (Mott et al. 2018). After sampling and washing leaf tissue to prevent the detection of POX activity from wounding (Minibayeva et al. 2009), we incubated the candidate MAMP with the tissue, and assayed for ROS production using 5-aminosalicylic acid. We first tested the candidate MAMPs in *Arabidopsis thaliana* and *N. benthamiana*, because they are better characterized systems for detecting ROS activity and because plants may differ in their ability to detect MAMPs, as seen with the recognition of EF-Tu by the EFR receptor (Lacombe et al. 2010). We used flg22, a well-characterized MAMP (Zipfel et al. 2004), as a positive control. At low concentrations of peptide (10 nM, 100 nM, or 1 µM), we were unable to detect POX activity in *Arabidopsis*. When tested at 10 µM, pksG and nuoD in Murashige and

Table 1. Candidate microbe-associated molecular patterns used in this article

Peptide name	PSS ^a	Peptide	Gene description
dnaG	3	KQIAQV TAKGE AECTAILIS	DNA primase
pksG	3	SILEI EDIT GIYTNDCMDFWR	Polyketide biosynthesis/hydroxymethylglutaryl-CoA synthase
lpxK	4	SGKKVL AFSGIADTE KFFTT	Tetraacyldisaccharide 4'kinase
thrS	3	SHEKARE LFESK EAYKVE	Threonine-tRNA ligase
nuoD	2	FKQRNVNIGTVKLEDAWG	NADH-quinone oxidoreductase subunit D
flgG	1	GSGVQISSVRNVY TQGE VVH	Flagellar basal-body rod protein
argG	2	IDFQRGDPIAINGQVMSPEVLLEQ	Arginosuccinate synthase
gyrA	2	AFVAFREEVVVR RTKYLL NKARD	DNA gyrase subunit A
priA	2	SAIYSQSCVVCSSGKMIACGFIER	Primosomal protein N/phosphoribosyl isomerase A
aidB	2	NQVHQPSDSL FALNQP PLFIGMN	Acyl-CoA dehydrogenase
uvrC	2	EISFFKQYGYKVKITVPKQ GEKR KI	UvrABC system protein C
fabB	2	HSLGAAGAQA IAIYCL IMMQERF	3-Oxoacyl-synthase 1/β-ketoacyl synthase
guaA	1	AFLIYEAIGINLTCVLVDHGFMRKN	GMP synthase

^a PSS = positively selected sites, shown in bold in peptide sequence.

Skoog (MS) media induced higher ROS production than MS media alone. However, the overall absorbance was still much lower than that induced by flg22 (Supplementary Fig. S2). At 20 μ M, we saw statistically significantly increased absorbance for nuoD, which was more comparable with that induced by flg22 (Fig. 1A). However, pksG displayed similar levels of POX activity when tested at 10 μ M or 20 μ M (Fig. 1A; Supplementary Fig. S2). We noticed that pksG contains 48% hydrophobic residues, which can impair its ability to dissolve in MS. Thus, we tested pksG in phosphate-buffered saline (PBS), amended with ammonium bicarbonate (PBS-A), which can improve peptide solubility. pksG triggered statistically significantly increased POX activity when tested at a lower concentration (5 μ M) in PBS-A buffer, presumably because it was more soluble. Interestingly, 1 μ M flg22 in PBS-A buffer showed decreased absorbance compared with 1 μ M flg22 in water (Fig. 1B).

Because peptides may be differentially recognized in hosts, we then tested these MAMP candidates in *N. benthamiana*. Our MAMPs elicited weaker responses in *N. benthamiana* compared with *Arabidopsis*. Only nuoD had slightly increased absorbance when tested at 30 μ M (Supplementary Fig. S2). At 50 μ M, nuoD showed consistently increased and statistically significant absorbance, comparable with that induced by flg22, while the other MAMP candidates showed only slightly increased absorbance (Fig. 1C). We also tested pksG or flg22 in the PBS-A buffer, and found that pksG triggered increased POX activity at 20 μ M, while 1 μ M flg22 elicited a similar response as in *Arabidopsis* (Fig. 1D).

To further confirm that nuoD and pksG induced ROS responses, we carried out luminol-based ROS assays. Only pksG elicited high levels of ROS production (approximately 200 relative light units [RLU]) (Fig. 2A, B, I, and J) in *Arabidopsis* and *N. benthamiana*, which were higher than those elicited by flg22 in PBS-A buffer (approximately 80 to 100 RLU)

Fig. 1. Some candidate microbe-associated molecular patterns (MAMPs) elicit reactive oxygen species production in the peroxidase (POX) assay in *Arabidopsis* and *Nicotiana benthamiana*. POX activity was measured after MAMP treatment. Asterisks indicate significant differences (mean $\pm$ standard deviation, $n = 10$). Statistical significance was assessed by t tests, where *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively. Each experiment was repeated three times with similar results. **A**, POX activity in *Arabidopsis* with Murashige and Skoog (MS) medium, 1 μ M flg22, or 20 μ M candidate MAMP. **B**, POX activity in *Arabidopsis* with H₂O, phosphate-buffered saline amended with ammonium bicarbonate (PBS-A) buffer, 5 μ M pksG, 1 μ M flg22 in PBS-A buffer, or 1 μ M flg22 in H₂O. **C**, POX activity in *Nicotiana benthamiana* with MS, 1 μ M flg22, or 50 μ M candidate MAMP. **D**, POX activity was measured after treatment in *N. benthamiana* with H₂O, PBS-A buffer, 20 μ M pksG, 1 μ M flg22 in PBS-A buffer, or 1 μ M flg22 in H₂O.

(Fig. 2C, D, I, and J). As expected, flg22 in water elicited high levels of ROS production (1,500 to 2,500 RLU) (Supplementary Fig. S3) in both species, indicating that the PBS-A buffer had a strong dampening effect on MAMP activity. We could not detect the ROS burst from treatment with nuoD within 60 min, even when we increased the peptide concentration to 200 μ M. To exclude the possibility that high concentrations of peptide could cause ROS production, we tested

thrS as a control. thrS carries three positively selected sites but it did not elicit an ROS burst when tested at 100 or 200 μ M in either species (Fig. 2E, F, I, and J) (data not shown). Thus, high peptide concentrations alone are not sufficient to trigger ROS responses. To exclude the possibility that our candidate peptides were contaminated with flg22, we tested pksG and nuoD in *Arabidopsis* ecotype Wassilewskija (Ws) (Supplementary Fig. S4), which is insensitive to flg22. pksG and

Fig. 2. pksG triggers the reactive oxygen species (ROS) burst in *Arabidopsis* and *Nicotiana benthamiana*. Luminol-based assay for ROS production in *Arabidopsis* or *N. benthamiana*. Peptides were added to leaf disks at 0 min, and relative light units (RLU) were measured for 60 min. Data are shown as mean $\pm$ standard deviation, $n = 6$. **A** and **B**, Treatment with 100 μ M pksG in phosphate-buffered saline amended with ammonium bicarbonate (PBS-A) buffer. **C** and **D**, Treatment with 1 μ M flg22 in PBS-A buffer. **E** and **F**, Treatment with 100 μ M thrS in PBS-A buffer. **G** and **H**, Treatment with PBS-A buffer. **I** and **J**, Total ROS production was calculated by integrating the area under the curve and normalizing the flg22 treatment to 100%. The average of four independent experiments is shown. Error bars represent standard error. Asterisks indicate statistically significant differences compared with the flg22 treatment. Statistical significance was assessed by t tests, where ** indicates $P < 0.01$.

nuoD both elicited an ROS burst in Ws, confirming that the activity arises from the peptide elicitors and is not due to contaminating flg22 peptide.

The differential responses to nuoD in the POX and luminol assays suggested to us that nuoD was a weak MAMP, with activity below the limit of detection in the luminol assay. Therefore, we wondered if the positively selected sites in nuoD were poorly recognized due to the peptide's secondary structure. We constructed several variants of nuoD that retained the positively selected "GT" sites but were extended toward the N terminus or C terminus of the protein (Fig. 3A). We then tested the nuoD variants again in *Arabidopsis* using the POX assay. At 20 μ M, nuoDII-25, with the GT site at the N terminus of the peptide, displayed increased absorbance compared with nuoD, whereas nuoDIV-25 and nuoDV-25, with C-terminal GT sites, induced lower absorbance than nuoD (Fig. 3B). nuoDIII-25 and nuoDVI-25, both of which had internal GT sites, induced less but still statistically significant increases in ROS production (Fig. 3B). Because nuoDII-25 elicited the strongest ROS production, we then tested it in the luminol assay. At 200 μ M, nuoDII-25 induced an ROS burst within 30 min in *Arabidopsis* (Supplementary Fig. S5A, C, and E). In *N. benthamiana*, only nuoD and nuoDII-25 induced statistically significant increases in ROS production in the POX assay (Fig. 3C). Using the luminol assay in *N. benthamiana*, nuoDII-25 showed a slightly delayed ROS burst peaking at 60 min (Supplementary Fig. S5B, D, and F). Thus, based on ROS output alone, nuoD and nuoDII-25 are quite weak MAMPs.

Taken together, these results indicate that pksG, nuoD, and nuoDII-25 can act as MAMPs in *Arabidopsis* and *N. benthamiana*, and elicit different levels of ROS production.

MAMP flg22 triggers a second ROS burst while candidate MAMPs do not.

ROS responses are typically detected for short periods (within 60 min) after peptide treatment. Several groups have shown that MAMPs can elicit a delayed ROS response that is much weaker than the initial ROS burst (Segonzac et al. 2011;

Shang-Guan et al. 2018). Because even high concentrations of some of our peptides did not elicit a rapid ROS burst in *Arabidopsis* or *N. benthamiana*, we performed time-course experiments for ROS production 12 h after peptide treatment. As expected, flg22 displayed an initial, very strong ROS burst within 1 h and a second, long-lasting ROS burst approximately 2 to 4 h after MAMP application in *N. benthamiana* but not in *Arabidopsis* (Supplementary Fig. S6A). Therefore we focused on *N. benthamiana* to investigate the second ROS burst.

Although ROS are largely produced in the apoplast (Mignolet-Spruyt et al. 2016), they can also be produced in intracellular compartments, including the chloroplast, peroxisome, and mitochondria (Camejo et al. 2016). To determine whether the second ROS burst was produced inside or outside of the cell, we employed a cell membrane-impermeable derivative of luminol, isoluminol (Shang-Guan et al. 2018), which allowed us to measure ROS production in the apoplast. We adjusted the isoluminol concentration relative to luminol to achieve a similar magnitude of flg22-triggered apoplastic ROS burst (Supplementary Fig. S6A and B). Using isoluminol as substrate, flg22 showed only a single, rapidly appearing peak within 60 min (Supplementary Fig. S6B), indicating that the second ROS burst occurred in intracellular compartments. Having established that we could detect the second ROS burst in *N. benthamiana*, we then tested our candidate MAMPs. However, none of the candidate peptides triggered a second ROS burst in *N. benthamiana* (Supplementary Fig. S7).

MAMPs elicit ROS production in citrus species.

Because some of our candidate MAMPs could trigger ROS production in *Arabidopsis* or *N. benthamiana*, we then turned to assaying ROS production in citrus species. Citrus species exhibit differing levels of ROS production in response to MAMPs such as flagellin from *Xanthomonas citri* (Pitino et al. 2015). Therefore, we selected a range of citrus species based on reports of HLB tolerance (Mexican key lime, Australian finger lime, and Eureka lemon), HLB susceptibility (Clementine mandarin, Minneola tangelo, Rio Red grapefruit, and Valencia

Fig. 3. nuoD variants exhibit different levels of reactive oxygen species production. **A**, nuoD variants with the positively selected "GT" sites (underlined), extended toward the N terminus or C terminus of the protein. **B**, Total peroxidase (POX) activity was measured after treatment with nuoD variants (20 μ M) in *Arabidopsis*. **C**, POX assay with nuoD variants (50 μ M) in *Nicotiana benthamiana*, as in part B. Data are shown as mean $\pm$ standard deviation, $n = 6$. Statistical significance was assessed by t tests, where *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively. Each experiment was repeated three times with similar results.

sweet orange), or mixed reports of susceptibility (Kumquat, Kishu mandarin, and mandarinquat) (Bowman et al. 2016; Folimonova et al. 2009; McCollum et al. 2016). However, it should be noted that Eureka lemon and Mexican key lime have also been reported to be susceptible under field conditions

(Flores-Sánchez et al. 2017; Ramadugu et al. 2016). We first endeavored to establish POX and luminol assays in citrus in response to flg22 because it is a very strong MAMP. Despite multiple attempts, we were unable to see any activity in the POX assay with flg22 in citrus and, thus, focused on the

Fig. 4. Microbe-associated molecular pattern flg22 induced varying levels of reactive oxygen species (ROS) production in citrus species using the luminol assay. Luminol-based assay for flg22-triggered (blue) or water-induced (red) ROS production. The following citrus species were tested: Kishu mandarin, Kumquat, Rio Red grapefruit, Australian finger lime, Mexican key lime, clementine mandarin, mandarinquat, Valencia sweet orange, Eureka lemon, and Minneola tangelo. Relative light units (RLU) were monitored for 60 min. The data are shown as mean $\pm$ standard deviation, $n = 6$. Each experiment was repeated three times with similar results.

luminol assay. Using the luminol assay, we found that different citrus species varied in their ability to recognize flg22 and induce ROS production (Fig. 4; Supplementary Fig. S8). We observed high levels of ROS production (400 to 500 RLU) in kumquat and Australian finger lime and moderate ROS production (100 to 200 RLU) in Kishu mandarin, Rio Red grapefruit, Mexican key lime, clementine mandarin, mandarinquat, Valencia sweet orange, and Eureka lemon (Fig. 4; Supplementary Fig. S8). We were unable to detect any ROS production in Minneola tangelo. In most species, ROS production peaked at approximately 15 to 20 min, except Mexican key lime, which peaked at 25 min, and Minneola Tangelo, which did not respond.

We then tested our two best candidate MAMPs: nuoD (and the nuoDII-25 variant) and pksG. In Australian finger lime, Eureka lemon, and Kumquat, only pksG triggered responses that were stronger than or equal to the responses induced by flg22 in PBS-A buffer (Fig. 5). In the other citrus species, pksG did not trigger ROS production (Supplementary Fig. S9), and nuoD and nuoDII-25 did not trigger ROS production in any of the tested citrus species (Supplementary Fig. S10). To confirm these data and visualize the in situ accumulation of hydrogen peroxide (H_2O_2), we employed nitroblue tetrazolium (NBT) staining in Australian finger lime and Minneola tangelo, because these two species displayed differing levels of ROS production. NBT precipitates in the presence of H_2O_2 and,

Fig. 5. pksG triggers varying levels of reactive oxygen species (ROS) production in citrus using the luminol assay. Luminol-based assay for pksG or flg22-triggered ROS production. Relative light units (RLU) were monitored for 60 min. Data are shown as mean $\pm$ standard deviation, $n = 6$. Each experiment was repeated three times with similar results. **A**, Australian finger lime, **B**, Eureka lemon, and **C**, Kumquat treated with 100 μ M pksG in phosphate-buffered saline amended with ammonium bicarbonate (PBS-A) buffer. **D**, Australian finger lime **E**, Eureka lemon, and **F**, Kumquat treated with 1 μ M flg22 in PBS-A buffer. **G**, Australian finger lime **H**, Eureka lemon, and **I**, Kumquat treated with PBS-A buffer. **J**, **K**, and **L**, Total ROS production was calculated by integrating the area under the curve and normalizing the flg22 treatment to 100%. The average of four independent experiments is shown. Error bars represent standard error. Asterisks indicate statistically significant differences compared with the flg22 treatment. Statistical significance was assessed by t tests, where * and ** indicate $P < 0.05$ and 0.01, respectively.

therefore, should localize at the sites where H_2O_2 is generated (Wohlgenuth et al. 2002). We compared NBT staining 24 h after infiltration of pksG or flg22 into leaves. We observed strong NBT staining in Australian finger lime leaves infiltrated with flg22 (in H_2O or PBS-A buffer) or pksG, and much weaker punctate staining in leaves infiltrated with H_2O , PBS-A buffer, or thrS peptide (Fig. 6A). Minneola tangelo displayed little NBT staining in response to flg22 or pksG treatment in PBS-A buffer, comparable with the H_2O , PBS-A buffer, or thrS peptide treatments (Fig. 6B). Quantification of NBT staining in three leaves through ImageJ analyses confirmed these results (Fig. 6C and D).

pksG induces defense gene expression in citrus.

To further demonstrate that MTI is induced, we analyzed the expression of selected defense genes after Australian finger lime or Minneola tangelo were treated with pksG or flg22. We selected MTI marker genes *WRKY22* and *GST1* and pathogenesis-related gene *PR1* for expression analysis because these genes have previously been shown to be responsive to MAMP treatment in citrus (Shi et al. 2015, 2018). We carried out quantitative reverse-transcription PCR (qRT-PCR) to determine transcript abundance of *WRKY22*, *GST1*, and *PR1*. In Australian finger lime, *PR1* gene expression decreased when treated with flg22 for 24 h but was unaffected by pksG treatment (Fig. 7A). *WRKY22* expression was strongly induced by pksG compared with flg22 (Fig. 7B). *GST1* expression was unaffected after treatment with flg22 or pksG (Fig. 7C). As expected, based on our ROS assays (Fig. 6; Supplementary Fig. S9), no statistically significant differences in expression were observed for *PR1*, *WRKY22*, or *GST1* marker genes in Minneola tangelo (Fig. 7D, E, and F).

pksG elicits immune responses to *P. syringae*.

P. syringae has been used to evaluate the effects of putative MAMPs on MTI (Hao et al. 2017; Lewis et al. 2015), because ‘Ca. L. asiaticus’ disease symptoms take many years to develop

in citrus. Therefore, to test whether pksG could elicit MTI, we carried out bacterial growth assays using the plant-pathogenic bacterium *P. syringae* pv. *tomato* DC3000 in *Arabidopsis*. We pretreated *Arabidopsis* with flg22 or pksG 1 day prior to infiltration with strain DC3000. In plants that were pretreated with H_2O or PBS-A buffer, strain DC3000 grew to approximately 6.5 logs after 2 days (Fig. 8). In plants that were pretreated with flg22 in water, strain DC3000 grew to approximately 1 log less, to approximately 5.5 logs (Fig. 8), as previously observed (Zeng et al. 2011). In plants that were pretreated with flg22 in PBS-A buffer or pksG in PBS-A buffer, strain DC3000 grew to approximately 0.5 log less, to approximately 5.5 logs (Fig. 8). Therefore, pksG elicits a level of MTI response similar to that of flg22 in PBS-A buffer in *Arabidopsis* infected with *P. syringae*. This indicates that pksG can elicit MTI and protect plants against a pathogenic bacterium such as *P. syringae*.

DISCUSSION

HLB pathogens present many challenges because they are unculturable and cause devastating disease in citrus. Therefore, we pursued a comparative genomics approach to identify putative MAMPs in ‘Ca. Liberibacter’ genomes. This strategy has been effective in identifying MAMPs in *P. syringae* and *R. solanacearum* (Eckshain-Levi et al. 2018; McCann et al. 2012; Mott et al. 2016). From 13 candidate MAMPs, we identified 2 that trigger ROS production in *Arabidopsis* and *N. benthamiana*, and 1 of these also triggers ROS production in citrus. Our data suggest that evolutionary approaches may be effective strategies to identify MAMPs for crop protection.

The initial MAMP-induced ROS burst occurs in the apoplast and is a rapid and transient response (Baker and Orlandi 1995; Lamb and Dixon 1997). nuoD (and the nuoD variant nuoDII-25) triggered the ROS burst in *Arabidopsis* and *N. benthamiana*

Fig. 6. Histochemical staining with nitroblue tetrazolium (NBT) for detection of in situ accumulation of hydrogen peroxide (H_2O_2). Accumulation of H_2O_2 in citrus leaves, as detected by NBT staining. The dark blue color indicates strong H_2O_2 production. Leaves were treated with H_2O , phosphate-buffered saline amended with ammonium bicarbonate (PBS-A) buffer, 1 μ M flg22 in PBS-A buffer, 1 μ M flg22 in H_2O , 100 μ M pksG in PBS-A buffer, or 100 μ M thrS in PBS-A buffer. Representative leaves are shown from NBT staining after treatment in **A**, Australian finger lime or **B**, Minneola tangelo. Scale bars indicates 1 cm. **C** and **D**, Quantification of the NBT-stained area as a proportion of the total leaf area from three leaves.

(Figs. 1 and 3) but not in citrus (Supplementary Fig. S10). The full-length NuoD protein is a subunit of the NADH-quinone oxidoreductase complex that translocates protons for energy production as part of the respiratory chain (Sinha et al. 2015), and genes encoding the NADH-quinone oxidoreductase complex are widespread in bacteria (Degli Esposti 2015; Spero et al. 2015). NuoD is predicted to be localized to the plasma membrane. The nuoD peptide was clearly a much weaker inducer of MTI than flg22, which is known to be a highly potent MAMP. However, high concentrations of peptide were not sufficient to trigger MTI (Fig. 2), and our peptides were not contaminated with flg22 (Supplementary Fig. S4). For nuoD, the position of the positively selected sites affected the level of ROS production (Fig. 3). Extending the peptide toward the N terminus of the protein (i.e., in nuoDIV-25 or nuoDV-25) resulted in the loss of ROS production (Fig. 3). Because ROS production was restored in nuoD or nuoDVI-25, the GT sites could be more accessible in these peptides, or part of the C-terminal region of the peptide might also contribute to MTI.

Our other MAMP, pksG, triggered the ROS burst in *Arabidopsis*, *N. benthamiana*, and citrus (Figs. 1, 2, and 5), and still elicited a response in the Ws ecotype of *Arabidopsis* that does not respond to flg22 (Supplementary Fig. S4). The pksG peptide is found in a polyketide synthase, which is a class

of enzymes producing medicinally important secondary metabolites that can also contribute to bacterial communication (Palazzotto and Weber 2018). PksG is functionally annotated as a hydroxymethylglutaryl-CoA synthase, and has been found in diverse bacteria and archaea (Calderone et al. 2006; Makarova and Koonin 2003; Piel et al. 2004). The PksG protein is predicted to be present in the cytoplasm; thus, the peptide might be exposed as bacterial populations turn over. Due to the hydrophobicity of pksG, we found that a basic PBS buffer (PBS-A) was critical for its solubilization. Interestingly, in the PBS-A buffer, pksG could induce levels of ROS production similar to that of flg22, at low peptide concentrations (5 μ M for pksG versus 1 μ M for flg22) (Fig. 1). We infer that less pksG peptide was sufficient to elicit a response in PBS-A buffer because it was more soluble in this buffer compared than in H₂O. It is unclear whether the pksG epitope would be exposed in planta, because it would depend on the three-dimensional folding of the full protein, and the plant environment is acidic.

We found that flg22 could trigger a second long-lasting ROS burst 2 to 5 h after MAMP treatment in *N. benthamiana* but not in *Arabidopsis* (Supplementary Fig. S6A). The biphasic accumulation of ROS has been known for many years (Atkinson et al. 1990; Keppler et al. 1989), and occurs in

Fig. 7. Responses of defense-associated genes in huanglongbing (HLB)-tolerant and susceptible cultivars. **A to C**, Australian finger lime or **D to F**, Minneola tangelo were infiltrated with H₂O, phosphate-buffered saline amended with ammonium bicarbonate (PBS-A) buffer, 1 μ M flg22 in PBS-A buffer, 1 μ M flg22 in H₂O, 100 μ M pksG in PBS-A buffer, or 100 μ M thrS in PBS-A buffer. Microbe-associated molecular pattern-triggered immunity (MTI) marker gene expression was evaluated 24 h postinfiltration, and quantified by quantitative reverse-transcription PCR with gene-specific primers. *PR1*, *WRKY22*, and *GST1* were selected as MTI marker genes, and *GAPC2* was used as the reference gene (Mafra et al. 2013). An asterisk indicates significant differences ($P < 0.05$) between the treatment and control. Bars are means $\pm$ standard deviation. Data are shown from three biological replicates.

Arabidopsis in response to lipopolysaccharide treatment (Shang-Guan et al. 2018). However, none of our candidate MAMPs induced the second ROS burst in *N. benthamiana* (Supplementary Fig. S7).

We employed flg22 from *P. aeruginosa* as a strong positive control for MTI responses, because it is the standard MAMP in the field. Using flg22, we established luminol assays for ROS production in citrus species, which will be a useful tool for future research (Fig. 4; Supplementary Fig. S8). flg22 triggered different levels of ROS production in citrus species (Fig. 4; Supplementary Fig. S8). The ROS bursts in citrus species varied from 150 to 400 RLU, which is lower than that observed in other plants such as *Arabidopsis* and *N. benthamiana*. However, similar levels of ROS production have previously been observed in citrus species (Pitino et al. 2015; Shi et al. 2015). Recent transcriptomic studies demonstrated that genes involved in biotic stress and signaling were upregulated in HLB-tolerant citrus compared with HLB-susceptible citrus (Shi et al. 2018; Wang et al. 2016; Yu et al. 2017), suggesting that immunity is enhanced in HLB-tolerant varieties. However, we found that there was not a strong correlation between flg22-triggered ROS production and responses in cultivars that are purported to be resistant or tolerant to HLB. Although pksG exhibited similar or stronger ROS production compared with that induced by flg22 (Figs. 4, 5, and 6), pksG and flg22 treatment resulted in different effects on MTI marker gene expression (Fig. 7). As previously observed in HLB or citrus canker-tolerant varieties (Pitino et al. 2015; Shi et al. 2015, 2018), flg22 treatment resulted in reduced *PR1* gene expression compared with the buffer controls (Fig. 7). These effects were specific to Australian finger lime because pksG and flg22 did not result in NBT staining in *Minneola tangelo* (Fig. 6). In addition, neither peptide caused differential MTI marker gene expression (Fig. 7) in *Minneola tangelo*. pksG treatment resulted in greater *WRKY22* expression in Australian finger lime compared with *Minneola tangelo* (Fig. 7). Yu and colleagues (2017) demonstrated

that *WRKY* genes were differentially expressed in HLB-tolerant rough lemon compared with HLB-susceptible sweet orange in an RNA sequencing experiment in '*Ca. L. asiaticus*'-infected citrus. *WRKY* genes can connect microbe-associated molecular pattern perception to defense gene expression and hormone regulation, and affect disease outcomes (Eulgem and Somssich 2007). Although we were unable to test for the effect of pksG on '*Ca. L. asiaticus*' infection due to the very lengthy time-scales necessary for disease, we demonstrated that pksG can induce MTI and reduce growth of *P. syringae* in *Arabidopsis* (Fig. 8), which further supports that it is a bona fide MAMP. Our data suggest that specific species of citrus may recognize pksG and flg22 by different mechanisms. Other studies have shown that MAMPs can have differing effects on Ca^{2+} influx and gene expression (Aslam et al. 2009; Lecourieux et al. 2005; Segonzac et al. 2011). In addition, after MAMP perception and Ca^{2+} influx, parallel signaling pathways in *N. benthamiana* trigger the ROS burst versus MAPK activation and MAMP-induced gene expression (Segonzac et al. 2011).

Genome sequence analysis indicates that '*Ca. L. asiaticus*' encodes most flagellar genes which could function as MAMPs (Bendix and Lewis 2018; Wang et al. 2017; Zou et al. 2012;). Indeed, flg22 from '*Ca. L. asiaticus*' has been shown to act as a weak MAMP in citrus (Shi et al. 2018) compared with flg22 from *X. citri* pv. *citri*, which causes citrus canker (Shi et al. 2015). '*Ca. L. asiaticus*' flg22 triggers rapid ROS production and induces the expression of MTI marker genes in moderately HLB-tolerant species (Shi et al. 2015, 2018; Zou et al. 2012). However, it is not clear which PRR recognizes '*Ca. L. asiaticus*' flg22 because PRRs are not well characterized in citrus. Transient expression of citrus FLS2 from resistant Nagami kumquat (FmFLS2-1) or Sun Chu Sha mandarin (CrFLS2-2) was able to enhance MTI, suggesting that these proteins may function as flagellin receptors in citrus (Shi et al. 2016). Transcriptional analysis has shown that an LysM RLK (CHITIN ELICITOR RECEPTOR KINASE 1) and other RLKs are induced in '*Ca. L. asiaticus*'-infected plants (Aritua et al. 2013; Mafra et al. 2013). However, it is unknown whether these act as PRRs. An open question is how the perception of the MAMP could occur at the surface of the cell membrane, when the bacterium is present in intracellular spaces. Under natural conditions, MTI is not effective in preventing '*Ca. L. asiaticus*' infection, perhaps because its effectors overcome MTI (Clark et al. 2018), as has been seen for other plant pathogens (Block and Alfano 2011; Lewis et al. 2009; Wang et al. 2017). Alternatively, perhaps MTI is not induced due to the low titer of '*Ca. L. asiaticus*', which might keep MAMPs at a very low level (Selvaraj et al. 2018), or its phloem niche, which might restrict the recognition of MAMPs by PRRs (Aslam et al. 2009). Our data demonstrate that specific MAMPs can stimulate significant immune responses. If these MAMPs are delivered in sufficient quantity to the right tissues, this could result in stronger MTI that might be sufficient to limit infection.

Our data show that nuoD induces ROS production and pksG induces MTI at a very early step of the signaling cascade. Fragments of flagellin and other MAMPs have been successfully used to enhance resistance in crops such as tobacco, wheat, sunflower, beet, rice, and tomato (Hao et al. 2014; Nandeeskumar et al. 2008; Quintana-Rodriguez et al. 2018). Expression of candidate MAMPs under pathogen-induced phloem-specific promoters could be a means to trigger strong MTI after perception of the pathogen. This approach combined with new MAMPs could be used as a means to boost immunity in citrus and to prevent HLB infection.

Fig. 8. Pretreatment with pksG induces microbe-associated molecular pattern-triggered immunity to *Pseudomonas syringae* in *Arabidopsis*. H₂O, phosphate-buffered saline amended with ammonium bicarbonate (PBS-A) buffer, 10 μ M pksG (in PBS-A buffer), or 1 μ M flg22 (in H₂O or PBS-A buffer) were infiltrated into leaves of 5-week-old *Arabidopsis* plants with a needleless syringe. After 24 h, the same leaves were syringe infiltrated with *P. syringae* pv. *tomato* DC3000 at 1×10^5 CFU/ml. Bacterial growth was determined at 1 h (Day 0) and 2 days (Day 2) postinoculation. The log average of six individuals is shown. Error bars represent standard error. Asterisks indicate statistically significant differences compared with the appropriate buffer control. Statistical significance was assessed by *t* tests, where * and ** indicate $P < 0.05$ and 0.01 , respectively.

MATERIALS AND METHODS

Plant materials and growth conditions.

Arabidopsis plants (Col-0 ecotype and Ws ecotype) were grown in a growth chamber at 22°C with cycles of 9 h of light (130 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 15 h of darkness. *N. benthamiana* plants were grown at 22°C with cycles of 14 h of light (150 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 10 h of darkness. Three-year-old citrus plants (from Four Winds Growers) were grown in a greenhouse at 23°C. Mexican key lime, Australian finger lime, Eureka lemon, Clementine mandarin, Minneola tangelo, Rio Red grapefruit, Valencia sweet orange, Kumquat, Kishu mandarin, and mandarinquat plants were pruned to encourage new flush and young, fully expanded leaves were used for luminol-based plate assays.

Core-genome prediction and selection analysis.

The analysis was performed using sequences from ‘*Ca. L. asiaticus*’, ‘*Ca. L. americanus*’, ‘*Ca. L. africanus*’, ‘*Ca. L. solanacearum*’, and a nonpathogenic relative, *L. crescens* (Fagen et al. 2014; Naranjo et al. 2019). The ‘*Ca. L. asiaticus*’ strains were gpxsy (GCF_000346595.1), psy62 (GCF_000023765.2), A4 (GCF_000590865.2), Ishi-1 (GCF_000829355.1), HHCA (GCF_000724755.2), FL17 (GCF_000820625.1), and YCPsy (GCF_001296945.1). The ‘*Ca. L. americanus*’ strains were PW-SP (GCF_000350385.1) and Sao Paulo (GCF_000496595.1). The ‘*Ca. L. africanus*’ strain was PTSAPSY (GCF_001021085). The ‘*Ca. L. solanacearum*’ strains were Clso-ZC1 (GCF_000183665.1), R1 (GCF_000756225.1), HenneA (GCF_000968075.1), LsoNZ1 (GCF_000968085.1), and RSTM (GCF_001414235.1). The *L. crescens* strains were BT-0 (GCF_001543305.1) and BT-1 (GCF_000325745.1). Prokka was used to annotate the genomes and produce GFF3 files (Seemann 2014). These files were used as input for Roary to identify core and accessory genes in the pan-genome from related species (Page et al. 2015). Core gene sets were first generated at 95% Blastp cutoff for each species: ‘*Ca. L. asiaticus*’, ‘*Ca. L. americanus*’, ‘*Ca. L. africanus*’, ‘*Ca. L. solanacearum*’, or *L. crescens*. Roary uses the blast results as input to the Markov Cluster algorithm, which clusters related proteins, to identify homologous genes (Enright et al. 2002; Page et al. 2015). Selection analysis was performed using the codeml program in the PAML 4.8 application suite. The core genome alignment and tree produced by Roary were used as input files. The program ete evol was used to run codeml on the full core genomes. The codeml models M1 versus M2 or M7 versus M8 were compared using likelihood ratio tests in order to identify positive selection on sites. Bayes Empirical Bayes estimates with a posterior probability of >0.5 from the positive selection models M2 and M8 were used to identify positively selected sites.

After positively selected codons were identified, Artemis was used to identify which ‘*Ca. L. asiaticus*’ genes were under selection. These genes were compiled into a list and organized according to how many positive selection sites were present in each. In addition, the selection sites were evaluated for their proximity to gaps and stop codons in the sequence; those closer to such sites were considered less reliable. The strongest candidates were those with a higher number of reliable positive selection sites. To refine the list of candidates in ‘*Ca. L. asiaticus*’ under positive selection, a second round of analysis was carried out to identify new core gene sets for different groupings of ‘*Ca. L. asiaticus*’ with the related species. These groupings were ‘*Ca. L. asiaticus*’ and ‘*Ca. L. americanus*’, ‘*Ca. L. asiaticus*’ and ‘*Ca. L. africanus*’, ‘*Ca. L. asiaticus*’ and ‘*Ca. L. solanacearum*’, or ‘*Ca. L. asiaticus*’ and *L. crescens*, with 80% Blastp cutoff.

Peptides.

The predicted MAMPs were designed from the ‘*Ca. Libribacter*’ genome sequences. The peptides were designed

to encompass the positively selected sites and to include more antigenic regions of the proteins as predicted by the Kolaskar-Tongaonkar method in CLC Bio, version 6.9.1 (Kolaskar and Tongaonkar 1990). The flg22 sequence (QRLSTGSRINSAKDDAAGLQIA) is from the *P. aeruginosa* genome, and is standardly used as a strong positive control for MTI. All peptides at >95% purity were purchased from the Bio Basic Inc. or Biomer Technology LLC. All peptides except pksG were dissolved in water, because they were highly soluble in water. Because pksG was not soluble in water, it was dissolved in PBS-A buffer as a 400 μM stock solution and sonicated for 5 min (20 s of sonication and 30 s of break). PBS-A buffer was composed of PBS buffer at pH 7.4 supplemented with 0.2 mM NH_4HCO_3 as a 1:1 mixture. pksG working solutions (5 and 100 μM) were diluted from the stock solution with distilled H_2O .

ROS production assays.

The POX assay in *Arabidopsis* and *N. benthamiana* was performed as previously described (Mott et al. 2016). In brief, leaf disks (5 mm in diameter) were obtained from young leaves by coring and incubated with 50 μl of 1 $\times$ MS buffer alone or supplemented with different peptides in a 96-well plate. After a 20-h incubation, the leaf disks were removed and each well received 50 μl of a 1-mg/ml solution of 5-aminosalicylic acid (A79809; Sigma-Aldrich), pH 6.0, with 0.01% H_2O_2 . The reaction was allowed to proceed for 3 min and stopped by the addition of 2 N NaOH prior to reading the optical density at 600 nm on a Tecan Spark 10M plate reader. The luminol assay was performed as previously described (Baker and Mock 2004). In brief, leaf disks (5 mm in diameter) were obtained from young leaves by coring and incubated overnight in a 96-well plate with water. The following day, 200 μM luminol, horseradish POX at 20 $\mu\text{g}/\text{ml}$, and peptides were added to each well. Isoluminol (600 μM) was used to achieve a similar magnitude for the flg22-triggered apoplastic ROS burst. Luminescence was measured using the Tecan Spark 10M plate reader.

Histochemical staining of ROS.

Peptides, distilled water, or buffer were infiltrated into the abaxial surface of the leaf using a 1-ml needleless syringe. Infiltrated leaves were detached and placed in freshly prepared NBT solutions at 1 mg/ml, followed by vacuum infiltration for 2 h and incubation in the dark for 24 h at 28°C with shaking. The leaves were cleared by boiling in 95% ethanol for 30 min and kept in 75% ethanol for 5 to 7 days until cleared.

qRT-PCR.

Peptides were infiltrated into the abaxial surface of the leaf using a 1-ml needleless syringe. Leaf tissue was collected 24 h after infiltration and RNA was extracted from infiltrated areas using the RNeasy plant mini kit (Qiagen). cDNA was synthesized as previously described (Chen et al. 2016). qRT-PCR was performed using CFX96 real-time PCR detection system (Bio-Rad) with SYBR Green PCR Master Mix. The thermocycler program was as follows: 95°C for 30 s, followed by 40 cycles alternating between 5 s at 95°C and 15 s at 60°C. Three biological replicates were performed for each sample. The primers are shown in Supplementary Table S2.

Induced resistance assays.

Induced resistance assays were performed as previously described (Boutrot et al. 2010). Briefly, H_2O , PBS-A buffer, 10 μM pksG (in PBS-A buffer), or 1 μM flg22 (in H_2O or PBS-A buffer) were infiltrated into leaves of 5-week-old *Arabidopsis* plants with a needleless syringe. After 24 h, the same leaves were

syringe infiltrated with *P. syringae* pv. *tomato* DC3000 at 1×10^5 CFU/ml. Bacterial growth was determined 2 days postinoculation by harvesting four disks (1 cm² each) of tissue, grinding in 10 mM MgCl₂, and plating on King's B medium with rifampicin and cycloheximide, as previously described (Lewis et al. 2008).

Statistical analysis.

The data were analyzed with a one-way analysis of variance at a significance level of $P < 0.05$ followed by a multiple comparisons of means using Tukey's post hoc test. Error bars indicate standard deviation. Data were statistically analyzed using GraphPad Prism 8 software.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ete evol: <http://etetoolkit.org/documentation/ete-evol/>
Artemis: <https://www.sanger.ac.uk/science/tools/artemis>

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