A Secreted ‘*Candidatus Liberibacter asiaticus*’ Peroxiredoxin Simultaneously Suppresses Both Localized and Systemic Innate Immune Responses In Planta

Mukesh Jain, Alejandra Munoz-Bodnar, Shujian Zhang, and Dean W. Gabriel[†]

Department of Plant Pathology, University of Florida, Gainesville, FL 32611, U.S.A.

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The oxidative (H₂O₂) burst is a seminal feature of the basal plant defense response to attempted pathogen invasions. In ‘*Candidatus Liberibacter asiaticus*’ UF506, expression of the SC2 prophage-encoded secreted peroxidase (F489_gp15) increases bacterial fitness and delays symptom progression in citrus. Two chromosomal 1-Cys peroxiredoxin genes, CLIBASIA_RS00940 (*Lasprx5*) and CLIBASIA_RS00445 (*Lasbcp*), are conserved among all sequenced ‘*Ca. L. asiaticus*’ strains, including those lacking prophages. Both LasBCP and LasDPrx5 have only a single conserved peroxidatic Cys (C_P/SH) and lack the resolving Cys (C_R/SH). *Lasprx5* appeared to be a house-keeping gene with similar moderate transcript abundance in both ‘*Ca. L. asiaticus*’-infected psyllids and citrus. By contrast, *Lasbcp* was expressed only in planta, similar to the expression of the SC2 peroxidase. Since ‘*Ca. L. asiaticus*’ is uncultured, *Lasbcp* and *Lasprx5* were functionally validated in a cultured surrogate species, *Liberibacter crescens*, and both genes significantly increased oxidative stress tolerance and cell viability in culture. LasBCP was nonclassically secreted and, in *L. crescens*, conferred 214-fold more resistance to *tert*-butyl hydroperoxide (*t*BOOH) than wild type. Transient overexpression of *Lasbcp* in tobacco suppressed H₂O₂-mediated transcriptional activation of *RbohB*, the key gatekeeper of the systemic plant defense signaling cascade. *Lasbcp* expression did not interfere with the perception of ‘*Ca. L. asiaticus*’ flagellin (flg22_{Las}) but interrupted the downstream activation of *RbohB* and stereotypical deposition of callose in tobacco. Critically, LasBCP also protected against *t*BOOH-induced peroxidative degradation of lipid membranes in planta, preventing subsequent accumulation of antimicrobial oxylipins that can also trigger the localized hypersensitive cell death response.

Huanglongbing (HLB), or citrus greening, has emerged as the single most devastating citrus disease worldwide. HLB is associated with three fastidious and uncultured α -proteobacteria; ‘*Candidatus Liberibacter asiaticus*’ in Asia and the Americas, ‘*Ca. Liberibacter americanus*’ in Brazil, and ‘*Ca. Liberibacter africanus*’ in Africa. ‘*Ca. L. asiaticus*’ is vectored and transmitted among citrus and some *Rutaceae* species by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Sternorrhyncha: Psyllidae) (Grafton-Cardwell et al. 2013). In psyllids, ‘*Ca. L. asiaticus*’ propagates both inter- and intracellularly as a systemic, circulative, and propagative endosymbiont across multiple organs. ‘*Ca. L. asiaticus*’ infection in psyllids doesn’t cause any overt disease symptoms, except for localized apoptosis in the midgut epithelium cells of the adult insects (Ghanim et al. 2017) but not in the nymphs (Mann et al. 2018).

By contrast, ‘*Ca. L. asiaticus*’ colonization of citrus phloem significantly compromises its transport functions. Altered nutrient and assimilate partitioning in citrus reduces fruit yield and quality and causes progressive decline and death of infected trees. Despite the complete genome sequencing of at least nine ‘*Ca. L. asiaticus*’ strains (Duan et al. 2009; Katoh et al. 2014; Wu et al. 2015; Zheng et al. 2015), genetic determinants of ‘*Ca. L. asiaticus*’ pathogenicity and host range in plants and its ability to systemically colonize insect hosts remain largely unknown. To date, all efforts to obtain sustained growth of ‘*Ca. L. asiaticus*’ in axenic cultures have failed, thus restricting functional genomics studies and subsequent development of effective chemical control methods (Jain et al. 2015; Pitino et al. 2016; Zou et al. 2012).

Recognition of conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by integral pathogen recognition receptors results in activation of pattern-triggered immunity or basal resistance in plants (Boutrot and Zipfel 2017; Choi and Klessig 2016). Compatible host-pathogen interactions are marked by low-amplitude, nonspecific apoptotic bursts of reactive oxygen species (ROS) (primarily H₂O₂) in response to PAMPs or DAMPs that are mediated through the action of respiratory burst oxidase homologs (RBOHs) (Baxter et al. 2014; Kadota et al. 2015). However, a second high-amplitude oxidative burst occurs during incompatible host-pathogen interactions, resulting in the establishment of effective disease resistance (Grant and Loake 2000). Successful infections by biotrophic plant pathogens are contingent upon detoxification of the first wave of ROS and active suppression of basal plant defenses (Kadota et al. 2015).

ROS (e.g., O₂^{•−}, H₂O₂, OH[•], ¹O₂) are byproducts of cellular metabolism, also serving as long distance signal transducers

Current address for Shujian Zhang: New Mexico Consortium Biolab, Los Alamos, NM 87544, U.S.A.

[†]Corresponding author: Dean W. Gabriel; E-mail: dgabr@ufl.edu

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within defense and developmental networks (Miller et al. 2009). ROS target polyunsaturated lipids, Cys residues, Fe-S clusters, and DNA (Imlay 2014). Nonenzymatic ROS-induced lipid peroxidation also results in accumulation of highly reactive α,β -unsaturated carbonyl compounds (reactive electrophilic species), primarily oxylipins. Oxylipins are various oxygenated derivatives of polyunsaturated fatty acids such as linoleic acid (18:2) and α -linolenic acid (18:3) and have well-known signaling functions in animals and in plants (Farmer and Mueller 2013). Oxylipins contribute to plant defense by overlapping effects on pathogens by direct antimicrobial activity, transcriptional activation of defense genes, and regulation of cell death (Prost et al. 2005).

Spatiotemporal regulation of ROS generation and detoxification pathways is critical for maintaining redox poise while preventing oxidation of cellular macromolecules (Schieber and Chandel 2014). The SC2 peroxidase (F489_gp15) in ‘*Ca. L. asiaticus*’ UF506 (Zhang et al. 2011) was functionally characterized as a secreted effector and was demonstrated to suppress H_2O_2 -mediated transcriptional activation of *RbohB* in planta, a prerequisite for innate plant immune defense or basal resistance (Jain et al. 2015). However, absence of the peroxidase in some ‘*Ca. L. asiaticus*’ strains lacking prophages, such as YCpsy (Wu et al. 2015), FL17 (Zheng et al. 2015), and Ishi-1 (Katoh et al. 2014), indicated the presence of an alternative ROS detoxification enzyme for these strains to survive in citrus.

Two chromosomal peroxiredoxin genes CLIBASIA_RS00940 (*Lasprx5*) and CLIBASIA_RS00445 (*Lasbcp*) are identically conserved among all nine ‘*Ca. L. asiaticus*’ strains sequenced to date, including the phage-less strains. CLIBASIA_RS00445 protein was recently purified to homogeneity and characterized to have a protective role against peroxide-mediated cell death and oxidative damage in vitro (Singh et al. 2017). Peroxiredoxin proteins (EC 1.11.1.15) are a diverse family of ubiquitous thioredoxin-scaffold enzymes with Cys-dependent and promiscuous peroxidase activity on hydrogen peroxide, peroxytrinitrate, and organic peroxide substrates. The specificities for the thiol-containing donor substrate vary considerably, comprising glutathione, trypanothione, thioredoxin, and trypanedoxin (Hofmann et al. 2002). Peroxiredoxins utilize either 2-Cys or 1-Cys reaction catalysis mechanisms, the latter of which is redox insensitive (Nelson et al. 2011). The broad distribution and high expression levels across all cell types is indicative of peroxiredoxins being an ancient and important enzyme family. *Escherichia coli* peroxiredoxin is one among the ten most abundant cellular proteins and the primary enzyme responsible for the reduction of endogenously generated H_2O_2 (Seaver and Imlay 2001).

The primary objective of this research was to identify critical virulence effectors of ‘*Ca. L. asiaticus*’ in citrus hosts. The strikingly lengthy incubation period between the time of initial infection and the first appearance of HLB symptoms was inexplicable without a mechanism of ‘*Ca. L. asiaticus*’-mediated ROS suppression. The discovery of ‘*Ca. L. asiaticus*’ strains lacking prophage-encoded peroxidases drew attention to the highly conserved chromosomal 1-Cys peroxiredoxins found in all pathogenic *Liberibacter* species, and prompted i) examination of the differences (if any) in the transcriptional profiles of putative ‘*Ca. L. asiaticus*’ peroxiredoxin genes *Lasprx5* and *Lasbcp* in psyllid and citrus hosts, ii) attempts to functionally validate LasPrx5 and LasBCP as peroxiredoxins, using *Liberibacter crescens* (Leonard et al. 2012), a culturable and genetically tenable surrogate species for uncultured *Liberibacter* species (Fleites et al. 2014; Jain et al. 2015), and iii) to determine a role, if any, for the (predicted) secreted LasBCP in dampening the H_2O_2 -mediated defense responses in planta.

RESULTS

Two chromosomally encoded peroxiredoxin genes in ‘*Ca. L. asiaticus*’ are conserved among all *Liberibacter* species.

Two putative peroxiredoxin genes, CLIBASIA_RS00940 (*Lasprx5*) and CLIBASIA_RS00445 (*Lasbcp*), are annotated in ‘*Ca. L. asiaticus*’ psy62 (Duan et al. 2009) and are 100% conserved among all ‘*Ca. L. asiaticus*’ strains sequenced to date. *Lasprx5* and *Lasbcp* showed 73 to 82% and 75 to 80% protein sequence identity, respectively, to peroxiredoxins annotated in other pathogenic *Ca. Liberibacter* species and several other α -proteobacteria, but only 65 and 60% sequence identity with *L. crescens* (Supplementary Figs. S1 and S2). *LasPrx5* encodes a PRX5-like peroxiredoxin, whereas *Lasbcp* encodes a bacterioferritin comigratory protein (BCP)-like peroxiredoxin. LasPrx5 and LasBCP each have a single peroxidatic Cys (C_P55/SH and C_P46/SH, respectively) contained within an absolutely conserved PXXX(T/S)XXC_P motif typical of Cys-based nonheme peroxidases. Both proteins lack the resolving cysteine (C_R/SH) residue. LasPrx5 and LasBCP display the well-conserved catalytic triad (T52/C55/R132 and T43/C46/R121, respectively), that utilizes the H-bond within the Thr hydroxyl group and the positive charge of the Arg sidechain for deprotonation of the catalytic Cys thiol (Wood et al. 2003). Additionally, a dimerization interface, characteristic of redox-insensitive atypical 2-Cys peroxiredoxins (P53, D85, F87, G107, S108, and R132) (Evrard et al. 2004), is also found within the amino acid sequence of LasPrx5. A similar dimerization interface was absent from LasBCP, characteristic of BCP-like peroxiredoxin subfamily members, the only peroxiredoxin proteins known to function as monomers.

Lasbcp is significantly upregulated in planta relative to the insect host.

Comparative expression analyses revealed that *Lasbcp* and *Lasprx5* were differentially expressed in ‘*Ca. L. asiaticus*’ infecting its citrus and psyllid hosts (Fig. 1). Similar and moderate transcript abundance was observed for *Lasprx5* in both ‘*Ca. L. asiaticus*’ hosts, in line with the predicted housekeeping functions of PRX5 homologs. However, the expression of *Lasbcp* was significantly downregulated and

Fig. 1. ‘*Candidatus Liberibacter asiaticus*’ peroxiredoxin gene *Lasbcp* is expressed in citrus but not in psyllids. Relative expression of peroxiredoxin genes *Lasbcp* and *Lasprx5* and the two endogenous housekeeping genes *LasgyrB* and *LasprfA* in citrus and psyllid hosts. Relative transcript abundance of each gene was normalized against its expression in ‘*Ca. L. asiaticus*’-infected citrus. The data presented are the means $\pm$ standard error for three independent experiments with four replicates. An asterisk indicates a significant difference between the means for *Lasbcp* ($P < 0.05$).

nearly undetectable in psyllids ($P = 0.000096$), similar to the low level of SC2_gp095 peroxidase expression reported previously in psyllids (Jain et al. 2015). The difference in expression levels of chromosomal genes *LasgyrB* and *LasprfA* in both hosts was not significant. The transcript abundance data were normalized against expression in ‘*Ca. L. asiaticus*’-infected citrus.

LasPrx5 and LasBCP are functional peroxiredoxins and mitigate oxidative stress in *L. crescens*.

Lasprx5 and *Lasbcp* were functionally characterized by heterologous expression in *L. crescens* BT-1, a culturable proxy for ‘*Ca. L. asiaticus*’. Both enzymes were individually cloned in the wide-host range (Rep *Bordatella*) shuttle vector pBBR1MCS-5 (Kovach et al. 1995) under control of a *lacZ* promoter and transformed into BT-1 cells. A protective role of ‘*Ca. L. asiaticus*’ peroxiredoxins and SC2 peroxidase in alleviation of menadione-generated intracellular ROS stress (Loor et al. 2010) was examined by staining BT-1 cells with the ROS-sensitive CellROX Deep Red reagent. Five-day-old BT-1 cells, transformed with the empty vector pBBR1MCS-5, pLF080 (*lacZ::F489_gp15*) (Jain et al. 2015), pSZ103 (*lacZ::Lasprx5*), or pSZ104 (*lacZ::Lasbcp*) were adjusted to an absorbance at 600 nm (Abs_{600}) = 1.0 (200 μ l) and were treated with 10 μ M menadione in the presence or absence of 50 μ M conoidin A, a covalent inhibitor that binds the active site Cys of peroxiredoxins (Nguyen et al. 2013). Menadione treatment resulted in a significant increase (15.6-fold) in fluorescence in BT-1/pBBR1MCS-5 cells, indicating greatly increased intracellular ROS stress (Fig. 2). By contrast, BT-1 cells expressing either the SC2 peroxidase or ‘*Ca. L. asiaticus*’ peroxiredoxins displayed only a 5.3- to 6.0-fold increase in fluorescence. In the presence of the peroxiredoxin inhibitor conoidin A, the menadione-induced increase in fluorescence was comparable in BT-1 cells carrying the empty vector, *Lasprx5*, or *Lasbcp*. By contrast, conoidin A had no effect on BT-1 cells carrying the SC2 peroxidase, confirming the predicted function of the LasPrx5 and LasBCP as peroxiredoxins and distinguishing these two enzymes from the SC2 peroxidase.

Fig. 2. ‘*Candidatus Liberibacter asiaticus*’ peroxiredoxins LasBCP and LasPrx5 both alleviated oxidative stress in *Liberibacter crescens* BT-1. *L. crescens* BT-1 cells (200 μ l, absorbance at 600 nm = 1.0) were transformed with the empty vector pBBR1MCS-5, pLF080 (*lacZ::F489_gp15*), pSZ103 (*lacZ::Lasprx5*), or pSZ104 (*lacZ::Lasbcp*) and were treated with reactive oxygen species (ROS) generator menadione (10 μ M) and peroxiredoxin inhibitor conoidin A (50 μ M), as indicated. Intracellular ROS were detected by the fluorescent stain CellROX Deep Red (Ex/Em maxima 640/665 nm). The data presented are the means $\pm$ standard error for three independent experiments with five replicates. Asterisks indicate significant differences between the means ($P < 0.05$).

LasPrx5 and LasBCP increased

L. crescens cell viability in culture.

Five-day-old *L. crescens* BT-1 cells carrying the empty vector pBBR1MCS-5, pLF080, pSZ103, or pSZ104 (adjusted to Abs_{600} = 0.5) were challenged with 100 μ M H_2O_2 or 50 μ M *tert*-butyl hydroperoxide (*tBOOH*), an organic peroxide, for 30 min and were plated for enumerating the colony-forming units (Fig. 3). Expression of either of the ‘*Ca. L. asiaticus*’ peroxiredoxins or the SC2 peroxidase in *L. crescens* BT-1 provided 1.3-fold higher cell viability in the absence of any exogenously provided oxidative stress, indicating that each of these enzymes increased ability to detoxify endogenously generated ROS as a result of aerobic respiration.

Even though *L. crescens* BT-1 carries two native peroxiredoxins (B488_RS02765 and B488_RS05115) and no known peroxidases, treatments of BT-1/pBBR1MCS-5 cells with the indicated amounts of H_2O_2 or *tBOOH* resulted in nearly complete loss of viability. BT-1/pSZ103 (*Lasprx5*) cells were 65-fold more resistant to exogenous H_2O_2 as compared with BT-1 cells harboring the empty vector. LasPrx5 conferred no detected protection against *tBOOH*. BT-1/pSZ104 (*Lasbcp*) cells were 36- and 214-fold more resistant to exogenous H_2O_2 and *tBOOH*, respectively, than BT-1/pBBR1MCS-5. Heterologous expression of the previously characterized SC2 peroxidase in BT-1/pLF080 cells was also compared in these viability assays; SC2 peroxidase conferred 17-fold enhanced resistance to exogenous H_2O_2 as compared with BT-1/pBBR1MCS-5. These results provide evidence that ‘*Ca. L. asiaticus*’ peroxiredoxins and the SC2 peroxidase play protective roles in redox homeostasis in *L. crescens* BT-1.

Lasprx5 and *Lasbcp* encode cytoplasmic and extracellular peroxiredoxins, respectively.

‘*Ca. L. asiaticus*’ peroxiredoxins were functionally corroborated by quantification of enzymatic degradation of H_2O_2 in spectrophotometric assays. *L. crescens* BT-1 cells transformed with either pSZ103 (*Lasprx5*) or pSZ104 (*Lasbcp*) displayed 1.5 and 2.3-fold higher peroxidase activity in cell lysates (4.3 ± 0.1 and 6.6 ± 1.1 mU, respectively) as compared with empty

Fig. 3. ‘*Candidatus Liberibacter asiaticus*’ peroxiredoxins LasBCP and LasPrx5 both enhanced viability of *Liberibacter crescens* BT-1 in culture. *L. crescens* BT-1 cells (1 ml, absorbance at 600 nm = 0.5) were transformed with the empty vector pBBR1MCS-5, pLF080 (*lacZ::F489_gp15*), pSZ103 (*lacZ::Lasprx5*), or pSZ104 (*lacZ::Lasbcp*), were treated with 100 μ M H_2O_2 or 50 μ M *tert*-butyl hydroperoxide (*tBOOH*) for 30 min, and were washed, serially diluted, and plated on selective BM7 media. The surviving bacterial colonies were scored after 3 weeks. The data presented are the means $\pm$ standard error for three independent experiments with two replicates. Asterisks indicate significant differences between the means ($P < 0.05$).

vector controls (2.9 ± 0.5 mU), assayed using 5-day-old BT-1 cell cultures adjusted to $\text{Abs}_{600} = 1.0$ (Fig. 4). BT-1/pLF080 cells expressing SC2 peroxidase also displayed significantly higher peroxidase activity (1.4-fold, 5.8 ± 0.3 mU) as compared with BT-1/pSZ103 (*Lasprx5*) cells. Neither *Lasprx5*- nor *Lasbcp*-encoded protein products were predicted to carry signal peptides for extracellular bacterial secretion. However, non-classical secretion potential was predicted by SecretomeP 2.0 (Bendtsen et al. 2005) for LasBCP (SecP score of 0.756256, cutoff 0.5). Consistent with the above predictions, BT-1/pSZ104 (*Lasbcp*) and BT-1/pLF080 (SC2 peroxidase) exhibited 10- and sixfold higher enzymatic activity in culture supernatants (3.0 ± 0.5 and 2.1 ± 0.2 mU, respectively) as compared with culture supernatants of *L. crescens* BT-1 carrying the empty vector (0.35 ± 0.06 mU). Enzyme activity in the supernatant of BT-1/pSZ103 (*Lasprx5*) cells was similar to that obtained from the empty vector control cells (0.73 ± 0.3 mU). A similar nonclassical secretion potential was predicted, using SecretomeP 2.0, for annotated BCP orthologs from other pathogenic *Liberibacter* species as well as for predicted BCP homologs from genera *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Xanthomonas*, *Pseudomonas*, *Erwinia*, and *Ralstonia*.

LasBCP suppressed transcriptional activation of *Rboh* in planta.

Given that LasBCP is a secreted protein and its expression is unique to the citrus host, its function and significance in planta were investigated. Expression levels of *NbRbohB* in tobacco (*Nicotiana benthamiana*) were examined following *Agrobacterium*-mediated transient expression of the *Lasbcp* peroxiredoxin from the binary plasmid pAMB004 (*CaMV35S::Lasbcp*) with or without coinfiltration of H_2O_2 . In the absence of peroxide induction, no significant change in *NbRbohB* transcript abundance was observed between 2 and 6 days post-infiltration (dpi) of *A. tumefaciens* GV2260 carrying either pCambia2301 (*CaMV35S::uidA*) or pAMB004 (Fig. 5A). Addition of (sublethal) 50 μM H_2O_2 to the suspension of GV2260/pCambia2301 used for infiltration caused a reproducibly sharp increase in *NbRbohB* transcript abundance between 2 and 6 dpi (Fig. 5B). However, transient expression of

Fig. 4. *Lasbcp* encoded an extracellular peroxiredoxin in *Liberibacter crescens* BT-1. Peroxidase activity was quantified in *L. crescens* BT-1 cells transformed with the empty vector pBBR1MCS-5, pLF080 (*lacZ::F489_gp15*), pSZ103 (*lacZ::Lasprx5*), or pSZ104 (*lacZ::Lasbcp*). Five-day-old cultures were adjusted to an absorbance at 600 nm = 1.0, and the enzyme activity was measured in 0.5 ml of cell lysates or 50 μl of culture supernatants. The data presented are the means $\pm$ standard error for three independent experiments with four replicates. Asterisks indicate significant differences between the means ($P < 0.05$).

Lasbcp in the presence of H_2O_2 significantly alleviated the sharp rise in *NbRbohB* transcriptional activity at 4 and 6 dpi ($P = 0.000181$ and 0.000693 , respectively) (Fig. 5B). These results provided unambiguous evidence that LasBCP detoxified H_2O_2 in planta and thereby suppressed transcriptional activation of *NbRbohB*.

LasBCP suppressed '*Ca. L. asiaticus*' flagellin (PAMP)-triggered immune responses in tobacco.

The effect of *Lasbcp* expression in tobacco on perception of the '*Ca. L. asiaticus*' flagellin peptide (flg22_{Las}) elicitor was examined by expression analyses of three PAMP perception and relay genes and response gene *NbRbohB* (Fig. 6). Infiltration of 40 μM flg22_{Las} in wild-type tobacco leaves resulted in a rapid increase in transcript abundance of flagellin sensing 2 (*NbFLS2*), BRI1-associated receptor kinase-1 (*NbBAK1*), and *Botrytis*-induced kinase-1 (*NbBIK1*) within 1 h postinfiltration (hpi). A similar increase in flg22_{Las}-induced *NbFLS2*, *NbBAK1*, and *NbBIK1* transcriptional activity was also observed in tobacco leaves expressing *Lasbcp* at 1 hpi. However, in tobacco leaves transiently expressing *Lasbcp*, *NbRbohB* transcription was dramatically repressed ($P = 0.000341$) as compared with the levels elicited by the flg22_{Las} peptide in wild-type tobacco leaves at 1 hpi.

The effect of *Lasbcp* expression in tobacco on callose deposition in response to perception of flg22_{Las} was also examined (Fig. 7). Significant callose deposition was seen in tobacco

Fig. 5. Transient expression of *Lasbcp* in tobacco suppressed H_2O_2 -induced transcriptional upregulation of defense-related gene *NbRbohB*. **A**, *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* GV2260 carrying the binary vectors pCambia2301 (*CaMV35S::uidA*) and pAMB004 (*CaMV35S::Lasbcp*) without added H_2O_2 and **B**, with 50 μM H_2O_2 added. Infiltrated tissues from three individual leaves were excised at 0, 2, 4, and 6 days postinfiltration (dpi) and were used for RNA extraction and quantitative reverse transcription-polymerase chain reaction analyses. *NbRbohB* expression was normalized against elongation factor 1 α (*NbEF1 α*) expression. The data presented are the means $\pm$ standard error for three independent experiments with three replicates. Asterisks indicate significant differences between the treatment means ($P < 0.05$).

leaf tissue responding to infiltration with 40 μ M flg22_{Las}. Callose deposition was almost completely suppressed in tobacco leaves infiltrated with the same level of flg22_{Las} by transient expression of *Lasbcp* in the same leaf region, while callose deposition was readily observed in the similarly treated tobacco leaves expressing β -glucuronidase (GUS) as the control. Taken together, these data provide unambiguous evidence that LasBCP did not interfere with flagellin signal perception (*NbFLS2* and *NbBAK1*) or relay (*NbBIK1*) events, but rather, interrupted H₂O₂-mediated activation of *NbRbohB*, leading to suppression of PAMP-triggered immune responses, including callose deposition.

LasBCP alleviated oxidative damage to membrane lipids in planta.

Tobacco leaf discs infiltrated with *A. tumefaciens* GV2260 (4 dpi) carrying the binary vectors pCambia2301 (*CaMV35S::uidA*), pAMB003 (*CaMV35S::Lasprx5*), and pAMB004 (*CaMV35S::Lasbcp*) were floated in Murashige Skoog (MS) medium (Murashige and Skoog 1962) containing 0, 25, and 50 mM *t*BOOH, for 2 days, for quantification of total chlorophyll content as a visual indicator of *t*BOOH-induced oxidative damage (Fig. 8A). These leaf disc senescence assays showed that 25- and 50-mM *t*BOOH treatments resulted in bleaching and significant loss of chlorophyll in GUS-expressing leaf discs (36 and 55% loss, respectively) as compared with leaves expressing either LasPrx5 (30 and 32% loss, respectively) or LasBCP (18 and 25% loss, respectively). These results indicated a role of both peroxiredoxin genes in mitigating ROS-induced damage to the photosynthetic apparatus and chloroplast membranes.

Oxidative damage to free and membrane lipids was quantified by determining the total malondialdehyde (MDA) (an oxylipin) content in tobacco leaf discs floated in MS medium containing *t*BOOH (Fig. 8B). No significant differences were observed in MDA content in tobacco leaf discs floated in 0 mM *t*BOOH and transiently expressing GUS, LasPrx5, or LasBCP (0.88, 0.93, and 1.05 nmole MDA per microgram of fresh weight, respectively). The 25- or 50-mM *t*BOOH treatments

resulted in a significant increase in MDA levels in tobacco leaf tissue expressing either GUS (17- and 20-fold, respectively) or LasPrx5 (11- and 16-fold, respectively), indicating no protective effect of LasPrx5 on lipid peroxidation. By contrast, LasBCP expression in tobacco leaf discs provided considerable protection against lipid peroxidation, resulting in a much lower 4.7- and 4.5-fold increase in MDA content when exposed to 25 or 50 mM *t*BOOH, respectively. Taken together, these data demonstrate a protective role of secreted LasBCP to benefit '*Ca. L. asiaticus*' survival and colonization by alleviating ROS-induced lipid peroxidation in host cells, thereby alleviating both the direct effects of antimicrobial oxylipins and also the indirect effects of subsequent host cell death response.

DISCUSSION

Upon initial contact with hosts, pathogenic biotrophic bacteria must deal with host detection of PAMPs by actively suppressing the host-generated oxidative burst, typically by use of a large repertoire of often-redundant ROS defensive genes encoding multiple catalases, peroxidases (Klotz and Loewen 2003), and peroxiredoxins (Dubbs and Mongkolsuk 2007). However, all *Liberibacter* genomes are highly reduced, with an average size of approximately 1.2 Mb (Duan et al. 2009; Leonard et al. 2012; Lin et al. 2011; Wulff et al. 2014), and encode only two highly conserved Prx5-like and BCP-like predicted peroxiredoxins. '*Ca. L. asiaticus*' strains with one or more prophages also encode an additional functional peroxidase (Jain et al. 2015; Zhang et al. 2011).

'*Ca. L. asiaticus*' peroxiredoxins *Lasprx5* and *Lasbcp* were functionally validated in the surrogate expression host *L. crescens* BT-1 (Leonard et al. 2012). *L. crescens* has its own pair of

Fig. 6. Transient expression of *Lasbcp* in tobacco inhibited transcriptional upregulation of *NbRbohB* despite the perception of pathogen-associated molecular pattern signal flg22_{Las}. *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* GV2260 carrying the binary vector pAMB004 (*CaMV35S::Lasbcp*). Four days later, the same leaves were infiltrated with 40 μ M flg22_{Las} peptide. Expression of pattern-triggered immunity genes *NbFLS2*, *NbBAK1*, *NbBIK1*, and *NbRbohB* 1 h postinfiltration (hpi) were normalized against elongation factor 1 α (*NbEF1 α*) expression and were calibrated against expression levels at 0 hpi. The data presented are the means $\pm$ standard error for three independent experiments with three replicates. An asterisk indicates a significant difference between the treatment means ($P < 0.05$).

Fig. 7. Transient expression of *Lasbcp* in tobacco suppressed flg22_{Las}-induced callose deposition. *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* GV2260 carrying the binary vector pCambia2301 (*CaMV35S::uidA*) or pAMB004 (*CaMV35S::Lasbcp*). Four days later, the same leaves were infiltrated with 40 μ M flg22_{Las} peptide. **A** and **B**, Callose deposition was visualized by UV epifluorescence in wild-type leaves expressing either **C** and **D**, β -glucuronidase or **E** and **F**, LasBCP, 24 h after flg22_{Las} infiltration. Magnification bars = 50 μ m.

(predicted secreted) peroxiredoxins, but these enzymes proved to be significantly less effective in mitigating oxidative stress than those from ‘*Ca. L. asiaticus*’ (Figs. 2, 3, and 4). Since *L. crescens* is not known to be pathogenic, such efficacy may not be needed. Similar to the previously described ‘*Ca. L. asiaticus*’ SC2 peroxidase (Jain et al. 2015), LasBCP was demonstrated to be a secreted, extracellular enzyme in *L. crescens*, despite lacking a typical Sec secretion signal. The secreted SC2 peroxidase was also shown to confer significantly enhanced peroxidase activity to culture supernatants as compared with wild-type *L. crescens* cells (Fig. 4). The two enzymes presumably act together within plant host cells to suppress the ROS burst.

Putative BCP-like peroxiredoxins from many other pathogenic or symbiotic species from genera including *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Xanthomonas*, *Pseudomonas*, *Erwinia*, and *Ralstonia* were also predicted by SecretomeP to be nonclassically secreted. However, to the best of our knowledge, this is the first report of evidence for a functional role of a bacterial BCP in modulation of host defense responses. In this work, LasBCP was functionally characterized as a pathogen-encoded extracellular peroxiredoxin that simultaneously compromised both RBOH-mediated systemic and oxylipin-mediated localized immunity in planta.

By contrast with secreted LasBCP, LasPrx5 was cytoplasmically retained (Fig. 4) but, nevertheless, conferred significant additional oxidative stress tolerance to *L. crescens* cells (Figs. 2 and 3). Also, in contrast with both the SC2 peroxidase (Jain et al. 2015) and *Lasbcp*, *Lasprx5* transcript abundance was similar in citrus as well as in the psyllid (Fig. 1). Both cytoplasmic localization and similar transcript abundance in very different hosts suggest that LasPrx5 has a general housekeeping function engaged in detoxification of endogenous ROS resulting from electron transport reactions (Cabiscol et al. 2000). LasPrx5 may also provide protection against host-generated peroxides and increase bacterial fitness and viability in host cells. Exogenous H_2O_2 can readily permeate bacterial cell walls, causing oxidation of Fe^{2+} to Fe^{3+} via the Fenton reaction, resulting in Zn^{2+} mismetallation within the active sites of Fe-dependent enzymes. This compromises Fe-dependent enzymatic activities, imposing metabolic bottlenecks and retarding cell growth and viability (Imlay 2014). Consistent with these observations, LasBCP and LasPrx5 both enhanced growth of *L. crescens* in culture (Fig. 3) and would likely increase resistance to exogenous H_2O_2 in the host cell.

An essential role for catalase and peroxidase enzymes in virulence is well-established for both fungal and bacterial plant pathogens, including *Ustilago maydis* in maize (Molina and Kahmann 2007), *Magnaporthe oryzae* in rice (Samalova et al. 2014), *Alternaria alternata* (Lin et al. 2009), *X. axonopodis* pv. *citri* (Tondo et al. 2010), ‘*Ca. L. asiaticus*’ (Jain et al. 2015) in citrus, and *X. campestris* pv. *campestris* in Chinese radish (Jittawuttipoka et al. 2009). By contrast with either peroxidases or catalases, peroxiredoxins are generally cytoplasmic or compartmentalized but rarely extracellular (Wood et al. 2003). Furthermore, Cys-based peroxiredoxin activity is dependent upon the presence of thiol donors such as glutathione and thioredoxin (Hofmann et al. 2002) within the same cellular compartment. Peroxiredoxins have only been reported as virulence factors of plant-pathogenic nematodes (Li et al. 2016) and of bacterial pathogens causing immunosuppression in animal hosts (Dons et al. 2014; Hillmann et al. 2016; Kaihami et al. 2014) but not, to our knowledge, of plant-pathogenic bacteria. To support phloem-limited intracellular growth of ‘*Ca. L. asiaticus*’, LasBCP must be secreted into the phloem sap with an abundant supply of thiol donors (Carella et al. 2016). Peroxiredoxins are catalytically more efficient than catalases or

peroxidases and are capable of degrading even sublethal micromolar levels of H_2O_2 (Seaver and Imlay 2001), thereby providing additional strategic advantage against the initial nonspecific low amplitude RBOH-mediated ROS burst (Baxter et al. 2014; Kadota et al. 2015).

Peroxide-induced activation of RBOH and generation of H_2O_2 via RBOH activity are imperative for systemic cell-to-cell autopropagation of the immune signaling cascade in plants (Kadota et al. 2015; Miller et al. 2009). Phloem sap and sieve tube elements serve as checkpoints for the generation, amplification, propagation, and transport of H_2O_2 (Van Bel and Gaupels 2004). *Lasbcp* is expressed only in planta and not in psyllid hosts (Fig. 1). We hypothesize that extracellular LasBCP eliminates RBOH-generated H_2O_2 and is an essential virulence factor that enables ‘*Ca. L. asiaticus*’ to evade early detection and clearing through innate immunity. ‘*Ca. L. asiaticus*’ eventually builds up significant titers resulting in systemic colonization of citrus phloem over several months to years, resulting in surprisingly long disease-incubation periods (Chiyaka et al. 2012). An increase in pathogen load eventually leads to a Zn deficit in ‘*Ca. L. asiaticus*’-infected citrus cells (Nwugo et al. 2013; Vahling-Armstrong et al. 2012), aggravating oxidative damage primarily through diminished antioxidant activity, enhanced RBOH-mediated superoxide generation,

Fig. 8. LasBCP alleviated oxidative damage to membrane lipids in tobacco. **A**, Leaf discs excised from fully expanded *Nicotiana benthamiana* leaves transiently expressing pCambia2301 (*CaMV35S::uidA*), pAMB003 (*CaMV35S::Lasprx5*), or pAMB004 (*CaMV35S::Lasbcp*) were floated for 2 days on liquid Murashige and Skoog medium containing 0, 25, or 50 mM *tert*-butyl hydroperoxide and were harvested for quantification of total chlorophyll (Chl *a+b*) and **B**, malondialdehyde (MDA) content. The data presented are the means $\pm$ standard error for three independent experiments with five replicates. Asterisks indicate significant differences between the treatment means ($P < 0.05$).

and disruption of mitochondrial electron transport and photosynthetic CO₂ fixation (Cakmak 2000; Eide 2011).

The 1-Cys LasBCP exhibited peroxidase activity against H₂O₂ and organic alkyl peroxides (Figs. 2, 3, and 4). LasBCP prevented activation of the RBOH signaling cascade (Figs. 5 and 6) and simultaneously suppressed oxidative deterioration of free and membrane-bound lipids in the host cell (Fig. 8), thereby preventing a host cell-death response. The entire suite of genes for flagellar biosynthesis is present in ‘*Ca. L. asiaticus*’ (Duan et al. 2009), and the PAMP activity of ‘*Ca. L. asiaticus*’ flagellin peptide flg22_{Las} has earlier been demonstrated in tobacco (Hao et al. 2014; Zou et al. 2012) as well as in citrus (Shi et al. 2017). Transient expression of *Lasbcp* in tobacco suppressed (exogenously supplied) H₂O₂-mediated transcriptional activation of *NbRbohB* (Fig. 5), suggesting a potentially crucial role for the ‘*Ca. L. asiaticus*’ secreted peroxidase in attenuation of host defense responses. Moreover, transient *Lasbcp* expression did not interfere with the perception of flg22_{Las} by FLS2, BAK1, or BIK1 but, instead, suppressed downstream *RbohB* autoactivation via an H₂O₂-mediated positive feedback loop (Fig. 6).

LasBCP, as a 1-Cys peroxidase, is ideally suited as a virulence effector for sustained suppression of plant ROS defenses. 1-Cys peroxidases are insensitive to redox regulation (reversible inactivation) through hyperoxidation of the peroxidic Cys (C_P/SO₂H) (Nelson et al. 2011). Oxidative inactivation of C_P/SH would promote localized buildup of H₂O₂ for participation in ROS signaling networks, a physiological function of 2-Cys peroxidases that would clearly not be useful for intracellular pathogens such as ‘*Ca. L. asiaticus*’. Suppression of RBOH activity leads to the reduction of disease resistance accompanied by elimination of the oxidative burst (Yoshioka et al. 2003). *Rboh* transcriptional activity in citrus leaves was downregulated following ‘*Ca. L. asiaticus*’ infection (Mafra et al. 2013). Furthermore, proteomic profiling of ‘*Ca. L. asiaticus*’-infected grapefruit leaves revealed decreased accumulation of several H₂O₂-scavenging as well as H₂O₂-responsive enzymes (Nwugo et al. 2013), providing additional evidence for active suppression of ROS signaling during ‘*Ca. L. asiaticus*’ infection. A decline in proline concentration in ‘*Ca. L. asiaticus*’-infected citrus fruits was also attributed to a defense-related H₂O₂ burst (Slisz et al. 2012).

In addition to providing H₂O₂ tolerance, LasBCP also provided a significant 214-fold increase in viability of *L. crescens* BT-1 cells exposed to tBOOH (Fig. 3). LasBCP prevented oxidative damage to plant membrane lipids and subsequent accumulation of oxylipins (Fig. 8). Oxylipins are formed in eukaryotic cells in response to a wide range of pathogens (García-Marcos et al. 2013; Sun et al. 2014). Both lipid peroxidation as well as lipase-based cleavage of membrane lipids and subsequent dioxygenation by lipoxygenases are stringently regulated within cells (Farmer and Mueller 2013). Lipoxygenase levels were significantly downregulated in ‘*Ca. L. asiaticus*’-infected citrus leaves (Fan et al. 2011; Nwugo et al. 2016). Furthermore, the increase in lipoxygenase substrates in citrus leaves caused by *Diaphorina citri* infestation was rapidly attenuated upon coinfection with ‘*Ca. L. asiaticus*’, indicating active suppression of the oxylipin-mediated defense pathway (Killiny and Nehela 2017).

LasBCP is a uniquely suitable and, likely, critical effector for suppressing host defenses in support of an intracellular lifestyle, because it is secreted inside the plant cell cytoplasm and has a high affinity for organic peroxide radicals. Cytoplasmic LasPrx5 and secreted LasBCP peroxidases in ‘*Ca. L. asiaticus*’ appeared to be important for both endogenous and host-generated ROS defense, respectively. LasBCP suppressed H₂O₂-mediated systemic defense signaling cascades in planta and, more

importantly, also provided protection against peroxidative damage to free and membrane-bound lipids and subsequent accumulation of oxylipins. All pathogenic ‘*Ca. Liberibacter* sp.’ are predicted to encode highly similar BCP-like enzymes, and all are predicted to be nonclassically secreted. Based on homology, we speculate that suppression of the accumulation of oxylipin, which exhibits antimicrobial activity and elicits hypersensitive host cell death, is critical for survival and phloem colonization not only for ‘*Ca. L. asiaticus*’, but for all pathogenic *Liberibacter* species. These BCP-like enzymes are promising targets for chemical inactivation and development of therapeutic chemical controls of several severe plant diseases, including HLB.

MATERIALS AND METHODS

Plant material and insect samples.

‘*Ca. L. asiaticus*’-infected leaf samples were excised from the curated citrus (*Citrus paradisi*) plants maintained in a quarantine greenhouse at the University of Florida, Gainesville, Florida. ‘*Ca. L. asiaticus*’-infected mixed-sex adult psyllids (reared on sweet orange, *C. sinensis*; approximately 80% infection density) were provided by D. Hall, United States Department of Agriculture Agricultural Research Service (USDA-ARS).

Bacterial strains and growth conditions.

The relevant characteristics, sources, and references for the bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *A. tumefaciens* strains were grown in Luria-Bertani medium at 37 and 28°C, respectively. *L. crescens* BT-1 was maintained on BM7 medium with gentle shaking at 150 rpm at 28°C (Fleites et al. 2014; Jain et al. 2015). Antibiotics were used as needed at the following concentrations (in µg/ml): ampicillin 100, kanamycin 50, rifampicin 75, and gentamycin 2.

Nucleic acid extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) from ‘*Ca. L. asiaticus*’-infected psyllids and citrus.

DNA was extracted from citrus leaf discs and whole psyllids using the DNeasy plant mini kit and a blood and tissue kit, respectively (Qiagen, Valencia, CA, U.S.A.). Presence of ‘*Ca. L. asiaticus*’ in the infected citrus samples was confirmed using conventional and nested PCR primer sets OI1/OI2c (Jagoueix et al. 1996) and CG03F/CG05R (Zhou et al. 2007) (Table 2). For RNA, midribs of PCR-confirmed ‘*Ca. L. asiaticus*’-infected leaves and psyllids (10 insects pooled together) were ground with a cold mortar and pestle in lysis buffer RLT (provided with the Qiagen RNeasy plant mini kit). RNA was extracted following the manufacturer protocol, was diluted with nuclease-free water to 200 ng µl⁻¹, and was cleaned with TURBO DNA-free (DNase) kit (Ambion, Austin, TX, U.S.A.). RT reactions were performed using 1 µg of RNA template (iScript Advanced cDNA synthesis kit; Bio-Rad, Hercules, CA, U.S.A.). qRT-PCR analyses were performed using a CFX96 Touch Real-Time PCR detection system (Bio-Rad) as described (Fleites et al. 2014; Jain et al. 2015). All the primers used are summarized in Table 2. The transcript abundance data were normalized against expression in ‘*Ca. L. asiaticus*’-infected citrus. Data analyses and Student’s *t* tests (*P* = 0.05) were performed using the Bio-Rad CFX Manager software package 3.0.

Construction of peroxidase expression vectors and transformation in *L. crescens* BT-1.

Lasprx5 and *Lasbcp* coding sequences were PCR-amplified using high-fidelity AccuPrime Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) and were directionally cloned in pSC-A

(StrataClone; Agilent Technologies, Santa Clara, CA, U.S.A.), using the primer pairs as described in Table 2. Following sequence confirmation, the cloned inserts were moved in wide-host range shuttle vector pBBR1MCS-5 (Kovach et al. 1995) to yield the plasmids pSZ103 (*lacZ::Lasprx5*) and pSZ104 (*lacZ::Lasbcp*). Electrocompetent *L. crescens* BT-1 cells were prepared and transformed with pSZ103 and pSZ104 as previously described (Fleites et al. 2014; Jain et al. 2015).

Bioinformatic analyses for potential secretion signals for *Lasprx5* and *Lasbcp*.

SignalP version 4.1 was used to identify potential secretion signals from protein sequences, using both default (discrimination

[D] score cutoff of 0.45) and sensitive (D score cutoff of 0.34) values. Prediction of nonclassical secretion was performed using SecretomeP (Bendtsen et al. 2005).

Measurement of ROS stress response and peroxide resistance assay.

For measurement of ROS detoxification response, 5-day-old *L. crescens* cells, transformed with the empty vector pBBR1MCS-5, pLF080 (Jain et al. 2015), pSZ103, or pSZ104 were adjusted to Abs₆₀₀ = 1.0, and a 200-μl cell suspension was treated with ROS generator menadione (10 μM), either in the presence or absence of peroxiredoxin inhibitor conoidin A (50 μM). CellROX Deep Red reagent (Molecular Probes, Eugene, OR,

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i> TOP10	F– <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara leu</i>) 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Sm ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>Liberibacter crescens</i>	Strain BT-1, originally isolated from mountain papaya	Leonard et al. 2012
<i>Agrobacterium tumefaciens</i> GV2260	C58, Rif ^R , pGV2260 (pTiB6S3AT-DNA, Cb ^R), octopine type	McBride and Summerfelt 1990
pSC-A	4.3 kb; polymerase chain reaction cloning vector, Ap ^R , Kn ^R	Agilent
pBBR1MCS-5	Rep <i>Bordetella</i> , <i>lacZ</i> ⁺ , Gm ^R	Kovach et al. 1995
pCambia2301	11.6 kb; pPZP family binary vector, <i>aadA</i> (bacterial selection), <i>nptII</i> (plant selection), enhanced 35S promoter, castor bean catalase intron, <i>uidA</i> reporter with 6xHis-tag	Hajdukiewicz et al. 1994
pLF080	pUFR071::F489_gp15::6xHis-tag	Jain et al. 2015
pSZ103	pBBR1MCS-5:: <i>Lasprx5</i>	This study
pSZ104	pBBR1MCS-5:: <i>Lasbcp</i>	This study
pAMB003	<i>Lasprx5</i> directionally cloned in pCambia2301 replacing the <i>uidA</i> coding sequence	This study
pAMB004	<i>Lasbcp</i> directionally cloned in pCambia2301 replacing the <i>uidA</i> coding sequence	This study

^a Sm^R, Rif^R, Cb^R, Ap^R, Kn^R, and Gm^R = streptomycin, rifampicin, carbenicillin, ampicillin, kanamycin, and gentamycin resistance.

Table 2. List of primers used in this study

Primer	Sequence (5'→3')	Reference
Las confirmation		
OI1	GCGCGTATGCAATACGAGCGGC	Jagoueix et al. 1996
OI2c	GCCTCGCGACTTCGCAACCCAT	
CG03F	RGGGAAAGATTTTATGGAG	Zhou et al. 2007
CG05R	GAAATATAYCATCTCTGATATCGT	
Quantitative reverse transcription-polymerase chain reaction		
SZ30 (<i>Lasprx5</i> F)	GAGCTCCATAACGCCAAGAA	This study
SZ31 (<i>Lasprx5</i> R)	TGATGGTTCGGGCGAATTTA	
SZ34 (<i>Lasbcp</i> F)	GTCAACTATCCTTATCGGCATCTC	This study
SZ35 (<i>Lasbcp</i> R)	CTCATCGGCAAGCAGTGTAATA	
<i>Lasprf</i> AF	TGTCTGAATCGCCTTCTGTC	Fleites et al. 2014
<i>Lasprf</i> AR	GATCACCGATGACAGTATGC	
<i>Lasgyr</i> BF	TTGAACAAGCTGTAAATTTCTGG	Fleites et al. 2014
<i>Lasgyr</i> BR	ATCTGTTTGCCAATTTAGAAGC	
<i>NbFSL2</i> F	TTCTGTCTCTTTCCGGGTAATG	This study
<i>NbFSL2</i> R	TCCCTTATGCTTGAAGGAATGT	
<i>NbBAK1</i> F	GGTAGAGGTGGATTTGGTAAGG	This study
<i>NbBAK1</i> R	CACCTTGAGTACGTTCTCTTT	
<i>NbBIK1</i> F	CGGATAGTGTGTTGGGAGAAG	This study
<i>NbBIK1</i> R	GCCCTCTGGTTCAATCTCTTA	
<i>NbRboh</i> BF	TTTCTCTGAGGTTTGCCAGCCACCACCTAA	Hao et al. 2014
<i>NbRboh</i> BR	GCCTTCATGTTGTTGACAATGCTCTTTAACA	
<i>NbEF1α</i> F	GACCACTGAAGTTGGATCTGTTG	Hao et al. 2014
<i>NbEF1α</i> R	TAGCACCAGTTGGGTCTTCTT	
Peroxiredoxin expression vectors		
SZ28 (<i>Lasprx5</i> F)	<u>GGATCC</u> AGGAGTATGAAATGATCCGATTTCAAATAC	This study
SZ29 (<i>Lasprx5</i> R)	<u>AAGCTT</u> ctattttttactttctgaataac	
SZ32 (<i>Lasbcp</i> F)	<u>GGATCC</u> AGGAGaaatcacatgacatctttatctgtg	This study
SZ33 (<i>Lasbcp</i> R)	<u>AAGCTT</u> TTATTGTTTAAAGATTTTACCATC	
Peroxiredoxin binary vectors		
AMB36 (<i>Lasbcp</i> F)	GCTGACGACTACCAGTCATGACATCTTTATCTGTG	This study
AMB37 (<i>Lasbcp</i> R)	GCGACGCGACGTGTTATTGTTTAAAGGATTTTACC	
AMB39 (<i>Lasprx5</i> F)	GCTGACGACTACCAGTCATGATCCGATTTCAAATAC	This study
AMB40 (<i>Lasprx5</i> R)	GCGACGCGACGTGCTATTTTACTTTCTCG	

U.S.A.) was added at a final concentration of 5 μ M, and the cells were incubated for 30 min at 28°C. Subsequently, the cells were washed three times with cold phosphate-buffered saline and the fluorescence was measured (Ex/Em maxima 640/665 nm). For examining the peroxide tolerance, 5-day-old *L. crescens* cells transformed with pLF080, pSZ104, and pSZ105 were harvested and resuspended in BM7 medium, to a density of Abs₆₀₀=0.5 and 1 ml of resuspended bacteria were treated with 100 μ M H₂O₂ or 50 μ M tBOOH for 30 min, were washed twice in BM7 medium, were serially diluted, and were plated on selective BM7 media. The surviving bacterial colonies were scored after 3 weeks.

Peroxidase activity determination.

Enzymatic peroxidase activity was measured using the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen). Aliquots of 5-day-old *L. crescens* cultures (1.0 ml, adjusted to Abs₆₀₀ = 1.0) were harvested by centrifugation and were resuspended in 50 μ l of 0.25 M sodium phosphate buffer (pH 7.4). An equal volume of 100 μ M AmplexRed reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 2 mM H₂O₂ was added to the bacterial suspension and was incubated for 30 min in the dark. The development of red-chromogenic oxidation product resorufin was measured at 560 nm. The enzyme activity was normalized against a standard curve obtained for horseradish peroxidase; 1 U enzyme activity is defined as the amount of enzyme that will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 and 20°C.

Agrobacterium-mediated transient expression of ‘*Ca. L. asiaticus*’ peroxiredoxin genes in tobacco.

Lasprx5 and *Lasbcp* were PCR-amplified from ‘*Ca. L. asiaticus*’-infected psyllid DNA and were cloned in pCR2.1-TOPO (Invitrogen), using the primer pairs described in Table 2. Following sequence verification, *Lasprx5* and *Lasbcp* were directionally subcloned into pCambia2301 (*CaMV35S::uidA*) between *AdhI* and *PmII* sites, replacing *uidA* (Hajdukiewicz et al. 1994). The resulting plasmids pAMB003 (*CaMV35S::Lasprx5*) and pAMB004 (*CaMV35S::Lasbcp*) were mobilized into *A. tumefaciens* GV2260 via electroporation. *Agrobacterium*-mediated transient expression assays in tobacco (*N. benthamiana*) were performed as described previously (Hao et al. 2014). Synthetic flg22_{Las} peptide (DRVSSGLRVSDAADNAAWWSIA) (Life Tein Company, South Plainfield, NJ, U.S.A.) was diluted in water to a final concentration of 40 μ M and was infiltrated into tobacco leaves with a 1-ml needleless syringe. The infiltrated leaf tissues were sampled for RNA extraction, cDNA synthesis, and RT-qPCR analysis as described (Jain et al. 2015).

Callose deposition assay.

Callose deposition in flg22_{Las}-infiltrated leaves was detected with aniline staining as described previously (Hao et al. 2014). Briefly, the tissue was cleared and dehydrated in 100% ethanol. Cleared leaves were washed with distilled water and were 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 was observed under ultraviolet epifluorescence (LSM5 Pascal confocal laser scanning microscope; Carl Zeiss Microscopy LLC, Thornwood, NY, U.S.A.).

Quantification of oxidative membrane injury.

Leaf discs from fully expanded tobacco leaves transiently expressing pCambia2301, pAMB003, or pAMB004 were floated on liquid MS basal medium (Murashige and Skoog 1962) containing 0 to 50 mM tBOOH. Total chlorophyll content in the leaf discs was estimated after 2 days, according to the procedure of Arnon (1949). The lipid peroxidation reaction

end product, i.e., MDA, was measured using the MDA assay kit (Sigma-Aldrich, St. Louis). MDA reacts with thiobarbituric acid to form a fluorimetric product (Ex/Em maxima 532/553 nm).

Statistical analyses.

All data were analyzed using SAS for Windows, version 9.2 (SAS Institute Inc, Cary, NC, U.S.A.) and Student *t* tests were performed to separate treatment means. A probability of 5% was used to determine statistically significant differences.

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