Plant hairy roots enable high throughput identification of antimicrobials against *Candidatus Liberibacter* spp.

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A major bottleneck in identifying therapies to control citrus greening and other devastating plant diseases caused by fastidious pathogens is our inability to culture the pathogens in defined media or axenic cultures. As such, conventional approaches for antimicrobial evaluation (genetic or chemical) rely on time-consuming, low-throughput and inherently variable whole-plant assays. Here, we report that plant hairy roots support the growth of fastidious pathogens like *Candidatus Liberibacter* spp., the presumptive causal agents of citrus greening, potato zebra chip and tomato vein greening diseases. Importantly, we leverage the microbial hairy roots for rapid, reproducible efficacy screening of multiple therapies. We identify six antimicrobial peptides, two plant immune regulators and eight chemicals which inhibit *Candidatus Liberibacter* spp. in plant tissues. The antimicrobials, either singly or in combination, can be used as near- and long-term therapies to control citrus greening, potato zebra chip and tomato vein greening diseases.
Fastidious and obligate phytopathogens cause devastating diseases in several food and commodity crops; for example, the phloem-limited bacteria *Candidatus Liberibacter* spp. cause severe yield losses in the Solanaceae [potato (*Solanum tuberosum* L.), tomato (*S. lycopersicum* L.), pepper (*Capsicum annuum* L.), and tobacco (*Nicotiana tabacum* L.)] and Rutaceae (*Citrus* spp.) families. Potato zebra chip (ZC) disease, putatively caused by *Candidatus Liberibacter* solanacearum (*Clos*), is transmitted by an insect vector, the potato psyllid (*Bactericera cockerelli* (Sulc)); Hemiptera: Triozidae) since its discovery in 1994, *ZC* has been documented in several commercial potato-growing regions of the United States, Mexico, Central America, Australia, and New Zealand, causing yield losses of up to 94%<sup>3,4</sup>. Similarly, citrus greening or huanglongbing (HLB), putatively caused by *Candidatus Liberibacter* asiaticus (*CLas*), is the most devastating disease threatening citrus production worldwide; its insect vector is the Asian citrus psyllid (*Diaphorina citri* Kuwayama [Hemiptera: Liviidae])<sup>5</sup>. Between 2006 and 2011, HLB caused losses of more than US$4.5 billion in the US state of Florida alone<sup>6</sup>. CLAs and *CLso* share some commonalities, such as their unculturable nature, genomic attributes, and lifestyles as psyllid-vectored and phloem-limited bacteria<sup>7–10</sup>. These and other diseases caused by fastidious plant pathogens are major threats to global agriculture and food security.

A challenge in identifying therapies against CLAs and *CLso* is our inability to culture or maintain them outside their plant and insect hosts. Over 99% of all microorganisms are estimated to be unculturable in the laboratory<sup>11</sup>. Attempts have been made to customize artificial growth media and culture conditions to culture such microbes, including specialized modifications to growth medium composition and growth conditions tailored to the particular microbe based on what we know about its natural growth environment (e.g., temperature, pH, incubation time, inoculum size, and CO₂/O₂ ratio), coculturing with other microbes, and growth in biofilms or other simulated environments<sup>11–21</sup>. Although these approaches have allowed some success in mono- or cocultures as means to propagate fastidious pathogens but they have provided little contribution to downstream applications such as the screening of antimicrobials against target pathogens, and therefore are not broadly utilized. Because of the constraints that exist in culturing fastidious pathogens in defined media and evaluating potential therapies, we explored alternative strategies.

Hairy roots are neoplastic growth that forms on plant tissues after wounding and infection with the soil bacterium *Rhizobium rhizogenes* (previously *Agrobacterium rhizogenes*)<sup>22,23</sup>. *R. rhizogenes* introduces its root-inducing transfer DNA (RT-DNA), encoding the root locus (*rolA*, *rolB*, *rolC*, and *rolD*) genes, into the plant genome. Expression of *rol* genes in planta modulates auxin homeostasis and induce hairy root initiation and proliferation in vitro; thus, hairy root cultures do not require the addition of phytohormones. Hairy roots are anatomically and metabolically similar to normal roots, and possess intact xylem and phloem vasculature connected to the source explant<sup>24–26</sup>. They constitute a valuable tool in plant functional biology, biotechnology, metabolic engineering, molecular pharming, and studies of root–rhizosphere interactions<sup>26–29</sup>. Classical microbiological techniques developed early in the 19th century, cultured animal and mammalian viruses in host cells, tissues, and embryonated eggs<sup>30–36</sup>. In a similar manner, we hypothesized that plant hairy roots could be suitable for propagating fastidious pathogens.

In this work, we show that hairy roots support the accumulation of fastidious plant bacteria, *Candidatus Liberibacter* spp. Furthermore, using the microbial hairy roots, we conduct antimicrobial screening leading to the discovery of multiple genetic and chemical therapies that effectively suppress *Candidatus Liberibacter* spp. in plant tissues.

**Results**

**Hairy roots support propagation of fastidious pathogens.** Since *Candidatus Liberibacter* spp. (CLso and CLas) are phloem-limited pathogens, we tested whether plant hairy roots, which possess intact vasculature<sup>24–26</sup>, could sustain their growth. Hairy roots are anatomically and metabolically similar to normal roots and can be induced in a variety of plants using *R. rhizogenes*<sup>24–26</sup>. To test our hypothesis, we generated ex vivo and in planta hairy roots from CLso- and CLas-infected potato, tomato and citrus explant tissues (Fig. 1a, b and Supplementary Fig. 1). We used *R. rhizogenes* harboring a binary T-DNA vector with a green fluorescent protein (GFP) marker to induce hairy root growth.

Fluorescence microscopy analysis confirmed the production and authenticity of the transgenic hairy roots, which were distinguished from the adventitious roots by their GFP signal (Fig. 1c, d). The transformation efficiency of the hairy root induction, defined by the percentage of GFP-positive hairy roots among all roots generated, was ~70–90% in potato and tomato and ~50–70% in citrus.

The genes, *rolB* and *rolC*, encoded by the Ri T-DNA of *R. rhizogenes*, were amplified using a polymerase chain reaction (PCR) and used as additional markers to confirm the transformation of the hairy roots (Fig. 1e, f). PCR amplification using diagnostic markers specific to CLso (16S rRNA)<sup>37,38</sup> and CLas (*A2/J5* and RNR)<sup>39,40</sup> readily detected these pathogens in the infected potato and citrus hairy roots, but not in the uninfected hairy roots (Fig. 1e, f). Similar results were obtained with CLso in a second host, tomato (Supplementary Fig. 3). The identity of CLso and CLAs in the hairy roots was confirmed by Sanger sequencing and Basic Local Alignment Search Tool (BLAST) analysis of the respective PCR amplicons for similarity to the NCBI nucleotide sequence database (Supplementary Fig. 8). Copy number and bacterial growth curve estimation by quantitative (q) PCR analysis further showed that CLso and CLAs could be maintained in the potato and citrus hairy roots for at least 28 and 120 days, respectively (Fig. 1g, h).

Next, we performed transmission electron microscopy (TEM) to visualize CLso/CLas and probe the anatomy of the hairy roots. Unlike PCR/Sanger sequencing or immuno-labeling approaches, TEM alone is not a confirmatory test to identify pathogens. Unfortunately, immuno-labeling is technically challenging to perform for CLso/CLas detection, primarily due to limitations of existing antibodies (only available for CLas) and the low-titers and highly variable nature of CLso/CLas accumulation in infected plant tissues<sup>41</sup>. Thus far, CLas immuno-labeling was primarily successful in insect-vector<sup>42</sup>. Nevertheless, our TEM analysis showed multiple rounds- and bacilliform-shaped bacteria-like cells in the infected potato and citrus hairy roots, but not in healthy hairy roots (Supplementary Fig. 9), along with notable symptoms of vascular deterioration and collapse, presumably caused by CLso/CLas<sup>43,44</sup>. The morphology and the vascular degeneration phenotypes were reminiscent of those reported for CLas-infected citrus<sup>43,44</sup>. Furthermore, the lack of other microbes in healthy tissues suggests that the normal microbiome is well-below the detection limits of TEM hence the bacteria-like cells observed in the infected tissues are associated with CLso/CLas infection. Combined with the Sanger-sequencing confirmatory analysis, our results sufficiently demonstrate the presence of CLso/CLas in the hairy roots and suggest that the CLso/CLas can be maintained in plant hairy roots like normal infected plant roots.
**Fig. 1** Candidatus Liberibacter solanacearum and Candidatus Liberibacter asiaticus in potato and citrus hairy roots. **a, b** Hairy roots (indicated by arrows) from Candidatus Liberibacter solanacearum (CLso)-infected potato (**a**) and Candidatus Liberibacter asiaticus (CLas)-infected citrus explants (**b**). **c, d** Visual confirmation of GFP expression in hairy roots by fluorescence microscopy. Scale bars, 1 cm. **e, f** Detection of CLso and CLas in the hairy roots by PCR amplification of diagnostic markers specific to CLso (16S rDNA) and CLas (rplK04/J5 and RNR). GFP, rolB, and rolC encoded on the Ti and Ri plasmids, respectively, and co-transformed into the hairy roots, were used as additional markers for hairy root authenticity; RPL2 and GAPC2 are endogenous potato and citrus genes, respectively, used as genomic DNA controls for PCR. “L” and “HR” indicate leaf and hairy root samples, respectively. “+” indicates positive controls used for the respective PCR amplifications. **g, h** Temporal growth curves of CLso and CLas in hairy root samples collected at different days in propagation are plotted, whereas the approximate genome equivalents (GE) per nanogram of root genomic DNA are indicated above the bar graph columns. Error bars represent ± standard error of mean (n = 4 and n = 3 for **g** and **h**, respectively). Uncropped raw agarose gel images used to prepare **e** and **f** are presented in Supplementary Fig. 10. Source data underlying Fig. 1g, h are provided as a Source Data file.
Genetic evaluation using microbial hairy roots. Hairy roots are functional plant tissues that mimic the pathogen’s native host root environment. Hence, we leveraged them to conduct functional genetic screening to identify therapeutics that can inhibit *Candidatus Liberibacter* spp. Because CLas and CLso are closely related to each other, we focused mainly on CLso-hairy potato roots for functional genetic screening due to the faster growth of potato hairy roots compared to citrus hairy roots. First, we evaluated whether gain-of-function genetic studies could be performed using the microbial hairy root system. *Nonexpresser of pathogenesis-related gene 1 (NPR1)* from *Arabidopsis thaliana* (AtNPR1 and AT1G64280) is a well-known broad-spectrum immune modulator that is effective against diverse pathogens. NPR1 is a receptor of the defense hormone salicylic acid (SA) and functions as a transcriptional activator of pathogenesis-related (PR) genes in plant defense and systemic acquired resistance (SAR) responses. We set out to evaluate whether any NPR1 ortholog(s) in *Solanaceae* could confer tolerance to *Candidatus Liberibacter* spp. By phylogenetic analysis (Supplementary Fig. 2) with AtNPR1 as a reference, we identified an orthologous gene from tomato (SlNPR1) and cloned the coding sequences into a binary vector containing a GFP reporter gene (Fig. 2a). Then we transformed each NPR1 construct and an empty vector control containing only GFP into unchallenged (healthy) or CLso-infected (CLso) explant tissue for hairy root induction using *Rhizogenes* (*Fig. 2b*)46–49. Finally, we performed molecular diagnostics on three biological replicates of each type of hairy roots, 30 days after transformation, to confirm the presence of GFP and NPR1 expression in the respective hairy roots (*Fig. 2c*). Supplementary Fig. 3, followed by a quantitative polymerase chain reaction (qPCR)-based estimation of the CLso titers (*Fig. 2d*). The heterologous expression of SlNPR1 significantly (*p ≤ 0.01*) decreased CLso titers (>90%) in the hairy roots compared to the hairy roots transformed with the empty vector (*Fig. 2*).

To determine the mechanism whereby NPR1 suppressed *Candidatus Liberibacter* spp. in potato, we monitored the changes in the downstream expression of two PR genes (PR-1 like and PR-3 like). Expression of these PR genes was significantly higher in both SlNPR1- and AtNPR1-expressing hairy roots under healthy conditions than in empty-vector-transformed controls (*Fig. 2e*). These expression levels were further greatly amplified upon CLso infection in both SlNPR1- and AtNPR1-expressing hairy roots when compared to vector-alone control (*Fig. 2e*). Expression of a WRKY transcription factor (WRKY6-like) involved in NPR1-mediated PR gene activation50,51 was also strongly induced in SlNPR1- and AtNPR1-expressing hairy roots (*Fig. 2e*). Compared to healthy hairy roots, CLso-infected hairy roots had significantly higher levels of free SA (*Fig. 2f*), as determined by liquid chromatography–mass spectrometry (LC–MS/MS). However, despite the activation of downstream defense-related genes, SA levels were significantly lower in SlNPR1- and AtNPR1-expressing hairy roots than in empty vector controls, under both healthy and CLso-infected conditions (*Fig. 2f*).

In addition to evaluating plant immune modulators, we tested the efficacy of eight putative broad-spectrum antimicrobial peptides (AMPs) isolated from spinach (*Spinacia oleracea* L.) that show some inhibitory activity against other Gram-positive and Gram-negative bacteria52–54. Briefly, we cloned the coding sequences of each peptide into the binary vectors and transformed them into the CLso-potato and CLas-citrus explant tissues for hairy root induction and analyzed them in a manner similar to NPR1 evaluation (*Fig. 2b–d*). The results of the antimicrobial assays showed that five of the eight AMPs (denoted AMP1–AMP5) significantly (*p ≤ 0.05 or 0.01*) reduced CLso and CLas titers (~70–98%) in the hairy roots compared to the controls transformed with the empty vector containing GFP alone (*Fig. 2g*). Conversely, AMP6 and AMP8 appear to slightly increase CLso and CLas titers, possibly by inhibiting other unknown competitive microbes present in the hairy roots.

Genome editing using microbial hairy roots. Next, we evaluated whether the microbial hairy root system could be used to perform genome-editing studies. For proof-of-concept, we evaluated the editing of a stably integrated GFP gene in a genetically engineered potato. To test this hypothesis, we generated constructs containing Cas9 alone (control) or Cas9–sgGFP. We then used these to transform healthy or CLso-containing explant tissues for hairy root induction, as described above (*Fig. 3a, b*). Fluorescence microscopy imaging of the transformed hairy roots showed a clear loss of GFP fluorescence (from the transgene) (*Fig. 3c, d*) in hairy roots transformed with Cas9–sgGFP, but not in Cas9 only (control). Amplicon sequencing of the target site confirmed successful editing had occurred in Cas9–sgGFP hairy roots, but not in Cas9 only roots. Frequencies of indels detected were ~86 and 100% in the healthy and CLso hairy roots, respectively (*Fig. 3c–f*). In addition, we sought to edit an endogenous gene, NPR3, in potato. Unlike NPR1, its homologs NPR3 and NPR4 function antagonistically to suppress plant defense in Arabidopsis, and their levels and activities are tightly regulated via complex biochemical interactions in order to maintain defense homeostasis55–57. Therefore, it seemed possible that reducing endogenous NPR3 activity in *Solanaceae* could impart plant tolerance for CLso. To test this hypothesis, we identified the potato ortholog of AtNPR3 (SINPR3) and generated constructs containing Cas9 alone (control) or Cas9–sgNPR3. We then used these to transform healthy or CLso-containing explant tissues for hairy root induction, as described above (Supplementary Fig. 4a, b). Amplicon sequencing of the target site confirmed that successful editing had occurred in the Cas9–sgNPR3 hairy roots, but not the Cas9 control hairy roots (Supplementary Fig. 4c), albeit at a lower rate of ~40%, which was not unexpected given the heterozygous autotetraploid nature of potato (*2n = 4x = 48*), which means there are four copies of each endogenous gene. Nevertheless, when we evaluated the CLso levels of three biological replicates at 30 days post transformation using qPCR, we found that the Cas9–sgNPR3 hairy roots had significantly (*p ≤ 0.01*) lower CLso titers (>90%) than the control hairy roots (Supplementary Fig. 4d). The suppression of CLso titers was strongly associated with induced expression of NPR1 and several defense marker genes (WRKY6-like, PR-1 like, and PR-3 like) in the Cas9–sgNPR3-edited hairy roots (Supplementary Fig. 4e). Similar activation of plant defenses was observed in *Theobroma cacao* wherein CRISPR editing of ~27% alleles of NPR3 was sufficient to enhance resistance to *Phytophthora tropicalis*58. The observed resistance even when partial NPR3 alleles are removed could be due to the systemic nature of the SAR response, whereby a defense signal originated from the NPR3-edited cells is perpetuated to non-edited cells, that ultimately limits the pathogen accumulation. Alternatively, it is possible that native NPR3 activity is affected by gene dosage or haploinsufficiency59–61 and/or dominant-negatively inhibited by the truncated/aberrant NPR3 proteins resulting from the edited NPR3 alleles62. Regardless of the exact mechanisms of the NPR3 action, these results offer proof-of-concept for conducting genome-editing experiments using the microbial hairy root system and uncover NPR3 as a potential target for CRISPR editing to gain resistance to *Candidatus Liberibacter* spp.

High throughput in vitro therapeutic screening. The use of trans/cis-genic-based or genome-editing strategies is a stable, long-term solution to combat diseases; however, it is often a
precarious one, owing to the tremendous regulatory burden associated with agricultural use of genetically modified crops, even those produced by CRISPR/Cas. Thus, we also sought to find short- to intermediate-term solutions, and we therefore conducted high-throughput screening of small-molecule inhibitors among a diverse collection of bioactive compounds approved for use in United States and other countries (Microsource Discovery Systems, Inc.)\textsuperscript{63,64}. For this, we developed an in vitro multi-well plate assay using the microbial hairy roots, which is scalable from 12- to 48- to 96-well layout. Briefly, we generated microbial hairy roots with CLso and CLas, transferred them to a multi-well plate containing growth medium to maintain the hairy roots. The figures show the relative bacterial titers and SA levels in different treatments.

**Figure a:** Diagram of the gene expression pattern of the multi-well plate assay.

**Figure b:** Images of plant samples showing healthy and infected states under different gene treatments.

**Figure c:** Images showing the control and different treated plant samples.

**Figure d:** Graph showing the relative CLso titer (%) in different treatments.

**Figure e:** Graph showing the fold change in various PR-like and WRKY-like gene expressions.

**Figure f:** Graph showing the SA levels (nmol/g FW) in different treatments.

**Figure g:** Graph showing the relative bacterial titer (%) in different treatments.
roots, and then added small molecules of interest to the assay plates and incubated the hairy roots for 72 h (Fig. 4a). We performed a primary screening with ~220 compounds in CLso hairy roots, using two biological replicates per compound (10 µM) along with untreated or solvent-alone controls (DMSO, 0.1% v/v). By molecular diagnostics, we identified nine molecules that inhibited CLso titers by >50–70% when compared to the untreated controls (Supplementary Dataset 1). We retested these nine molecules using five biological replicates, alongside a reference antibiotic, tetracycline (TC, 250 ppm), that was previously reported to moderately inhibit CLas in planta.65 We also screened the efficacy of the nine compounds (25 µM) and tetracycline (TC, 500 ppm) in CLas hairy roots. Seven out of the nine test compounds, as well as tetracycline, consistently showed statistically significant (p ≤ 0.05 or 0.01) inhibition of CLso (Fig. 4b). Four compounds showed statistically significant (p ≤ 0.05 or 0.01) inhibition of CLas, on par with tetracycline (Fig. 4c). Three compounds inhibited both CLso and CLas (#3, #8 and #9) (Fig. 4b, c). In general, the active compounds appear to possess diverse structures and bioactive profiles (Fig. 4d–m): three of them were indexed as antibacterial, whereas others were classified as antifungal, antiprotozoal, hemostatic, anthistaminic, anti-diabetic, and mydriatic drugs targeting various biological processes. Note that since the retested nine molecules were originally prescreened in CLso-potato hairy roots (Supplementary Data 1), it is very likely that further re-screening of the entire library using CLas-citrus hairy roots would identify additional hits.

Besides screening small molecules, we also tested whether the in vitro hairy root bioassay setup could be used for evaluating small peptides (~50 aa). For this, we tested the efficacy of the two AMPs (AMP2 and AMP5; Fig. 2g) by direct vacuum infiltration of the recombinant peptides (5 and 10 µg/ml) into the CLas-citrus microbial hairy roots, in a manner similar to the small molecules (Fig. 4a). Molecular diagnostics performed after 72 h of treatment revealed that both AMPs showed statistically significant (p ≤ 0.05 or 0.01) dose-dependent inhibition of CLas (Supplementary Fig. 5) compared to untreated controls.

Delivery of small molecules into the plant phloem, especially in hardy perennial citrus trees, is a major challenge. The most practical and common approach is foliar spraying; however, this was shown to be relatively ineffective in citrus as a means to deliver bactericides such as oxytetracycline to inhibit CLas.65 Multiple research groups are evaluating alternative approaches, such as trunk injection and/or nanoparticle-based systems.65,66 Potato plants are relatively easier to handle and are amenable to foliar spraying techniques. Thus, to determine the in planta efficacy of the three molecules that showed good inhibitory activity against both CLso and CLas (#3, #8, and #9) in the hairy root assays (Fig. 4), we used the CLso-potato plant system. First, we repeated efficacy assays in CLso-potato microbial hairy roots, testing a broader range of concentrations (0, 5, 10, 25, and 50 µM) (Supplementary Fig. 6) to establish a general dose-response curve. Based on these results, 10 and 25 µM concentrations were chosen for in planta foliar spraying experiments. Plants were sprayed with each compound twice a week, alongside untreated plants and tetracycline (250 ppm)-treated plants as negative and positive controls, respectively. Disease progression was monitored for 28 days post infection (dpi), by which time untreated plants showed the typical foliar disease symptoms such as chlorosis, necrosis, leaf curling and wilting, and were quite close to dying (Fig. 5a). In contrast, potatoes sprayed with any of the three test molecules showed a lower level of disease symptoms that was also dose-dependent, i.e., plants sprayed with 25 µM showed the least symptoms, followed by those sprayed with 10 µM and untreated plants, and the level of protection was on par with that from tetracycline treatment (Fig. 5a). The attenuated symptoms also correlated with lowered CLso titers in the respective treatment groups compared to untreated controls (Fig. 5b). Together, these experiments demonstrate that the identified compounds can inhibit Candidatus Liberibacter spp. in planta, and the efficacy results conform with the results of the hairy root bioassays.

Discussion

A recent consensus study by the US National Academy of Sciences (NAS) on the status of the citrus greening research and development concluded that there is no single effective therapy to fight the disease.67 This holds not only for citrus greening but also for other insect-vectored diseases caused by Candidatus Liberibacter spp.1 The NAS report also suggested deploying a combination of therapies and management practices to combat the disease.54,67–70 However, a major challenge in finding therapies has been the unculturable nature of Candidatus Liberibacter spp., which makes conventional gene- or chemical-therapy evaluation laborious and time-consuming. For instance, evaluating the efficacy of an AMP using either stable transformation or transient viral vectors (e.g., tobacco rattle virus and citrus tristeza virus) followed by disease challenges in mature plants could take from 6 months to several years.71–73 Similarly, screening of small-molecule inhibitors of active ingredients by foliar spraying, by stem injection, or by grafting using mature plants, is inherently variable due to the challenges associated with delivering the inhibitors to the bacteria that reside unevenly within the plant vasculature.65 Furthermore, the latter approaches are laborious and time-consuming, and hence not suitable for high-throughput studies.65,68,69 Researchers have also resorted to using distantly related microbes as surrogates.74–77 However, these results may or may not be reliable or extrapolatable to the target pathogen, owing to differences in pathosystems and virulence mechanisms (or lack thereof).
Fig. 3 Evaluation of genome editing in microbial hairy roots. **a** Typical CRISPR-Cas9 gene editing construct. The underlined sequence is the target DNA site used in the sgRNA. **b–d** Transformation of Cas9 alone (control) and Cas9-sgGFP targeting stably expressed GFP transgene in healthy and *Candidatus Liberibacter solanacearum* (CLso) hairy roots. The loss of GFP fluorescence in Cas9-sgGFP, but not in Cas9 alone, indicates successful editing of the GFP transgene. Scale bars, 1 cm. The experiment was independently repeated two times, and all attempts of replication were successful. **e, f** Amplicon sequencing confirmed gene editing in the target site (GFP), as indicated by presence of indels in the Cas9-sgGFP hairy roots, but not in Cas9-alone hairy roots. Frequencies of indels detected were ~86 and 100% in the healthy and CLso hairy roots, respectively. Uncropped raw agarose gel images used to prepare Supplementary Fig. 3b are presented in Supplementary Fig. 11.
In a manner similar to the classical culture strategies for mammalian viruses in host cells, tissues, and embryonated eggs\textsuperscript{30–36}, our experiments demonstrate that plant hairy roots can be used for ex vivo propagation of fastidious pathogens like \textit{Candidatus Liberibacter} spp. We hypothesize similar strategies using insect-vector cell lines could be used for propagating \textit{Candidatus Liberibacter} spp. Although this is not an axenic culture system, the microbial hairy root system is highly versatile and enables faster genetic and chemical therapeutic screening, which led to the identification of multiple potential short- and long-term therapies to control CLso and CLas. As short-term therapies, we identified eight bioactive compounds that are quite diverse in their chemical structures and modes of action (Fig. 4, Supplementary Dataset 1). Toward long-term solutions, we identified six broad-spectrum AMPs and one positive and one negative plant defense regulator (\textit{Sinpr1} and \textit{Stinpr3}, respectively), that inhibit \textit{Candidatus Liberibacter} spp. in plant tissues. All these, either individually or in combinations, could be further deployed in the host plants to develop robust resistance against these phytopathogens.

We also show that the microbial hairy root system can be used to obtain mechanistic insights into gene function. Characterization of \textit{Sinpr1} and \textit{AtNpr1} function in potato hairy roots revealed that \textit{Npr1} confers tolerance to CLso by activating \textit{PR} gene expression, consistent with its known role as a transcriptional co-activator in other plant–microbe interactions. Interestingly, \textit{Sinpr1} and \textit{AtNpr1}-expressing hairy roots accumulate SA to a lesser degree than empty vector controls. There are multiple scenarios that could explain this. First, it is possible that SA accumulation in \textit{Npr1} overexpressors is directly (positively) correlated to the levels of \textit{Npr1}–expressing hairy roots. Untreated (UT) and tetracycline (TC)-treated hairy roots (at 250 and 500 ppm, respectively, for the CLso and CLas assays) were used as positive and negative controls. The bacterial titers were estimated by qPCR after 72 h of treatment with each compound and plotted relative to those of untreated samples (set to 100%). Error bars represent \( \pm \) standard error of mean (\( n = 5 \)). \( p \) Values were calculated by two-sample t test (one-tailed) relative to untreated samples.  

\textbf{Fig. 4} High-throughput screening and identification of small molecules that confer tolerance to \textit{Candidatus Liberibacter} spp.  
\textbf{a–c} Multi-well microbial hairy root culture plates were used to conduct high-throughput screening of \( \sim \)220 compounds (Supplementary Dataset 1). The efficacy data for nine selected hit compounds were re-assayed at 10 and 25 \( \mu \)M, respectively, in \textbf{a} CLso and \textbf{b, c} CLas hairy roots. Untreated (UT) and tetracycline (TC)-treated hairy roots (at 250 and 500 ppm, respectively, for the CLso and CLas assays) were used as positive and negative controls. The bacterial titers were estimated by qPCR after 72 h of treatment with each compound and plotted relative to those of untreated samples (set to 100%). Error bars represent \( \pm \) standard error of mean (\( n = 5 \)). \( p \) Values were calculated by two-sample t test (one-tailed) relative to untreated samples.  

\textbf{d–m} Chemical structures of d tetracycline and the nine hits: e aminocaproic acid (#1), f carbinoxamine maleate (#2), g chloroxylenol (#3), h chloropropamide (#4), i chlortetracycline (#5), j cinoxacin (#6), k cortisone acetate (#7), l duartin (#8), and m cyclopentolate hydrochloride (#9). The structures of the different chemical compounds were retrieved from the ChemSpider database (http://www.chemspider.com/) (last accessed on 15 October 2020). Source data underlying Fig. 4b, c are provided as a Source Data file.
features that underpin native plant–microbe interactions, aspects that cannot be replicated in axenic culturing systems. A growing number of studies have indicated the pivotal roles of roots, root–rhizosphere interactions and root–microbe composition in plant health and disease, including citrus health and HLB progression94–99. For instance, in the majority of presymptomatic citrus trees, roots appear to be the first site of CLas detection, preceding leaves86. CLas also is more abundant and more consistently detectable in roots than in shoots, making root a preferred source tissue in which to perform disease diagnostics tests. Furthermore, roots act as the reservoir for CLas between periods of foliar flushing88 and to endure the seasonal fluctuations of high temperatures in summer months84. Relatively few groups have studied CLso root infections, but the levels and distribution of CLso are greater in roots than shoots, much as for CLas91. Given the significance of roots to CLas and CLso pathology, we posit that the hairy-root-based bioassays described here are more biologically relevant than surrogate pathogen and/or axenic culture systems74–76. This is also underscored by reproducible screening results with Arabidopsis NPR1, and a broad-spectrum bactericide (tetracycline) (Figs. 2 and 4), which inhibited Candidatus Liberibacter spp. in the microbial hairy root bioassays, much as they did in planta (Fig. 5)65,73. Moreover, hairy root bioassays circumvent challenges associated with inefficient delivery of compounds into the phloem of whole plants that can result in inconclusive results65. For all of these reasons, this approach is very well suited both for primary screening of potential antimicrobial therapies and for narrowing down potential hits.

The hairy-root propagation and antimicrobial screening approaches are relatively straightforward and do not require specialized equipment or apparatus11–19. The only prerequisite is that the host plant or variety should be amenable to R. rhizogenes-mediated hairy root transformation92. Depending on the host plant, the microbial hairy roots can be maintained for ~6 weeks (e.g., CLso-potato hairy roots) or >12 months (e.g., CLas-citrus hairy roots) (Fig. 1g, h). In our experience, this is mainly determined by the lifestyle of the host plant (annual vs. perennial), as well as its degree of tolerance for the pathogen. The latter is relevant because the hairy roots are living tissue matrices, and they eventually succumb to pathogen pressure and show vascular degeneration symptoms (Supplementary Fig. 9) in a manner similar to the host plant roots43,44. Nevertheless, the hairy root propagation timeframes we have demonstrated are more than sufficient to accomplish various antimicrobial screening experiments.

Another advantage of using the microbial hairy root system is that the screening can be accomplished four to six times faster than with conventional approaches68,69,71,72. For instance, small molecules or peptides can be screened using in vitro hairy root assays in ~72 h (Fig. 4), vs. several weeks to months when using conventional in planta approaches (Fig. 5). For evaluating larger proteins, in size range of NPR1, or those forming biochemical
complexes as in CRISPR–Cas9, we suggest using the genetic-based hairy root transformation approach (Fig. 2 and Supplementary Fig. 4). Caution must also be exercised to ensure that the small peptides being tested are appropriately folded and/or have the necessary post-translational modifications when produced via synthetic or recombinant techniques. Nevertheless, evaluation of genes/CRISPR targets using genetic constructs still can be accomplished in ~30 days in potatoes and ~90–120 days in citrus after transformation vs. several months to years, respectively, for conventional in planta techniques, thus facilitating faster screening and discovery of therapies. In conclusion, microbial hairy roots offer a versatile and robust system for the discovery of multiple therapies effective against fastidious pathogens. We suggest that small-molecule inhibitors, AMPs, and genes discovered in this study, either individually or in combination, can be further utilized in plants to control potato ZC and citrus greening diseases.

Methods

Plant and insect maintenance and disease challenges. Potato (Solanum tuberosum L. var. Atlantic) and tomato (Solanum lycopersicum L. var. annc) plants were propagated in a professional growth mix (Metro-Mix 360, Sun Gro Horticulture, Canada) and maintained in a growth chamber at 21–22 °C with a 14-h/10-h light/dark photoperiod and 50% relative humidity. Bacterica cockerelli (Sûc) psyllid colonies that were either CLS0 positive (haplotype B, the most virulent and prevalent haplotype in Texas) or negative were collected from citrus grown at the Bugdorm, Taiwan. The presence and haplotype of CLS in the psyllid colonies were confirmed using PCR, with primers specific to the unique sequence repeat (SSR) loci of CLS0 (SSR-F and SSR-R; Supplementary Table 1). To generate CLS0-infected plants, 1-month-old live plants were challenged with 1 mL of a 1:200 solution of 100 μL of infected psyllids per plant harboring CLS0. The psyllids were released onto the plants in an enclosed organza bag tied to a lower branch with fully expanded leaves and were allowed to feed for 1 week. Subsequently, the bags and psyllids were removed from the plant, and the unchallenged upper branches were sampled periodically using PCR, as described below, to confirm the systemic spread of CLS0 in the plants, which typically could be detected at around 14 days post-challenge.

Microbial hairy root production and molecular diagnostics. Both ex vivo and in planta approaches were evaluated to generate potato and tomato hairy roots using CLS0-infected plants as an inoculum. In the ex vivo approach, branches were excised from either healthy or CLS0-infected plants 21 days post-challenge and then dipped into a fresh culture of R. rhizogenes (American Type Culture Collection strain ATCC 58211) and vacuum infiltration was used to introduce the bacterial genome into the tissues. After 14 days in cultivation, leaves were surface sterilized with 2% bleach, followed by a 20 min rinse in sterile distilled water and a 20 min rinse in Sorenson phosphate buffer, and post-sterilization, leaves were washed in Sorenson phosphate buffer for 4 h at room temperature. To generate the citrus hairy roots, CLS0-positive branches, confirmed using PCR, were collected from either HLB-graft-infected or naturally infected citrus trees (Rio Red grapefruit [C. × aurantium L. var. racemosa (Risso & Pot.)] or sour orange [C. × aurantium L.]) maintained in the research plots of Texas A&M University Kingsville Citrus Center, Weslaco, TX, USA (26°10′N, 98°57′W). Alternatively, uninfected citrus explants from glasshouse-grown trees could be used for hairy root induction, followed by exposure to the Asian citrus psyllid (Diaphorina citri) to transmit CLS0, as it takes longer to passage CLS0 into hairy roots by the later approach. In either approach, for hairy root induction citrus explants were inoculated with R. rhizogenes using a syringe, after which the stems were wrapped with foil to maintain humidity and placed in a growth chamber. To generate the potato hairy roots, each potato explant was excised, and the cut ends were immersed in a solution of (OD600 = 0.5), followed by vacuum infiltration for ~30 min at ~30 psi. All explants were subsequently transferred to the inert vermiculite matrix in a humidity dome tray with frequent misting to maintain high humidity. In the in planta approach, R. rhizogenes (OD600 = 0.5) was directly inoculated into the stems of live plants using a syringe, after which the stems were wrapped with foil to maintain humidity and placed in a growth chamber. To confirm the systemic presence and copy number of either the CLS0 or the rollerB gene40 were reported per 1 ng of DNA extracted from healthy hairy roots for further qPCR analysis. The qPCR analysis was performed as described in the material and methods. The log10 of the copy number was plotted against the threshold cycle (Ct) to establish a standard curve (Supplementary Fig. 7). The standard curve regression equations below, to confirm the systemic spread of CLS0 in the plants, which typically could be detected at around 14 days post-challenge.

Transmission electron microscopy. Healthy and CLS0/CLS0-infected hairy roots were surface sterilized with 2% bleach, followed by five washes with sterile distilled water. Hairy-root tips (~3 cm) were excised and fixed with 3% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for overnight at 4 °C. Fixed roots were washed in Sorenson phosphate buffer, and post-fixed in 2% osmium tetroxide (w/v) in the same buffer for 4 h at room temperature. All samples were washed in Sorenson phosphate buffer and dehydrated in 10% acetone (v/v), followed by infiltration and embedding in Spurr’s resin over 3 days. Embedded roots were sectioned (100 nm) and mounted on a formvar-coated copper grids (200 mesh), and stained with 2% aqueous uranyl acetate (w/v) and Reynolds lead citrate. Samples were examined under a FEI Morgagni 268 transmission electron microscope (FEI, Netherlands).

SA quantification. One hundred milligrams of hairy root tissue was mixed with 500 μL 2% sodium thiosulfate extraction buffer (1% propanol/water/HEC [2:1:1:0.02 vol/vol]) containing 500 nM dCas9 (Sigma-Aldrich, St. Louis, MO, USA). Zirconia beads were added to the samples, and they were homogenized at 6000 rpm for 1 min at room temperature in a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The samples were then agitated for 30 min at 4 °C under darkness. Afterwards, 500 μL dichloromethane was added to each sample and the samples were again agitated for 30 min at 4 °C in darkness before being centrifuged at 17,000g for 5 min. The lower organic layer of each sample was transferred to a glass vial for evaporation under nitrogen gas. Samples were resuspended in 150 μL methanol, transferred to 1.5-mL microcentrifuge tubes and stored in a –20 °C freezer overnight. The samples were then centrifuged at 5,000×g for 15 min, and the supernatant of each sample was transferred into an autosampler vial for LC–MS/MS analysis. An Ascentis Express C-18 Column (3 cm × 2.1 mm, 2.7 μm) (Sigma-Aldrich, St. Louis, MO, USA) was coupled to an API 3200 LC–MS/MS (Sciex, Framingham, MA, USA) using electrospray ionization with multiple reaction monitoring was used to detect [m/z = 140.0 ± 10] of the infected roots (C1-04O1) and d9-SV/Δ3 (C1-03O1). The injection volume was 10 μL, and the 500 μL mobile phase consisted of Solution A (0.2% acetic acid in water) and Solution B (0.2% acetic acid in acetonitrile) with a
gradient consisting of (time—%B): 0.5–10%, 1.0–20%, 21.0–70%, 24.6–100%, 24.8–10%, 29–stop. SA was quantified by comparison against the isotopically labeled internal standard, d6-SA (St. Louis, MO, USA).

Antimicrobial efficacy testing using microbial hairy roots. The phylogenetic analysis of Arabidopsis, potato, and tomato NPR genes was performed using the Maximum Likelihood statistical method and Jones–Taylor–Thornton substitution model using MEGA89. For the transformation of the antimicrobial genes and CRISPR constructs into the Cls-adapter or Cls-citrus hairy roots, binary vectors containing either AINPR1-, SINPR1-, or SINPR3-CRISPR-Cas9 constructs or the appropriate empty vector (pGFP alone) were transformed into R. rhizogenes and used for the induction of hairy roots using pre-injected explant source material, as described above. About 30 days (for Cls-potato) and 90 days (Clas-citrus) after the transformation, the hairy roots were visually screened for GFP under a fluorescence dissection microscope. Three independent biological replicates comprising randomly selected positive hairy roots, were collected from the controls and treatments, flash-frozen in liquid nitrogen and stored at −80°C until use for molecular diagnostics.

High-throughput screening of small molecules was performed using ~220 compounds from the Spectrum bioactive collection (Microsource Discovery Systems, Inc., CT, USA)8,9. Primary screening was performed with two biological replicates of each compound (10 µM in aqueous DMSO, 0.1% v/v), along with untreated and solvent-alone negative controls (DMSO, 0.1% v/v) against Cls. Briefly, ~200 mg of the Cls-containing hairy roots were surface sterilized with 2% bleach and then washed five times with sterile distilled water. The hairy roots were transferred into multi-well plates containing Gamborg’s B-5 medium with 1% sucrose. The compounds were then added to the plate, and the medium was vacuum infiltrated at ~20 psi to facilitate the penetration of the molecules into the hairy root matrixes. The hairy roots were incubated on a 3D platform rotator (Thermo Scientific) at 25°C and 80 rpm in the dark for 48 h. The hairy root tissue samples were sampled after 72 h, flash-frozen in liquid nitrogen and stored at −80°C in preparation for further processing. The most effective compounds identified in the primary screening were retested at both 10 and 25 µM, using five biological replicates each, against both Cls and Clas, along with tetracycline as a positive control. The structures of the different chemical compounds were retrieved from the ChemSpider database (http://www.chemspider.com/) (last accessed on 15 October 2020). Direct protein infiltration assays were performed in the same manner as for small molecules, but with purified recombinant peptides (Shifa Biomedical Corp., PA, USA) corresponding to AMP2 and AMP5.

In planta efficacy trials. Three-week-old potato (var. Atlantic) plants were challenged with Cls-containing hydroids. Briefly, five hydroids carrying Cls were restricted to the lower branch of each plant and centrifuged using an organza drawing bag tied around the branch. After 1 week, the bags containing hydroids were clamped off the plant to end inoculation and prevent the escape of the newly emerging hydroids and/or nymphs. All the challenged plants were treated with either 0 (untreated), 10 or 25 µM of the selected compounds and tetracycline (250 µM) twice a week for 4 weeks. To enhance penetration into leaf tissues, the hydroid-containing hairs were clipped off the plant to end inoculation and prevent the escape of the newly emerging hydroids. Four biological replicate plants were monitored weekly, and tissue samples were collected (as described above) at 28 dpi (days post inoculation) for molecular diagnostics as described below.

Plasmid design and amplicon sequencing. To obtain the full-length NPR1 coding sequences, the total RNA was extracted from 3-week-old Arabidopsis (A. thaliana; ecotype Col-0) and tomato seedlings and used for CDNA synthesis with Superscript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer’s instructions. The Arabidopsis NPR1 (1,783-bp) (AF152271) and tomato NPR1 (1,731 bp) coding sequences (Supplementary Table 1) were amplified with Plasmid DNA polymerase (New England Biolabs, Ipswich, MA, USA) from the template CDNA using primers containing the EcoRI restriction site at the 5′ end of the forward primer, and BamHI site at the 3′ end of the reverse primer (Supplementary Table 1). The PCR products were then digested with EcoRI and BamHI, and cloned into a binary vector containing the GFP reporter gene (pBin19-mGFP) under the control of a double-enhanced CaMV 35S promoter (DE35S-P) and the 35S terminator (35S-T). All the nucleotide sequences were confirmed using Sanger DNA sequencing before being transformed into R. rhizogenes to generate hairy roots.

For the CRISPR experiments, multiple single guide RNA (sgRNA) targets were designed for the GFP and NPR3 gene sequences (PGSC0003DMG04000281) using the CRISPR-P design toolset. The sequences of the top sgRNA hits are provided in Fig. 3a and Supplementary Fig. 4a. The respective sgRNA targets were then synthesized and transfected into tomato and potato expressing GFP under a double-enhanced CaMV 35S promoter (pBIN-mGFP) under the control of a double-enhanced CaMV 35S promoter (DE35S-P) and the 35S terminator (35S-T). The sequences of the top sgRNA hits are provided in Fig. 3a and Supplementary Fig. 4a. The respective sgRNA targets were then synthesized and transfected into tomato and potato expressing GFP under a double-enhanced CaMV 35S promoter (pBIN-mGFP) under the control of a double-enhanced CaMV 35S promoter (DE35S-P) and the 35S terminator (35S-T). The sequences of the top sgRNA hits are provided in Fig. 3a and Supplementary Fig. 4a. The respective sgRNA targets were then synthesized and transfected into tomato and potato expressing GFP under a double-enhanced CaMV 35S promoter (pBIN-mGFP) under the control of a double-enhanced CaMV 35S promoter (DE35S-P) and the 35S terminator (35S-T). The sequences of the top sgRNA hits are provided in Fig. 3a and Supplementary Fig. 4a. The respective sgRNA targets were then synthesized and transfected into tomato and potato expressing GFP under a double-enhanced CaMV 35S promoter (pBIN-mGFP) under the control of a double-enhanced CaMV 35S promoter (DE35S-P) and the 35S terminator (35S-T). The sequences of the top sgRNA hits are provided in Fig. 3a and Supplementary Fig. 4a. The respective sgRNA targets were then synthesized and transfected into tomato and potato expressing GFP under a double-enhanced CaMV 35S promoter (pBIN-mGFP) under the control of a double-enhanced CaMV 35S promoter (DE35S-P) and the 35S terminator (35S-T). The sequences of the top sgRNA hits are provided in Fig. 3a and Supplementary Fig. 4a. The respective sgRNA targets were then synthesized and transfected into tomato and potato expressing GFP under a double-enhanced CaMV 35S promoter (pBIN-mGFP) under the control of a double-enhanced CaMV 35S promoter (DE35S-P) and the 35S terminator (35S-T).
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Author contributions
K.K.M. and S.I. developed the concept and designed the experiments. S.I., M.R., S.P., P.N., M.G., R.R., D.R., C.L., Z.G., and D.A. performed the experiments and conducted the data analysis. S.I., A.L., M.K., M.S., I.B., C.A., M.S.I., and K.K.M. supervised the study. S.I., M.R., and K.K.M. prepared the paper and all authors reviewed the paper.

Competing interests
The Texas A&M University System has filed patent applications for the microbial hairy root system [15/353,645 and 16/54,178, status pending]. K.K.M., S.I., M.R., S.P., and P.N. are co-inventors on the related disclosures and patents. Southern Gardens Citrus (Glewiston, FL) has licensed rights in these patents and patent applications from The Texas A&M University System. A disclosure of invention for the compounds screened using this system and a U.S. provisional patent application (63031962) has been submitted. Southern Gardens Citrus also provided matching funds to the Foundation for Food and Agricultural Research New Innovator Award (2018-534,299) to K.K.M. Co-author M.S.I. (Director of Research and Business Development, Southern Gardens Citrus) contributed to the study design for screening antimicrobial peptides, data analysis, and preparation of the manuscript. The remaining authors declare no competing interests.

Additional information
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