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Small RNA Profiling Reveals Phosphorus Deficiency as a Contributing Factor in Symptom Expression for Citrus Huanglongbing Disease

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ABSTRACT Huanglongbing (HLB) is a devastating citrus disease that is associated with bacteria of the genus '*Candidatus* Liberibacter' (*Ca.* L.). Powerful diagnostic tools and management strategies are desired to control HLB. Host small RNAs (sRNA) play a vital role in regulating host responses to pathogen infection and are used as early diagnostic markers for many human diseases, including cancers. To determine whether citrus sRNAs regulate host responses to HLB, sRNAs were profiled from *Citrus sinensis* 10 and 14 weeks post grafting with *Ca.* L. asiaticus (Las)-positive or healthy tissue. Ten new microRNAs (miRNAs), 76 conserved miRNAs, and many small interfering RNAs (siRNAs) were discovered. Several miRNAs and siRNAs were highly induced by Las infection, and can be potentially developed into early diagnosis markers of HLB. miR399, which is induced by phosphorus starvation in other plant species, was induced specifically by infection of Las but not *Spiroplasma citri* that causes citrus stubborn—a disease with symptoms similar to HLB. We found a 35% reduction of phosphorus in Las-positive citrus trees compared to healthy trees. Applying phosphorus oxyanion solutions to HLB-positive sweet orange trees reduced HLB symptom severity and significantly improved fruit production during a 3-year field trial in south-west Florida. Our molecular, physiological, and field data suggest that phosphorus deficiency is linked to HLB disease symptomology.

Key words: Huanglongbing; small RNA; miRNA399; disease diagnosis; phosphorus deficiency.

INTRODUCTION

Huanglongbing (HLB), also called citrus greening, is one of the most destructive citrus diseases threatening the global citrus industry. HLB was first reported in Asia about a century ago, then in South Africa and South America (Gottwald, 2010). In the Americas, HLB was discovered in Sao Paulo, Brazil, in 2004, and subsequently in Florida and Texas, and more recently in California in 2012 (Stokstad, 2012). HLB is associated with phloem-restricted Gram-negative bacteria that belong to the genus *Candidatus* Liberibacter (*Ca.* L.) and are vectored by insects of the Psyllidae family (Bove, 2006). At least three members of the *Ca.* L. genus have been associated with HLB: *Ca.* L. africanus (Laf), *Ca.* L. americanus (Lam), and *Ca.* L. asiaticus (Las). In the US alone, annual HLB management-related expenses have totaled about 1.2 billion dollars (Perez et al., 2011), and it has been estimated that HLB management has increased citrus production costs by 40–50% in affected areas (Stokstad, 2012). Current strategies for HLB management consist of control of the psyllid vectors, identification and removal of infected trees, and the use of pathogen-tested citrus nursery stocks for replants and new orchards.

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Although advances have been made in sensitive HLB diagnostics (Lin et al., 2010), the current polymerase chain reaction (PCR)-based techniques are dependent on detection of the DNA of the presumed causal bacteria and are reliable when bacterial cells or DNA is present in the tested sample. In some cases, disease symptoms are not present. This can be problematic sometimes, since the titer and distribution of the HLB-associated bacteria within citrus trees, types of tissue infected, and degree of disease progression are variable, especially in recently infected trees (Bove, 2006; Li et al., 2007; Tatineni et al., 2008; Li et al., 2009; Folimonova and Achor, 2010). Thus, it is important to develop new diagnostic markers that can detect infection independent of pathogen presence in a tested sample and prior to symptom development.

Symptoms of HLB include blotchy mottled leaves, sections of yellow and underdeveloped vegetative growth, premature fruit drop, and in some cases small, off-flavored fruit with aborted seeds. The mechanisms of HLB pathogenesis and host responses are largely unknown. In the citrus relative *Poncirus trifoliata* and some of its hybrids that are used as rootstocks, tolerance has been observed (Folimonova et al., 2009; Albrecht and Bowman, 2011), but no HLB-resistant citrus scion cultivars have been identified. Many genes are differentially expressed between healthy and Las-positive plants (Albrecht and Bowman, 2008, 2012), manifesting the host's effort against the bacterial infection.

Previous studies in systems such as *Arabidopsis* have demonstrated that some host small RNAs (sRNAs) are rapidly and specifically induced by pathogen infection and contribute to the gene expression reprogramming in host defense responses (Katiyar-Agarwal et al., 2006; Navarro et al., 2006; Katiyar-Agarwal et al., 2007; Wang et al., 2011; Zhang et al., 2011b). Similarly, specific panels of human miRNAs have been used for early diagnosis of many human diseases, including various cancers (Iorio and Croce, 2009; Fabbri, 2010; Ferracin et al., 2010). In this study, we profiled sRNAs from both Las-positive and healthy sweet orange (*Citrus sinensis* (L.) Osbeck) plants and identified a panel of microRNAs (miRNAs) and small interfering RNAs (siRNAs) that were highly induced by Las infection. These sRNAs can potentially be developed into early diagnosis markers. Induction of miR399 in Las-positive trees corresponds to our finding that Las-positive plants had severe phosphorus (P) deficiency. MiR399 induction is associated with phosphorus deficiency in *Arabidopsis* and other species (Fujii et al., 2005; Bari et al., 2006; Lin et al., 2008). We further demonstrated that application of phosphorous solutions to Las-positive plants significantly reduced HLB symptoms and improved fruit yield in a three-year field trial.

RESULTS

Profiling sRNAs in Las-Positive and Healthy Sweet Orange

To study the expression of citrus sRNAs in response to HLB, we grafted greenhouse-grown healthy sweet orange plants with healthy or Las-positive bark or leaf pieces. Both donor and receptor trees tested negative for other graft-transmissible pathogens of citrus. Samples were collected at 10 and 14 weeks post inoculation/grafting (wpi) for small RNA profiling. Because Las is not always detectable by PCR at such early stages of infection, leaves were collected continuously at later time points for PCR examination to ensure that the tissue used for small RNA libraries was from trees that became Las-positive. sRNAs ranging from 18 to 28 nucleotides (nt) were isolated, cloned, and sequenced (Supplemental Table 1). To map the sRNA reads to expressed citrus genes, 582334 citrus EST sequences from NCBI (www.ncbi.nlm.nih.gov/) were assembled. Deep-sequencing data revealed 810K, 834K, 909K, and 831K total reads from the healthy 10 wpi, Las-infected (HLB-positive) 10 wpi, healthy 14 wpi, and Las-infected 14 wpi libraries, respectively. Among them, 465K, 459K, 530K, and 469K reads were mapped to assembled citrus transcripts, while allowing a maximum of two mismatches. According to their sequence homology and secondary precursor structures, the identified *C. sinensis* sRNAs were grouped into miRNAs and siRNAs. Reads were classified as miRNAs if they matched conserved miRNAs (allowing up to two mismatches) from other plant species (top 20 plant species in miRBase; Supplemental Table 2), or if their precursor RNAs could form stem-loop structures. Our data showed that about 38% of the sRNA were miRNAs, while the other 62% were siRNAs due to lack of miRNA characteristics (Supplemental Table 1). We named them csi-miRNAs and csi-siRNAs in this study, since they were identified from *C. sinensis*. As in other species, most of the csi-miRNAs (>95%) were 21 nt in length and predominantly favored a U (Uridine; >98%) at the 5′ end (Supplemental Figure 1A and 1B), while csi-siRNA species were mainly 24 nt (58%) and 21 nt (23%) in length (Supplemental Figure 1A), most of which started with either an A (Adenine; >40%) or a U (>30%) (Supplemental Figure 1B). We found 76 conserved csi-miRNAs and focused on 27 of them that had more than 10 reads/million total matched reads in this study (Supplemental Table 2). We also found 10 new csi-miRNAs (csi-miRNA5001 to csi-miRNA5010) that have not been reported previously and have miRNA characteristics with clear stem-loop precursor structures (Supplemental Table 3). The majority of the novel csi-miRNAs, such as csi-miRNA5001, 5002, 5003, 5004, 5005, and 5009, could be detected by small RNA Northern blot analysis (Supplemental Figure 2).

Identifying Differentially Expressed csi-sRNAs upon Las Infection

Some sRNAs in *Arabidopsis* and other species are specifically elicited upon pathogen infection and play an important role in regulating host responses (Katiyar-Agarwal et al., 2006; Navarro et al., 2006; Katiyar-Agarwal et al., 2007; Katiyar-Agarwal and Jin, 2010; Zhang et al., 2011b). To investigate whether expression of csi-sRNAs is altered upon Las infection, we compared csi-sRNA levels in healthy and Las-positive samples. By using a fourfold-change cut-off, we found that csi-miRNAs such as csi-miR159, 399, and 393 were induced

at both 10 and 14 wpi, whereas csi-miR396 was suppressed at both 10 and 14 wpi (Figure 1A and 1B). In addition, the newly identified csi-miR5010 was induced fourfold at 10 wpi, while csi-miR408, 171, 403, 167, 3951, and the newly identified csi-miR5008 were down-regulated at 10 wpi (Figure 1A). csi-miR160, 394, 398, and the new csi-miR5010 and 5001 were down-regulated at 14 wpi (Figure 1B). Because the deep-sequencing method involved adaptor-ligation and PCR steps that may introduce bias (Zhang et al., 2011a), we validated the deep-sequencing results with sRNA Northern blot analysis on several selected miRNAs and siRNAs. As shown in Figure 2, csi-miR399 and csi-miR159 showed significant induction in Las-positive samples, whereas csi-miR393 did not. Similarly, expression of a majority but not all of the down-regulated miRNAs identified from deep sequencing was validated by Northern blot analysis (Supplemental Figure 3), supporting the notion that caution should be taken when extracting quantitative information from deep-sequencing results.

Many csi-siRNAs were also differentially expressed between healthy and Las-positive samples (Figure 1C and 1D, and Supplemental Table 4). By using a fourfold-change cut-off, we found that 16 siRNAs were up-regulated in Las-positive samples compared to healthy controls at both 10 and 14 wpi, whereas six and three siRNAs were induced only at 10 and 14 wpi, respectively. Twelve siRNAs were down-regulated by HLB in both 10 and 14 wpi, and nine siRNAs were only down-regulated in one of the two time points. Computational analysis predicted potential gene targets for many of these siRNAs, including genes that specify disease resistance proteins, kinases, and transcription factors, which are likely to regulate gene expression during plant responses to Las infection (Supplemental Table 5).

Induction of csi-miR399 Is Specific to HLB Infection

To determine whether the induction of csi-miR399 and csi-miR159 was specific to HLB, we tested their expression after inoculation with *Spiroplasma citri*, a phloem-limited bacterial pathogen that causes citrus stubborn disease. Although citrus stubborn has symptoms similar to HLB, such as chlorotic foliage patterns, lower fruit yield, and smaller fruits with aborted seeds (Daniels, 1983), no noticeable alteration of csi-miR399 and csi-miR159 expression levels was observed (Figure 3A). In *Arabidopsis*, miR159 is induced by the bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 carrying an avirulent effector, *avrRpt2* (Zhang et al., 2011a), whereas the miR399 level was not altered (Figure 3B), suggesting that induction of miR399 is rather specific to Las infection but not to infections of other pathogens tested. We also examined csi-miR399 expression levels in other HLB-positive citrus varieties. 'Valencia' and 'Hamlin' sweet oranges and 'Duncan' grapefruit (*C. paradisi* Macfadyen) were graft-inoculated with Las and samples were collected 12–15 months after inoculation. 'Sun Chu Sha' mandarin (*C. reticulata* Blanco) trees were inoculated by Asian citrus psyllids (ACP, *Diaphorina citri*) and samples were collected 3–4 months post inoculation. As shown in Figure 2B, expression of csi-miR399 is induced 3–30-fold in all tested HLB-positive citrus trees, confirming the concurrence of Las infection and csi-miR399 induction.

Induction of csi-miR399 Led to the Discovery of Phosphorus Deficiency in Las-Positive Plants

MiR399 is induced by phosphorus (P) deficiency and is involved in the regulation of P. homeostasis and signaling in *Arabidopsis* (Fujii et al., 2005; Lin et al., 2008; Hsieh et al., 2009), rapeseed (Buhtz et al., 2008), and pumpkin (Pant et al., 2008). We examined the levels of mineral elements, such as phosphorus (%), potassium (%), zinc (ppm), and carbon (%),

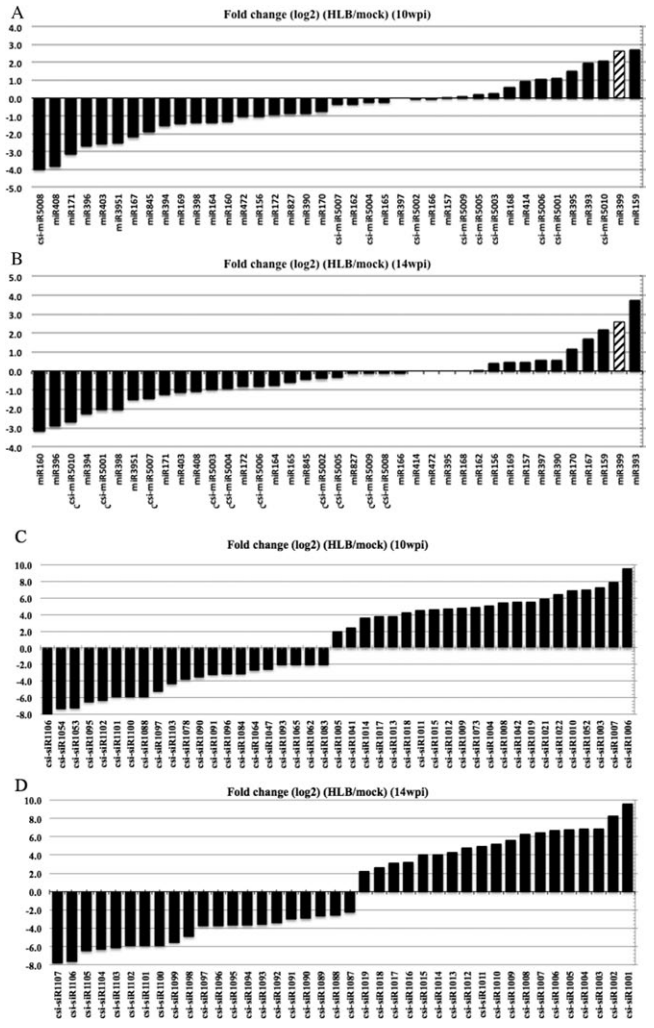

Figure 1. Some csi-miRNAs and csi-siRNAs Were Differentially Expressed in Las-Positive and Las-Negative Plants.
Expression levels at 10 wpi **(A)** and 14 wpi **(B)** are presented as fold changes (log2) in Las-treated samples over corresponding healthy samples. The csi-miRNA IDs are listed at the bottom while the fold change is at the left. '+1' indicates a twofold induction; '–1' indicates a twofold reduction; HLB, Las-infected; wpi, weeks post inoculation. Red bar highlights miR399, which is involved in *C. sinensis* P homeostasis.
(C, D) Relative expression levels of some csi-siRNAs that changed more than fourfold at 10 and 14 wpi, respectively.

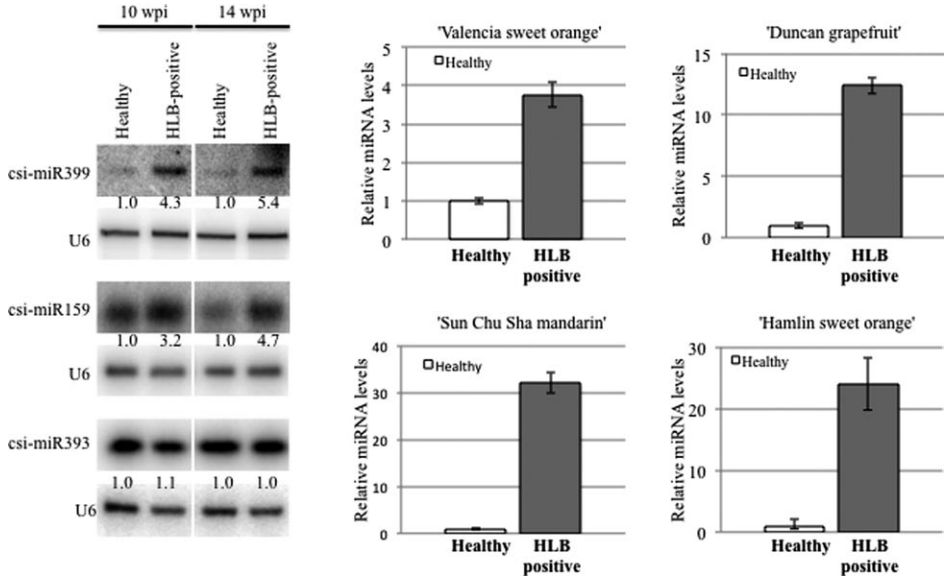

Figure 2. csi-miR399 and csi-miR159 Were Induced in Las-Positive Plants.
(A) Expression levels of some csi-miRNAs were examined by Northern hybridization. Total RNA was extracted from healthy and Las-infected plants at 10 and 14 wpi, as indicated at the top. 100 μg of total RNA (15 μg for miR159) was loaded into each well. Each blot was hybridized with a probe reverse complementary to the target small RNA, as indicated to the left. U6 was used as an internal control for quantification, shown below as relative abundance (RA). The RA of healthy samples was assigned to 1.
(B) Expression level of csi-miR399 was examined using real-time PCR. RNAs were extracted from Las-infected 'Valencia' sweet orange, 'Duncan' grapefruit, 'Sun Chu Sha' mandarin, and 'Hamlin' sweet orange plants. Relative transcript levels were measured by real-time RT–PCR. Csi-actin was used as an internal control. Standard deviations were calculated from three technical replicates. Similar results were obtained from three biological replicates.

in leaves from both healthy and Las-positive trees using optical emission spectrometry. The P level in leaves of Las-positive plants was only about 65% of that in healthy plants (Figure 4), suggesting that Las infection is associated with P starvation in the citrus plants, which in turn may contribute to the HLB symptom expression. Reduced P content in Las-positive plants was also observed in another study (Mann et al., 2012). We did not observe changes in the levels of potassium, carbon, and zinc, and similar results were obtained from a second set of healthy and Las-positive trees. Zinc deficiency was suspected because foliar symptoms typical of zinc deficiency are very often present on Las-positive trees.

Las-positive plants displayed significant induction of csi-miR399 and a reduced level of P. In *Arabidopsis*, miR399 targets a gene encoding a putative ubiquitin-conjugating enzyme (PHO2), which is important for degrading P transporter proteins (PT) (Fujii et al., 2005; Bari et al., 2006; Lin et al., 2008). To determine whether the regulatory mechanism of P homeostasis mediated by miR399 is conserved in citrus, we searched the NCBI EST database and found *C. sinensis* orthologs of *PHO2*,

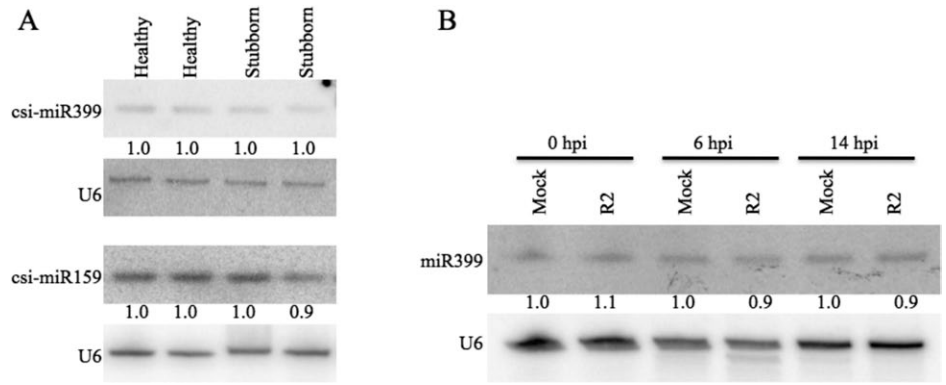

Figure 3. Infection of *Spiroplasma citri* or *Pseudomonas syringae* Had No Obvious Effect on the Expression of csi-miR399 in Citrus and *Arabidopsis*, Respectively.
(A) Total RNA was extracted from healthy and *S. citri*-infected *Citrus sinensis*, and subjected to Northern blot analysis as in Figure 2. Stubborn, *S. citri*-infected.
(B) In *Arabidopsis*, miR399 expression was not affected by *Pst* (*avrRpt2*) infection. R2, *avrRpt2* treated; hpi, hours post inoculation.

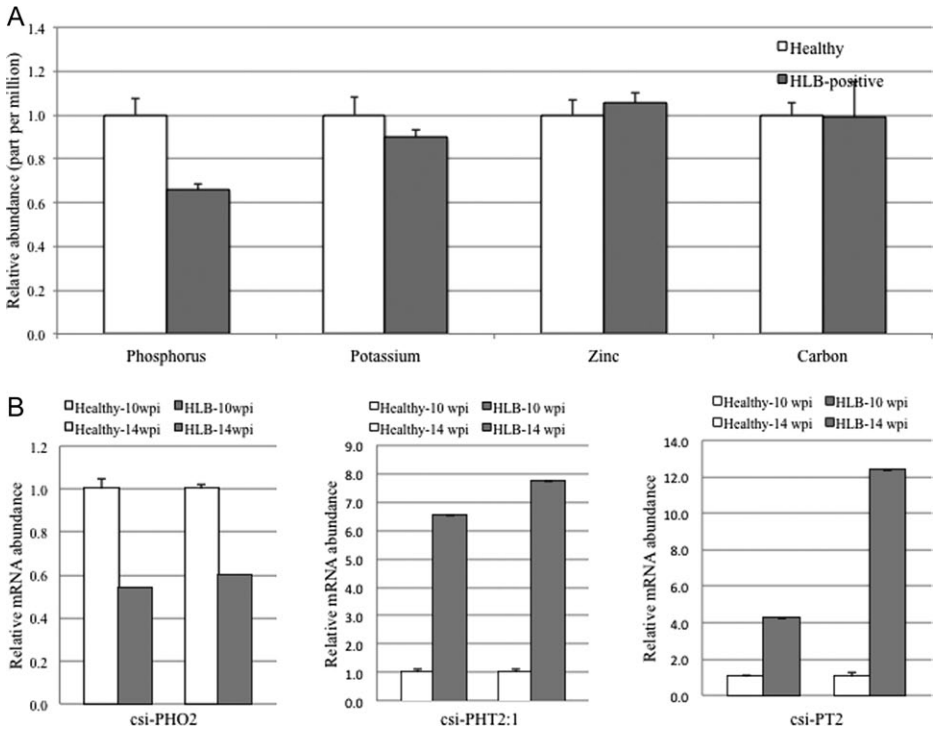

Figure 4. Las-Positive Plants Had Reduced P Content Compared to Negative Control Plants and miR399-Mediated Regulation of P Homeostasis Is Conserved in Citrus.

(A) Foliar levels of P (%), K (%), and Zn (ppm) were measured by ICP–OES spectrometry (IRIS 1000 HR Duo, ThermoElemental). Carbon (C; %) was analyzed using a NC 2100 analyzer (CE Elantech, Inc.). 15–20 leaves were collected from each of five sweet orange trees for analysis. Leaves from five non-infected trees were collected for comparison. Levels of each element in non-infected samples were assigned a value of 1. Experiments were repeated twice and similar results were obtained.

(B) Expression of *csi-PHO2* was down-regulated and PTs were up-regulated in Las-positive plants as compared with the Las-negative control. Expression levels of *csi-PHO2* (gi|38053156), *csi-PT2* (gi|56530333), and *csi-PHT2;1* (gi|219250130) relative to citrus *actin* (gi|45420693) were determined by quantitative real-time PCR (mean ± SE). Expression level of untreated samples is assigned to 1. Experiments were repeated three times and similar results were obtained.

PT1, and *PT2*. We examined the expression level of *csi-PHO2*, *csi-PHT2;1* (ortholog of *Arabidopsis PT1*), and *csi-PT2* in the Las-negative and Las-positive plants (Poirier and Bucher, 2002). As expected, expression of *csi-PHO2* was down-regulated and *csi-PHT2;1* and *csi-PT2* were induced in Las-positive plants as compared with the control, indicating that miR399-mediated regulation is conserved in citrus (Figure 4B).

Application of Inorganic P Solution Alleviated P Limitations and Improved the Appearance and Fruit Production of Las-Positive Trees

Since miR399-induction in Las-positive plants suppresses *PHO2* and subsequently induces PTs, thus pre-adapting trees to transport more P to compensate P deficiency, we hypothesized that applying P solutions to the Las-positive trees would reduce HLB symptoms and improve tree performance. We applied P solutions (56% phosphorous oxyanion and polyoxyanion solution, plus potassium nitrate (KNO_3) and citrus spray oil) to Las-positive Hamlin sweet orange trees in a field trial in southwest Florida three times per year beginning in 2008 for more than three years. These trees were confirmed as HLB-positive by real-time PCR (Supplemental Table 6). As controls, we also applied only KNO_3 and citrus spray oil (mock treatment) to other Las-infected trees in the same location. After 2 years of P solution treatment, the Las-infected trees displayed reduced HLB symptoms (Figure 5A and 5B). Compared with the mock-treated plants, the P-treated trees had a greener appearance and more vigorous growth. Fruit yield (mean; kilogram/tree) from five replicates of three-tree plots indicated that P treatment increased fruit yield approximately twofold compared with the mock treatment (Table 1). Our data showed that the reduced disease symptoms were not due to application of potassium and nitrogen since the mock-treated plants also received KNO_3. Even though P treatment did not cure HLB, namely trees were still Las-positive (Supplemental Table 6), continuous treatment has helped maintain tree productivity and vigor for 3 years until the time this manuscript was written.

DISCUSSION

As important global economic plants, citrus trees have been threatened by the very destructive disease HLB (Stokstad, 2012), which has now spread to almost all citrus-growing areas in the world. Early diagnosis of HLB is still challenging. It has

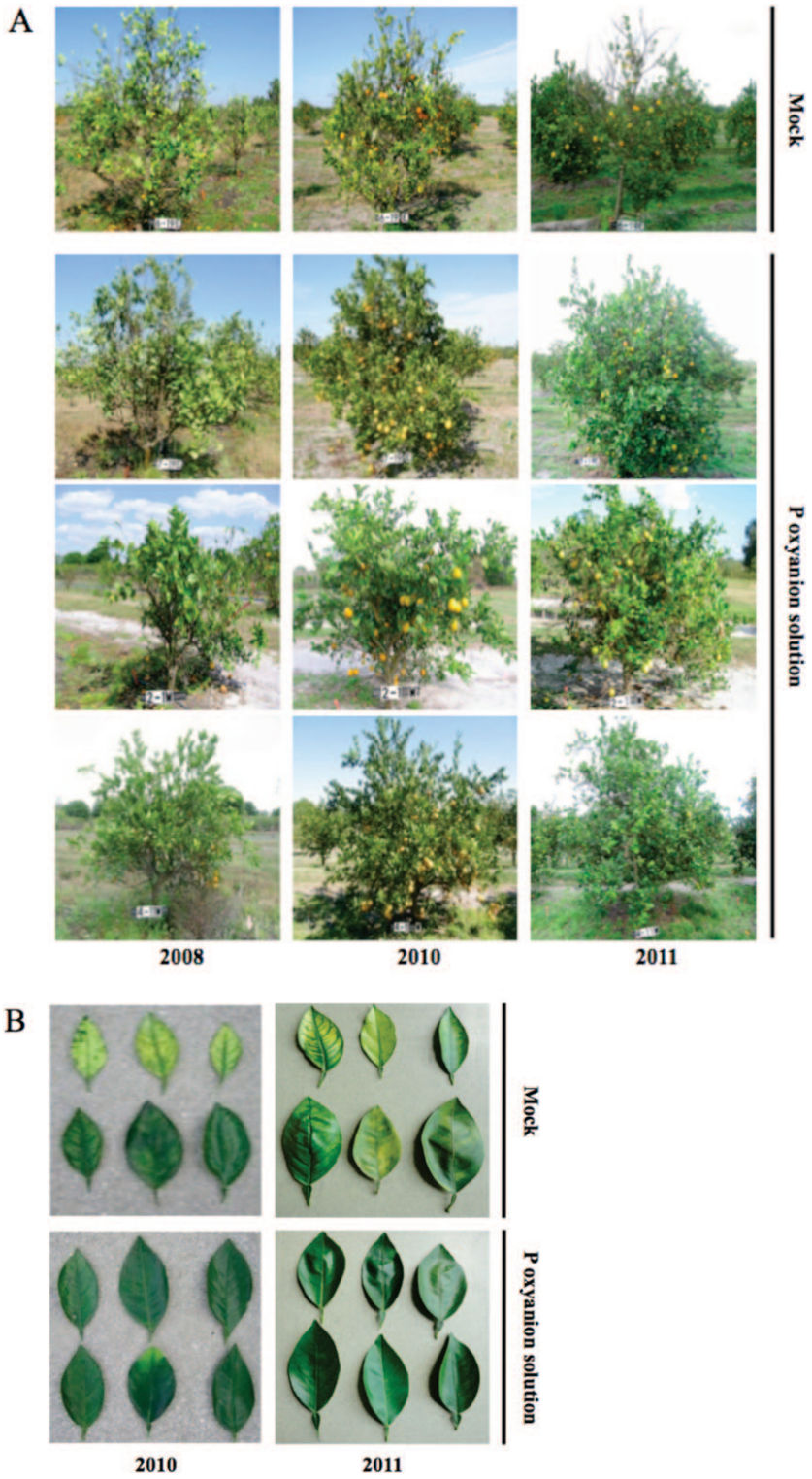

Figure 5. Applying P Solution Alleviated Disease Symptom of HLB and Increased the Fruit Yield of the Diseased Trees.
Phosphorus oxyanion solution was applied to Las-infected Las-positive sweet orange trees three times each year for 3 years. Application was synchronized with the initiation of new vegetative flushes in spring (March), summer (June), and fall (September). The treatment was a 3–18–20 liquid fertilizer of P oxyanion solution with 56% mono- and dipotassium salts of P acid (K-Phite, Plant Food Systems, Inc.), plus 3.8 kg of spray-grade potassium nitrate (KNO_3) and 18.9 L of 435 citrus spray oil. Control trees received KNO_3 and citrus spray oil only. Photos of trees with and without phosphorus oxyanion solution treatment are shown in **(A)**, and photos of random-picked leaves from control and phosphorus oxyanion solution-treated trees are shown in **(B)**. Treatments and the year the photos were taken are indicated on the right and bottom, respectively. Pictures were taken around the same time of the year and at the same location with similar camera settings.

Table 1. Fruit Yield of Huanglongbing-Positive 'Hamlin' Sweet Orange (*Citrus sinensis*) on Swingle citrumelo (*C. paradisi* Macf. X *Poncirus trifoliata* (L) Raf.) Rootstock after Phosphorus and Mock Treatments.

	Treatment[1]	
	Phosphorus	Mock
Year	Yield (kg)	Yield (kg)
2009	60.43 ± 1.22 a[2]	26.68 ± 5.19 b
2010	45.19 ± 1.44 a	21.39 ± 1.81 b
2011	49.13 ± 1.63 a	25.63 ± 1.42 b
Overall	51.58 ± 0.98 a	24.57 ± 1.867 b

[1] Phosphorus treatment: 3–18–20 liquid fertilizer of potassium solution of phosphorus oxyanion, with 56% mono- and dipotassium salts of phosphorus acid (K-Phite, Plant Food Systems, Inc.), plus 3.8 kg of spray-grade potassium nitrate (KNO_3), and 18.9 L of 435 citrus spray oil. Mock treatment: KNO_3 and spray oil at the same rate. Treatments were applied three times each year and timed with the initiation of the new vegetative flushes in spring (March), summer (June), and fall (September).

[2] Each value is the mean and standard deviation of five plot replicate plots of three trees per plot. Means in the same row followed by different letters were statistically significant at $p < 0.001$. Treatment effects were highly significant in each year and overall using a repeated measures general linear model analysis with years as the repeated factor.

been difficult to detect the associated bacteria *C.* L., because its concentrations and distribution in host plants is variable (Bove, 2006; Li et al., 2007, 2009; Folimonova and Achor, 2010). To prevent spread and achieve effective HLB management, accurate, rapid, and robust detection methodologies are highly desirable, especially pre-symptomatic diagnosis. Pathogen infection can induce rapid defense responses in host plants. Recent studies in both plant and animal systems have discovered that some small RNAs are rapidly and specifically induced by various pathogens and diseases (Katiyar-Agarwal and Jin, 2007; Fabbri, 2010; Ferracin et al., 2010). Human miRNAs have been developed for early diagnosis of many diseases, including various cancers (Iorio and Croce, 2009; Fabbri, 2010; Ferracin et al., 2010). In this study, we demonstrated the induction of a panel of sRNAs, including miR399, by Las infection. Further analysis of these sRNAs in other citrus-pathogen systems will help identify those that are induced specifically by HLB-associated bacteria, have low basal expression levels, and can move systemically, such as miR399, allowing them to be developed as early diagnosis biomarkers independently of pathogen DNA. This output is of great practical significance.

Citrus management costs have increased drastically during the last few years in HLB-affected areas, due in large part to the need for additional treatments to mitigate the effects of HLB (Stokstad, 2012). Therefore, more proactive management tools applicable to different sets of circumstances (i.e. HLB infestation levels, type of citrus industry and cultural practices, short- and long-term economic planning, etc.) must be developed. In the case of HLB exclusion and eradication, a panel of HLB-induced small RNAs and other host rapid responsive molecules could be developed into early diagnostic markers for HLB detection. Effective early diagnosis allows for more effective and accurate eradication practices which are typically one step behind the disease spread, since they are based on visual inspections for HLB symptoms and PCR targeting an elusive pathogen (Belasque et al., 2010). Application of P solutions to infected groves has resulted in improved growth and yields, as reported in China, Indonesia, and Florida (Lin, 1963; Pustika et al., 2008). Our data may have identified the molecular basis for these observations. The fact that miR399 is induced by P starvation in other (non-citrus) systems led us to examine the P level in Las-positive citrus. Indeed, the level of P was considerably reduced in Las-positive trees. Further analysis revealed that the miR399-mediated regulatory mechanism for P homeostasis is conserved in citrus. Las infection likely causes P deficiency that induces miR399 and subsequently down-regulates its target *PHO2*, which in turn causes increased expression of PTs. Up-regulation of PTs has equipped the plants for maximum P acquisition when available. Although no large-scale systematic studies have been performed and contradictory results have been reported for the effect of P on HLB-affected citrus trees (Gottwald et al., 2012; Mann et al., 2012), our results suggested that P deficiency is one important factor associated with the symptom expression of HLB. P plays an extremely important role in the cell because it is not only an essential structural element for DNA, RNA, phosphoproteins, phospholipids, and ATP, etc., but is also important for cellular processes, such as plant energy reactions and photosynthesis (Khorana, 1979; Brooks, 1986; Hammond and White, 2008). Although further work is needed, this study suggests that additional P application may help the infected trees to partially overcome the effects of Las infection and temporarily improve fruit yield. However, it is important to recognize that P application will not eliminate Las infection or significantly reduce the bacterial titer, and the surviving P-treated trees could still serve as sources of inoculum.

Finally, the most attractive long-term, sustainable, and desirable means of disease control has always been the utilization of natural host plant defense mechanisms. Although there is no known complete resistance in *Citrus* spp., tolerant citrus cultivars that have very mild or no obvious disease symptoms and reduced bacterial titer have been identified (Folimonova et al., 2009; Albrecht and Bowman, 2011). Our study contributed to the understanding of the molecular mechanisms of citrus natural defense against HLB and provided new insights into host responses to Las infection that may direct the engineering of stronger host resistance.

METHODS

Plant Material and Inoculation

To examine sRNA from healthy and Las-infected citrus, 2-year-old greenhouse-grown 'Navel' sweet orange scions on Cleopatra mandarin (*C. reticulata*) rootstocks were inoculated

by grafting a combination of three bark pieces or leaf pieces onto the rootstock portion of each plant. Nineteen plants were inoculated with tissue from infected greenhouse-grown 'Valencia' plants that were PCR-positive for *Ca.* L. asiaticus and symptomatic for HLB (Las-infected). As controls, five plants were mock-inoculated with pathogen-free tissue pieces obtained from healthy greenhouse-grown 'Valencia' plants. All plant material used for inoculation was confirmed to be free of *Citrus tristeza virus* by RT–PCR analysis. Plants were kept under natural light conditions at a temperature of 21–28°C. Las detection and HLB symptom evaluation were conducted in 4-week intervals beginning at 6 wpi. For sRNA library construction, three healthy plants (mock) and three Las-infected plants with similar patterns of disease progression were selected.

In the follow-up experiments, plants of additional citrus varieties namely 'Valencia' and 'Hamlin' sweet oranges, 'Duncan' grapefruit, and 'Sun Chu Sha' mandarin grown on Volkamer lemon (*C. limonia* Osbeck 'Volkameriana') rootstocks were used. 'Valencia' and 'Hamlin' sweet oranges and 'Duncan' grapefruit trees were graft-inoculated with Las-containing budwood as described above and samples were collected 12–15 months after inoculation. 'Sun Chu Sha' mandarin trees were first placed in a contained insect room with Las-positive ACP in the Citrus Research and Education Center in Lake Alfred, FL, for 4–5 weeks and kept in an ACP-free growth room for another 3–4 months before sample collection.

To examine *S. citri*-induced sRNAs, three budsticks, each with three nodes, were collected from *S. citri* -infected 'Spring Navel' sweet orange and side-grafted onto Madam Vinous sweet orange receptor plants in August 2008. The inoculated plants were kept seasonally in an air-conditioned greenhouse from October to May at 26–29°C and in a photoperiod of 16:8 (light/dark). From May to October, plants were maintained in a screen house under ambient conditions in Parlier, CA. The presence of *S. citri* was confirmed by culturing and by PCR using primers specific to the *S. citri* spiralin gene (Foissac et al., 1996; Yokomi and Sisterson, 2011). Three healthy and three infected plants were used for sRNA analysis.

To examine the expression level of miR399 in *Arabidopsis thaliana*, 4-week-old *Arabidopsis* ecotype Col-0 plants were leaf-infiltrated with *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 carrying an avirulent effector, *avrRpt*2, at a concentration of OD_{600} = 0.02 (suspended in 10 mM $MgCl_2$, equivalent to 2×10^7 cfu ml^{-1}) for 0, 6, or 14 h. For treatment control, 10 mM $MgCl_2$ was used.

sRNA Library Construction

Total RNA was extracted and resolved by a denaturing 14% polyacrylamide gel, from which 18–28 nt RNA fragments were recovered. The purified fragments were ligated to a 5' adaptor (GUUCAGAGUUCUACAGUCCGACGAUCAG), and a 3' adaptor (UCGUAUGCCGUCUUCUGCUUG) according to the Illumina protocol. The ligated fragments were gel-purified and reverse-transcribed (SuperScript II, Invitrogen). After PCR amplification for 15 cycles (98°C for 30 sec; 98°C for 10 sec; 60°C for 30 sec), the PCR products were gel-purified and sequenced according to the Illumina sRNA sequencing protocol (33 cycles). All the probes and primers used in this study are listed in Supplemental Table 7.

Sequence Analysis

The detailed sequence analysis is described in the Supplementary Data. Briefly, the adaptor-trimmed and quality-filtered sRNAs were clustered based on their mapping positions on the assembled EST sequences. The number of reads mapping to the sRNA clusters was used to estimate their expression. Conserved miRNAs were identified by sequence similarity searches against the registry of plant microRNAs. Furthermore, the novel miRNA precursor candidates were predicted by stem-loop structure prediction matched by sRNAs. The targets of conserved and novel citrus miRNAs were then identified by target prediction routines.

Mineral Measurement

For mineral analysis, 15–20 HLB symptomatic leaves were collected from each of five 2-year-old field-grown 'Hamlin' sweet orange trees located at the USHRL-USDA research farm in St Lucie County, FL. Trees were irrigated by micro-sprinklers three times a week in the absence of adequate rainfall. Fertilizer and pesticides were applied seasonally, or as needed, but did not include increased nutritional applications or aggressive psyllid control as currently practiced in many HLB-affected commercial citrus groves in Florida. Leaves from five non-infected (as determined by PCR analysis) 'Hamlin' trees at the same location were collected for comparison. Leaf tissue was washed for 15 sec in reverse osmosis-distilled (RO-D) water, 0.01% detergent (Citranox, Alconox), and 0.1 N HCl, followed by three more rinses in RO-D water and dried at 80°C for at least 24 h in a forced-air oven. The dry weight was recorded, and leaf tissue was milled to pass a 20-mesh screen. Leaf tissue (500 mg) was digested in 10 ml of concentrated HNO_3 (trace metal grade) at 300 psi and 170°C for 10 min in a MARS 5 microwave (CEM Corp.). Leaf digestates were brought to volume in 100-ml volumetric flasks and filtered through Whatman No. 41 filter paper (Maidstone). Foliar levels of phosphorous (P), potassium (K), and zinc (Zn) were determined by ICP–OES spectrometry (IRIS 1000 HR Duo, ThermoElemental). Carbon (C) was analyzed using a NC 2100 analyzer (CE Elantech.).

Application of Inorganic P Solutions

For phosphorus treatments, 6-year-old field-grown 'Hamlin' sweet orange grafted onto Swingle citrumelo (*Citrus paradisi* Macf. X *Poncirus trifoliata* (L) Raf.) rootstock was used. Trees were located at the University of Florida Research & Education Center in Immokalee. These trees were Las-positive tested by the HLB Diagnostic Lab. at the Southwest Florida Research

and Education Center (SWFREC) as described by Stansly et al. (2013). The nutrient sprays started with the spring flush in 2008. Applications were given three times each year and timed with the initiation of the new vegetative flushes in spring (March), summer (June), and fall (September). The spray treatment was a 3–18–20 liquid fertilizer of potassium solution of phosphorous oxyanion and polyoxyanion solution, with 56% mono- and dipotassium salts of phosphorous acid and polymers (K-Phite, Plant Food Systems, Inc.), plus 3.8 kg of spray-grade potassium nitrate (KNO_3) and 18.9 L of 435 citrus spray oil. Spray was applied to the foliage at a 0.23 L m^{-2} rate using a handgun until runoff. The control trees received KNO_3 and spray oil only (mock treatment). All trees received two ground-applied applications of a controlled-release 14–0–24 fertilizer to the soil each year. Systemic and pre-emerge herbicides were used for weed management. Other standard good grove management practices known to favor good production were used. Yields were collected at fruit maturity in 2009, 2010, and 2011. Plot mean yields were analyzed using the GLM model of SAS 9.2, with years as a repeated measure.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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SUMMARY

We identified several HLB-induced citrus small RNAs that can be potentially developed into early diagnostic markers of HLB. Induction of miR399 by Las led to the discovery that HLB-positive trees suffer from phosphorus starvation. Applying phosphorus solutions help reduce HLB symptoms.