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Metabolite Signature of *Candidatus Liberibacter asiaticus* Infection in Two Citrus Varieties

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Supporting Information

ABSTRACT: Huanglongbing (HLB), also known as Citrus Greening Disease, is caused by the bacterium '*Candidatus Liberibacter asiaticus*' (CLAs) and is a serious threat to the citrus industry. To understand the effect of CLAs infection on the citrus metabolome, juice from healthy ($n = 18$), HLB-asymptomatic ($n = 18$), and HLB-symptomatic Hamlin ($n = 18$), as well as from healthy ($n = 18$) and HLB-symptomatic ($n = 18$) Valencia sweet oranges (from southern and eastern Florida) were evaluated using ^1H NMR-based metabolomics. Differences in the concentration of several metabolites including phenylalanine, histidine, limonin, and synephrine between control or asymptomatic fruit and symptomatic fruit were observed regardless of the citrus variety or location. There were no clear differences between the metabolite profiles of Hamlin fruits classified by PCR as asymptomatic and control, suggesting that some of the control fruit may have been infected. Taken together, these data indicate that infection due to CLAs presents a strong metabolic response that is observed across different cultivars and regions, suggesting the potential for generation of metabolite-based biomarkers of CLAs infection.

KEYWORDS: citrus, biomarker, *Candidatus Liberibacter asiaticus* (CLAs), ^1H NMR (nuclear magnetic resonance), Huanglongbing, Citrus Greening Disease, metabolomics, sweet orange

■ INTRODUCTION

Huanglongbing (HLB), also known as Citrus Greening Disease, is responsible for approximately \$7 billion in losses to Florida's citrus industry,¹ and is caused by the Gram-negative, phloem-limited bacterium *Candidatus Liberibacter asiaticus* (CLAs). The bacterium is transmitted by the insect known as the Asian Citrus Psyllid (*Diaphorina citri* (ACP))^{2,3} or by grafting infected material.^{4–6} Infection by CLAs in the early stages results in no discernible difference in the outward appearance of the tree or fruit. The gold-standard for detection of the bacterium is polymerase chain reaction (PCR), which may detect the infection prior to outward symptoms developing (i.e., asymptomatic tree and/or fruit). When the tree eventually becomes symptomatic (which can take several years), the HLB disease presents itself and results in discolored leaves, misshapen and bitter fruit, 30–100% yield reductions, and, ultimately, early tree death.^{2,7,8} HLB is considered the most serious citrus disease worldwide, and there is no known cure.^{7,9} Prevention of disease spread is mostly targeted toward controlling the psyllid populations via trapping or application of pesticides, as well as removal of infected trees to reduce CLAs inoculum. Furthermore, application of enhanced nutrient programs has been shown to have no significant effect on improving tree health or fruit quality and yield.¹⁰ The disease affects all citrus varieties, although some cultivars are more susceptible than others.^{7,11,12} Since its discovery in Florida in 2005,¹³ HLB is now present in all Florida citrus growing counties,¹⁴ and the entire state of Florida and Georgia, as well as the entire commonwealth of Guam, are under quarantine for HLB by the USDA.¹⁵ HLB has also spread to other citrus-growing states, including Texas and California.^{15,16}

Following pathogen attack, plants initiate multiple defense responses against further injury,^{17–20} which includes changes in gene expression, plant signaling, and other biochemical and physiological modifications.^{18,19} These changes can be measured throughout the plant, providing clues for understanding a pathogen's effect on its host plant. Metabolomics, which characterizes the complete set of metabolites produced in an organism, can be employed to gain an overview of the changes that occur in a plant during injury. We have previously used ^1H NMR-based metabolomics to study the metabolite profile of Valencia sweet orange fruits collected from a single commercial orchard that included trees infected with CLAs.²¹ Differences between healthy, asymptomatic, and symptomatic fruit were found, which included decreased levels of proline and arginine in asymptomatic and symptomatic fruit as compared to control fruit, both of which are amino acids shown to normally accumulate in stressed plants. Additionally, phenylalanine was elevated in asymptomatic and symptomatic fruit, while sucrose, fructose, glucose, and the °Brix/acid ratio were decreased relative to control fruit.²¹ Phenylalanine is also reported to be involved in plant defense, playing a role in the phenylpropanoid biosynthetic pathway, in which salicylic acid is a product.^{22,23} In a separate study, juice from HLB-asymptomatic and symptomatic fruit also showed a lower °Brix/acid ratio, and the authors suggested that juice from symptomatic fruit was similar to that

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of immature fruit.²⁴ Juice from asymptomatic and symptomatic fruit has also been shown to contain more bitter compounds, but in concentrations lower than taste threshold values.^{14,24}

The value of Florida's sweet orange crop for 2011–2012 alone is reported to be \$1.2 billion. Hamlin and Valencia varieties make up 42.6% and 55%, respectively, of this value.²⁵ In our previous study, we evaluated the juices prepared from Valencia oranges of varying stages of CLAs infection and harvested from a single location.²¹ Additionally, we have examined the impact of environmental differences such as variations in elevation and soil depth on the metabolome of Mandarin oranges.²⁶ Recognizing that the state of HLB infection, as well as varietal and regional differences, could influence the metabolome of citrus juices, this study was undertaken to investigate the metabolite differences between healthy and infected fruit of two different citrus varieties grown in different regions. It is important to understand the impact of regional and varietal variation on the juice metabolome during infection, as this may provide insight into differences in response to infection depending on location or cultivar, and therefore help determine if these variables may confound the ability to detect disease using this method.

MATERIALS AND METHODS

Plant Materials. *Citrus sinensis* cultivars Valencia and Hamlin fruit samples were used in this study. The presence of CLAs bacteria in symptomatic and asymptomatic trees, and its absence in control trees, was confirmed by polymerase chain reaction (PCR) using standard methods as described.²⁷ Control Hamlin fruit samples were collected from CLAs[−] (PCR negative), and asymptomatic and symptomatic Hamlin fruit were collected from different CLAs⁺ (PCR positive) trees. Asymptomatic fruit were visually indistinguishable from control (CLAs[−]) fruit. A total of 54 Hamlin samples were collected: 18 control, 18 asymptomatic, and 18 symptomatic. Control Valencia fruit samples were collected from CLAs[−] (PCR negative) trees; symptomatic Valencia fruit were collected from CLAs⁺ (PCR positive) trees and showed visual symptoms of HLB. A total of 36 Valencia samples were collected: 18 control and 18 symptomatic.

All samples were collected from mature trees approximately 20 years old. In each region, fruit were gathered from up to three different blocks, and in each block, samples were taken from three different trees. The per-tree sample size consisted of 15–20 fruits that were juiced, aliquoted, and stored at −20 °C until used for analysis. Sampling of Valencia fruits was conducted from May 2009 to June 2009 and Hamlin fruits from December 2009 to January 2010. For each cultivar, trees were from commercial orchards in south and east Florida. For the Hamlin oranges, 36 samples came from east Florida (12 samples from each infection stage), and 18 came from south Florida (6 samples from each infection stage). For the Valencia oranges, 18 samples came from east Florida, and 18 came from south Florida (9 samples from each infection stage for both locations).

°Brix and Limonin Measurements and NMR Data Collection.

°Brix, limonin measurements, and NMR data collection were performed as previously described by Slisz et al.²¹ Briefly, °Brix was determined by measuring total soluble solids with a Rudolph Research model J257 automatic refractometer (Hackettstown, NJ). Limonin was measured by high-performance liquid chromatography (HPLC) and represents limonin concentration after a 48 h thaw at 4 °C to allow conversion of limoninoate A-ring lactone into limonin. A 50 × 2.0 mm Phenomenex Phenosphere-Next-4 μ m phenyl column with a guard column of the same material was used with solvent flow rate at 1.0 mL/min comprised of 70% 10 mM formic acid and 30% ACN.

For NMR analysis, juice samples were thawed, centrifuged, and then filtered (Nanosep Omega-3 3000 molecular weight cutoff (Pall Life Sciences, Ann Arbor, MI)). Internal standard containing 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-*d*₆ (DSS-*d*₆) in >98% D₂O and 0.2% NaN₃ (for a final DSS-*d*₆ concentration of 0.5 mM, 0.02% NaN₃,

Figure 1. Comparison of composition of both Hamlin (filled) and Valencia (open) orange juice samples. (a) PCA scores plot comparing control (green), asymptomatic (orange), and symptomatic (red) samples. Circle and square markers indicate south or east Florida origin, respectively. Principal component 1 (PC1) explains 45.1% of the variation, and PC2 explains 17.9% of the variation. $R^2X = 0.787$; $Q^2 = 0.658$. (b) Loadings plot corresponding to panel a.

and approximately 10% D₂O in each sample) was added to a mixture of 400 μ L of juice filtrate and 185 μ L of ultrapure H₂O to ensure a sample volume of 600 μ L required for NMR analysis. Sample pH was adjusted to 6.8 ± 0.1 using NaOH, and then 600 μ L of sample was transferred to 5 mm NMR tubes. NMR spectra were acquired at 298 K using the Bruker "noesypr1d" experiment on a Bruker Advance 600 MHz NMR spectrometer equipped with a SampleJet with the following acquisition parameters: 12 ppm sweep width, 2.5 s acquisition time with 2.5 s relaxation delay, and 100 ms mixing time. Water saturation was applied during the relaxation delay and mixing time. The resulting spectra were zero-filled to 128 000 data points, and an exponential apodization function corresponding to a line-broadening of 0.5 Hz was applied. Spectra were processed in Chenomx NMRSuite Processor v7.6.

Data Analysis. Metabolites in orange juice samples were identified and quantified using targeted profiling²⁸ with Chenomx NMR suite v7.6 (Chenomx Inc., Edmonton, Alberta, Canada). In total, 32 metabolites were assigned and quantified as previously described²¹ and included sugars (fructose, glucose, sucrose, and myo-inositol), amino acids (alanine, arginine, asparagine, aspartate, histidine, isoleucine, leucine, phenylalanine, proline, threonine, and valine), organic acids (citrate, succinate, malate, formate, ascorbate, and γ -aminobutyric acid (GABA), and others (adenosine, choline, ethanol, limonin glucoside, methanol, proline betaine, putrescine, quinic acid, trigonelline, synephrine, and an unknown metabolite with peaks at 0.8, 1.1, and 1.4 ppm). The concentration of limonin was determined using HPLC and was included in statistical analysis. For NMR measured metabolites, the profiled concentrations were corrected for dilution by multiplying the measured concentration by the final volume (650 μ L)

Figure 2. Comparison of composition of Valencia oranges. (a) PCA scores plot of control (green) and symptomatic (red) Valencia orange juice samples. PC1 explains 29% of the variation and PC2 23.3%. $R^2X = 0.812$; $Q^2 = 0.496$. (b) Loadings plot corresponding to panel a. (c) PLS-DA scores plot of Valencia samples classified by both region and infection status. Square and circle markers represent fruit from east and south Florida, respectively. R^2 and Q^2 were 0.587 and 0.532, respectively, and validation by random permutation (100 permutations) had y -intercepts for R^2 and Q^2 of 0.111 and -0.238 , respectively, and both had positive slopes. (d) Loadings plot corresponding to panel c.

and dividing by the volume of juice used (400 μ L). Final metabolite concentrations were subjected to $\log_{10}$ transformation to ensure normal distribution as described by Zhang et al.²⁶ Multivariate statistical data analyses, including principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA), were performed using SIMCA-P with mean centering and unit variance scaling. The quality of the models was judged by the goodness-of-fit parameter (R^2X or R^2Y) and predictive ability parameter (Q^2), as well as permutation testing (for the PLS-DA models), where each data point was randomly assigned a class 100 times, and the corresponding R^2Y and Q^2 calculated and compared to the original model. From the PLS-DA models, variable influence in projection (VIP), which is a weighted sum of squares of the PLS loading weights that takes into account the amount of explained Y -variation in each dimension, was used to assess those variables of significance for further scrutiny. Metabolites with a VIP value greater than 1 were selected for further discussion.²⁹ Significance testing, using Mann–Whitney test for Valencia samples, and Kruskal–Wallis test and Dunn’s multiple comparison test for Hamlin samples, was performed on raw untransformed data using GraphPad Prism with significance set at $\alpha = 0.05$. Kruskal–Wallis test and Dunn’s multiple comparison test were selected for the Hamlin samples due to unbalanced numbers in the selected sample groups.

RESULTS

We previously published on the metabolite composition of control and asymptomatic Valencia fruit.²¹ However, that study only examined one variety of fruit from one location in Florida. Here, we analyzed the composition of two citrus varieties from different growing regions in the context of infection by CLas (control (PCR CLas⁻), asymptomatic (PCR CLas⁺), and symptomatic (PCR CLas⁺) Hamlin fruit, as well as control

(PCR CLas⁻) and symptomatic (PCR CLas⁺) Valencia fruit). The contributions of variety and growing region (south or east Florida) were assessed to evaluate the impact of these variables on tree metabolic response to infection by CLas.

All Orange Samples. Multivariate analysis of all of the Hamlin and Valencia samples (Figure 1) showed clear separation by principal components analysis (PCA) ($R^2X = 0.787$; $Q^2 = 0.658$) based on their cultivar and infection status (healthy, HLB-asymptomatic or HLB-symptomatic). Samples were discriminated by cultivar along principal component 1 (PC1), and were separated by infection status along principal component 2 (PC2). These data show that orange cultivar represents the major difference in the fruit metabolome, followed by infection status. Many metabolites were higher in the Valencia juice samples including GABA, isoleucine, arginine, valine, and threonine. Ascorbate and formate were higher in the Hamlin juice samples (Figure 1b). To understand and compare the impact of CLas infection on each cultivar, statistical analyses were conducted independently on each variety and are shown below.

Valencia Samples. Figure 2a shows a PCA for Valencia fruit samples ($R^2X = 0.812$; $Q^2 = 0.496$). Sucrose, glucose, fructose, proline, ethanol, and putrescine were higher in concentration in control fruit as compared to symptomatic fruit (Figure 2b). Sucrose concentration was over 2-fold higher in control. Phenylalanine, histidine, limonin, synephrine, quinic acid, and asparagine were higher in symptomatic fruit (Figure 4 and Supporting Information Figure S1). The symptomatic fruit had over 2-fold higher phenylalanine and asparagine, and 2.6-fold higher limonin concentration. °Brix content was significantly

Table 1. °Brix for Hamlin and Valencia Juice Samples, Grouped by Infection Status^a

Hamlin				Valencia	
group 1	group 2	group 3	symptomatic	control	symptomatic
10.66 ab	12.05 a	12.90 a	8.73 b	12.18	8.79 ^b

^aComparisons are within each cultivar. For Hamlin, values with different letters are significant at $p < 0.05$. ^bFor Valencia, denotes significance at $p < 0.0001$.

lower in symptomatic Valencia oranges as compared to control oranges (Table 1).

To determine the impact of the growing region on metabolite composition, fruit classified by both their region and their infection status were compared using PLS-DA. Figure 2c shows the scores plot, and Figure 2d shows the loadings plot. Interestingly, although regional differences could be observed, the ability to differentiate fruit on the basis of infection status was clearly evident, as metabolites responsible for regional variation differed from those responsible for variation due to infection (Figure 2d).

Hamlin Samples. To investigate the influence of infection and growing region on metabolite composition of Hamlin orange samples, PLS-DA was employed (Figure 3a). A similar trend was observed for Hamlin oranges as was observed for Valencia oranges. The control and CLas⁺ symptomatic samples separated along component 1. The control and CLas⁺ asymptomatic samples clustered together on component 1, but some differentiation could be observed along component 2. The low Q^2 value (0.436), which represents the predictive ability of the model, reflects the overlap between the control and asymptomatic samples; however, permutation testing revealed a valid model. Although separation was not complete, a pattern emerged that appeared as mainly control fruit in the lower right-hand quadrant that transitioned to mostly CLas⁺ asymptomatic fruit in the upper right-hand quadrant. Proposed groupings based on this clustering are circled and labeled as groups 1–3 in Figure 3a. Distinctions in metabolite composition included higher ethanol and malate in group 1 (mostly control), and higher fructose, glucose, proline, and sucrose in group 3 (mostly asymptomatic) fruit (Figure 4). Malate and ethanol exhibited a stepwise decrease in concentration from group 1 to group 3 fruit (Supporting Information Figure S2). Fructose, glucose, sucrose, °Brix, and proline initially increased from group 2 (mixture of control and asymptomatic) to group 3 (mostly asymptomatic) fruit, and then decreased in symptomatic fruit. Sucrose was approximately 2-fold higher in group 3 fruit as compared to symptomatic fruit. Phenylalanine, histidine, synephrine, asparagine, GABA, choline, limonin, and quinic acid were higher in symptomatic fruit as compared to control and group 3 fruit (Supporting Information Figure S2). Phenylalanine was approximately 3.8-fold higher, histidine 3-fold higher, and asparagine 2-fold higher in symptomatic fruit as compared to group 3 fruit. °Brix was significantly lower in symptomatic fruit as compared to fruit classified as groups 2 and 3 (Table 1).

To understand how different growing regions contribute to Hamlin fruit metabolite composition during infection, the fruit were classified based on the combination of infection status and growing region (Figure 3b). As with the Valencia juice samples, juice samples from Hamlin oranges discriminated by infection status along component 1, and by region along component 2, and metabolites that characterized growing regions differed from those that characterized infection stage (Figure 3c).

Figure 3. Comparison of composition of Hamlin orange juice samples. (a) PLS-DA scores plot of control (green), asymptomatic (orange), and symptomatic (red) Hamlin samples. $R^2 = 0.537$ and $Q^2 = 0.436$; validation by random permutation (100 permutations) showed positive slopes for R^2 and Q^2 , and intercepts of 0.118 and -0.187 , respectively. (b) PLS-DA scores plot of Hamlin samples based on infection status (control (green), asymptomatic (orange), and symptomatic (red)) and the sample's growing region (south (circle) and east (square) Florida). R^2 and Q^2 are 0.33 and 0.292, respectively, and random permutation (100 permutations) revealed positive slopes with R^2 and Q^2 intercepts of 0.054 and -0.147 , respectively. (c) Loadings plot corresponding to panel b.

DISCUSSION

Analysis of Hamlin and Valencia oranges revealed that the two varieties are distinguishable from one another on the basis of their metabolite composition. Although the samples originated from varying locations and were from two cultivars, the ability

Figure 4. Metabolite concentration (average $\pm$ SEM) at varying states of CLAs infection in Hamlin and Valencia orange juice samples. Valencia samples are grouped according to whether they are control or symptomatic (CLAs⁻ or CLAs⁺ via PCR, respectively). ** $p < 0.01$ and **** $p < 0.0001$. Hamlin samples are classified as group 1, group 2, group 3, and symptomatic. Samples with shared letters/symbols indicate no significant difference between those samples at $p < 0.05$.

to discriminate samples on the basis of infection status was not impaired. The concentrations of phenylalanine, histidine, asparagine, and limonin were lower in the control and asymptomatic Valencia or Hamlin fruit as compared to symptomatic fruit in both varieties (Figure 4). This is in agreement with another study on juice,¹⁴ as well as our previous study in which we reported increases in amino acids such as asparagine, histidine, and phenylalanine in symptomatic Valencia oranges as compared to healthy and asymptomatic Valencia oranges.²¹

Examination of the PLS-DA (Figure 3a) of Hamlin fruit revealed a cluster of samples delineated as control fruit (PCR CLAs⁻) in the lower right quadrant, and a cluster of mostly asymptomatic fruit (PCR CLAs⁺) in the upper right quadrant. A mixture of control and asymptomatic samples was between the two. Similarity in metabolite composition among samples in groups 2 and 3 suggests that some of the controls were possible false negatives by PCR. PCR is a nucleic acid-based method that can be used to determine the presence or absence of a bacterium by testing for the presence of its genetic material. Indeed, PCR is prone to false negatives, as it requires a minimum bacterial titer for detection, and bacteria are not evenly distributed throughout the tree.^{30,31} Since detection of HLB in Florida in 2005, the entire state is now under quarantine for HLB and ACP.¹⁵ It is unknown how many truly CLAs⁻ trees remain in Florida. These results suggest a potential role for metabolomics to detect infection earlier than PCR. Detecting infection early and understanding the tree response to infection is important for developing treatment plans. These results will need to be further investigated with a broader range of citrus varieties to establish global metabolite biomarkers of CLAs infection.

Fructose, glucose, sucrose, and proline concentrations were significantly lower in symptomatic fruit as compared to control Valencia. However, differences in these metabolite concentrations between group 1 (primarily control fruit) and symptomatic Hamlin failed to reach significance. There may be different reasons for this discrepancy. First, there is a difference in sample size, with fewer samples in group 1 as compared to symptomatic fruit. Second, not all Valencia control samples may be truly control, and some could come from trees not yet testing positive for CLAs. While Hamlin fruit are harvested from October to January, Valencia fruit are late season fruit and harvested from March to June. Indeed, lower amplification of CLAs during the spring months has been reported,³² and thus the difference in harvest time may contribute to the presence of false negatives by PCR. However, because there are no asymptomatic Valencia samples in this study, we cannot attempt to tease out possible PCR false-negatives based on similarities in metabolite composition as we have done with the Hamlin samples, and it is unknown if the Valencia control samples may contain samples that are analogous to Hamlin groups 2 and 3.

Comparison of fructose, glucose, sucrose, and proline concentrations between group 3 and symptomatic Hamlin and control and symptomatic Valencia revealed similar trends (Figure 4). In the asymptomatic Hamlin samples (groups 2 and 3), these metabolites increase. Symptoms of HLB include starch accumulation in the leaves and phloem impairment.^{20,33,34} The decrease in sugars during symptomatic infection could reflect altered carbohydrate transport. Fructose and glucose have been shown to increase in tomatoes subjected to water and nematode stress,³⁵ and sucrose, fructose, and glucose accumulated in strawberries infected

with *Colletotrichum nymphaeae*.³⁶ Defensive mechanisms are costly,³⁷ and the influx of sugars during asymptomatic infection could indicate mobilization of plant defenses prior to symptom development. Thus, these metabolite concentrations measured in Hamlin group 1 could reflect true control concentrations, and those measured in groups 2 and 3 reflect changes in plant metabolism with early infection. Because there are no asymptomatic Valencia samples, however, it is unknown if a similar pattern can be observed during early infection in Valencia oranges.

Although we previously reported decreases in the concentrations of fructose, glucose, sucrose, and proline when comparing healthy and asymptomatic Valencia fruit,²¹ it is important to note that there were differences in the appearance of the asymptomatic fruit as compared to this study. This may explain why the results presented here for asymptomatic Hamlin fruit differ from those of asymptomatic Valencia fruit in the Slisz et al. paper.²¹ Indeed, the fruit in that article showed minor signs of HLB such as a slight reduction in average size and small amounts of greening. Here, the asymptomatic Hamlin fruit were visually indistinguishable from control fruit, although they were CLas⁺ by PCR. Unfortunately, no asymptomatic Valencia fruit were available for this study, and thus a difference between cultivars in how asymptomatic fruit differ in terms of metabolite content cannot be ruled out. Nonetheless, this brings to light a need for standardization of the term “asymptomatic” because it currently relies on subjective interpretation of visual symptoms. A chemical profile that classifies the fruit may be desirable to promote sampling uniformity and to reduce possible misunderstandings.

Although Hamlin and Valencia oranges are two distinct orange varieties harvested during different parts of the year, the presence of CLas has a substantial effect on the metabolite composition of the fruit. The overall pattern for change in many metabolites remained consistent in both Hamlin and Valencia cultivars. Asymptomatic Hamlin oranges revealed more details about nuances in metabolite changes during early infection. Although differences due to the tree location can be detected, this does not impair our ability to discriminate the fruit on the basis of their infection status.

In summary, the present data suggest that metabolomics may be useful to detect trees in the early stage of infection (asymptomatic), and differentiate from those in later stages (symptomatic).

■ ASSOCIATED CONTENT

■ Supporting Information

Comparisons of additional metabolites for control and symptomatic Valencia juice samples (Figure S1); comparison of additional metabolites for Hamlin juice samples based on infection status (Figure S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HLB, Huanglongbing; CLas, *Candidatus Liberibacter asiaticus*; ACP, Asian Citrus Psyllid; NMR, nuclear magnetic resonance; PCA, principal component analysis; PLS-DA, partial least-squares-discriminant analysis

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