

Communication

# Investigation of Citrus HLB Symptom Variations Associated with “*Candidatus Liberibacter asiaticus*” Strains Harboring Different Phages in Southern China

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**Abstract:** Huanglongbing (HLB) is a devastating disease affecting citrus production worldwide. In China, the disease is associated with an unculturable alpha-proteobacterium, “*Candidatus Liberibacter asiaticus*” (CLAs). Phages/prophages of CLAs have recently been identified through intensive genomic research. The phage information has facilitated research on CLAs biology such as population diversity and virulence gene identification. However, little is known about the roles of CLAs phages in HLB symptom development. Such research is challenging due to the unculturable nature of CLAs and the lack of laboratory strains that carry a single phage. In this study, CLAs strains singly carrying Type 1 phage (Type 1 CLAs) and Type 2 phage (Type 2 CLAs) were identified and maintained in an experimental screenhouse in southern China. The strains were characterized through next-generation sequencing (NGS). Then, each CLAs strain was inoculated into seedlings of three different citrus cultivars/species through graft transmission in a screenhouse in Guangdong, China. Symptom developments were recorded. All CLAs-infected cultivars showed HLB symptoms in seven months. In cultivar Nianju (*Citrus reticulata*), Type 1 CLAs caused pronounced yellowing symptoms and severe defoliation, whereas Type 2 CLAs caused typical Zn-deficiency-like symptoms. In contrast, symptoms from the two CLAs strains’ infections on cultivars Shatianyu (*C. maxima*), and Eureka lemon (*Citrus limon*) were more difficult to differentiate. Results from this study provide baseline information for future research to investigate the roles of CLAs phages in HLB symptom development.

**Keywords:** Huanglongbing (HLB); *Candidatus Liberibacter asiaticus*; phages; prophages; HLB symptoms; graft inoculation



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## 1. Introduction

Citrus Huanglongbing (HLB, yellow shoots disease) is one of the most destructive diseases affecting citrus production in China and around the world [1,2]. In China, HLB is associated with “*Candidatus Liberibacter asiaticus*” (CLAs), a phloem-limited fastidious alpha-proteobacterium transmitted by Asian citrus psyllid (ACP, *Diaphorina citri* Kuwayama). CLAs is currently not culturable in vitro. Most CLAs information is derived from genome sequencing and analyses [3–6]. One significant discovery was the identification of CLAs phages or prophages through metagenomic studies [7,8].

Phages are an important genetic element of bacteria and play critical roles in bacterial evolution and environmental adaptation, including pathogenesis [9]. A typical phage is composed of a proteinaceous capsid and a circular DNA genome. Prophage is the integration of a phage genome into the host bacterial chromosome. For the convenience of communication, the term phage and prophage are interchangeable in this manuscript. In CLAs, the chromosomal region is highly conserved, whereas the prophage region is highly

variable. This information has been used to establish prophage typing systems of CLas strains [10,11]. The biological functions of several phage genes have been studied [12,13]. However, the roles of CLas phages in HLB symptom development remain largely unknown.

In a disease, symptoms are the outcome of host–pathogen interactions. HLB symptoms are highly variable but could be grouped into (1) leaf evenly yellowing; (2) leaf mottling; and (3) Zn-deficiency-like [14]. Mechanisms on how CLas strains cause different HLB symptoms are not known. CLas phages have been one of the few research targets in this regard. Research in CLas is challenging due to its unculturable nature. Study on CLas phages is further complicated by the fact that a CLas strain often harbors more than one phage type as the case in US [7,11,15]. However, a survey in southern China revealed the predominance of single prophage (Type 1 or Type 2) in the CLas population [16]. This prompted us to initiate this research project to explore the use of single-phage CLas strains to address HLB biology questions.

In this study, we had two main objectives: (1) To collect and identify CLas strains carrying single phages. The CLas strains were characterized through PCR and next-generation sequencing (NGS); and (2) single-phage CLas strains were inoculated to citrus cultivars through grafting. Symptom developments were compared to establish preliminary associations between CLas phages and symptom variations under greenhouse conditions in Guangdong, China. Meanwhile, sensitivities of different citrus cultivars to CLas infections were documented.

## 2. Materials and Methods

### 2.1. Source of CLas Strains and Phage Types

An HLB survey was performed on a lemon orchard in Ruili City of Yunnan Province, China, in 2016. Budwoods were collected from HLB symptomatic trees of Eureka Lemon (*Citrus limonia* Osbeck) and grafted onto one-year-old Eureka lemon seedlings on trifoliolate orange (*Poncirus trifoliata* L.) rootstock. The grafted seedlings were maintained in an insect-free experimental greenhouse on the campus of South China Agricultural University, Guangzhou, China. From the successfully grafted seedlings, leaf samples showing typical HLB symptoms were collected for DNA extraction. Using the PCR primer sets (Table 1), one seedling was tested to contain only Type 1 phage (named as NJ5-T1), and another seedling was tested to contain only Type 2 phage (named as NJ8-T2).

**Table 1.** A list of PCR primers for detection of “*Candidatus Liberibacter asiaticus*” (CLas) and CLas phages used in this study.

Name		Sequence (5′-3′)	Size (bp)	Genomic Locus/Specificity	Reference
CLas-4G HLBr HLBp	Forward Reverse Probe	AGTCGAGCGGTATGCGAAT GCGTTATCCCGTAGAAAAAGGTAG FAM-AGACGGGTGAGTAACGCG-BHQ	78	16S rRNA gene/species	Bao et al. 2020 [17]
T1-2F T1-2R	Forward Reverse	TGGCTCGGGTTCAGGTAAAT AAGGGCGACGCATGTATTTC	975	Endolysin/Type 1 phage	Zheng et al. 2018 [8]
T1-3F T1-3R	Forward Reverse	CTCACTGCGTCTTGATTCGG CGAACGAGCGGTATGTTTGT	866	Hypothetical protein gene/Type 1 phage	Same as above
T2-2F T2-2R	Forward Reverse	ACCCTCGCACCATCATGTTA TCGTCTTGATTGGGCAGAGT	813	Hypothetical protein gene/Type 1 phage	Same as above
T2-3F T2-3R	Forward Reverse	ACAGTTAAGAGCCACGGTGA AAGACGTGGGTGTTATGGGT	918	Hypothetical protein gene/Type 1 phage	Same as above
891-1F 891-1R	Forward Reverse	CTGATCCTTTACCATGCCGC CAGCGAAACCGATCTTGAGG	950	hsdS/Type 3 phage	Same as above
891-2F 891-2R	Forward Reverse	ACCGCGATCTACCCGTAATT TGTGTTTTGCGAGTGAAGGG	884	hsdR/Type 3 phage	Same as above

For further validation of phage-type status, DNA from NJ5-T1 and NJ8-T2 was subjected to high-throughput sequencing (Illumina HiSeq, 100 × 2, Illumina Inc., San Diego, CA, USA). HiSeq reads of each sample were mapped to sequences of SC1 (NC\_019549.1, Type 1 phage), SC2 (NC\_019550.1, Type 2 phage), and P-JXGC-3 (KY661963.1, Type 3 phage) using QIAGEN CLC genomic workbench software (Version 10.1.1, <https://digitalinsights.qiagen.com/> (accessed on 3 November 2021)) with parameters: length fraction = 1.0, similarity fraction = 0.80, for CLAs phage type determination [18].

## 2.2. Graft Transmission Experiments

Three citrus cultivars, Nianju (*C. reticulata* Blanco), Shatianyu pomelo (*C. maxima* (Burm.) Merr.), Eureka lemon (*C. limonia* Osbeck), were selected. These cultivars were purposely selected due to their differences in citrus species. Seedlings were obtained from disease-free nurseries locally in Guangdong Province. Citrus seedlings were grown in grow bags with a size of 7.5 × 9 × 28 cm in sterilized organic soil. Nianju was on sour orange rootstocks (*C. aurantium* L.). Shatianyu pomelo and Eureka lemon were on trifoliolate orange rootstock. Seedlings 8 to 10 months old were used for graft inoculations with budwoods from NJ5-T1 or NJ8-T2 trees in October 2016. Each cultivar had six replications and one seedling received one bud. Two un-grafted plants in each cultivar served as non-inoculated controls.

Inoculated seedlings were maintained in an insect-free screenhouse. HLB symptom developments were monitored continuously (every ten days) through the growing season. Five months after grafting (March 2017), five or six leaf samples were collected monthly from each plant and continued to October 2017. Symptomatic and non-symptomatic fully expanded (mature) leaves on the new growth branches above the inoculation point were sampled. DNA was extracted from each sample and the titers of CLAs were determined using PCR.

## 2.3. PCR Assay

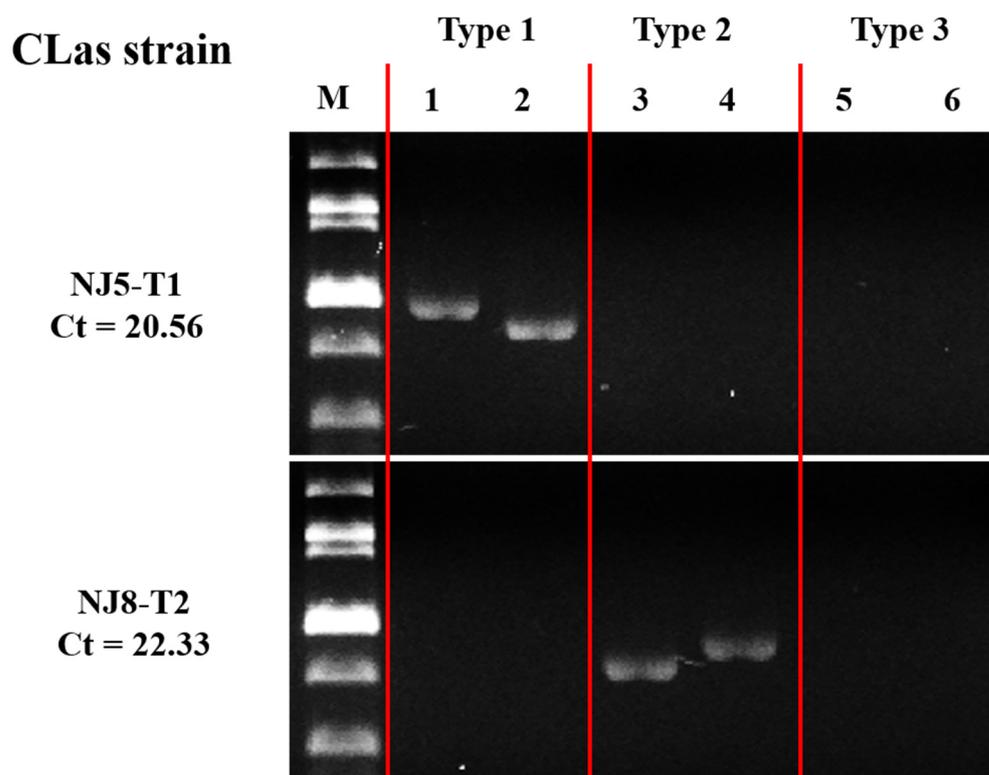
DNA of leaf midrib (200 mg) was extracted followed the instructions of the DNeasy plant Mini kit (QIAGEN, Shanghai, China). A TaqMan qPCR assay with primer set HLB4g-HLBr (17, Table 1) was used to detect and quantify CLAs titer. Standard agarose gel PCR was used for CLAs phage detection, with primer sets T1-2F/T1-2R and T1-3F/T1-3R for CLAs Type 1 phage, primer sets T2-2F/T2-2R and T2-3F/T2-3R for CLAs Type 2 phage, and primer sets 891-1F/891-1R, and 891-2F/891-2R for Type 3 phage [8] (Table 1).

TaqMan qPCR assays were performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). The published procedure with primer set and probe (CLAs-4G-HLBr-HLBrp, Table 1) [17] was followed. Briefly, the reaction mixture (20 µL) consisted of the following: 10 µL of Bestar<sup>®</sup> qPCR Master Mix (DBI<sup>®</sup> Bioscience, Ludwigshafen, Germany), 1 µL of DNA template (25 ng), 0.2 µL of TaqMan<sup>®</sup> probe (5 µM), and 0.4 µL of each forward and reverse primer (10 µM). The standard amplification procedure was: 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The data were analyzed using Bio-Rad CFX Manager 2.1 software with automated baseline settings and a manually set threshold at 0.1.

For standard agarose gel PCR, a previously published procedure [8] was followed. Briefly, PCR was performed on a Bio-Rad T100<sup>™</sup> Thermal Cycler (Bio-Rad, Hercules, CA, USA). The 25 µL reaction mixture contained 1 µL of DNA template, 0.4 µL of Taq DNA polymerase at 2.5 U/µL (Tiangen biotech co., Beijing, China), 2.5 µL of 2.5 mM deoxynucleotide triphosphates (dNTPs), 2.5 µL of 10 × DNA polymerase buffer, 0.5 µL of each forward and reverse primer (10 µM), and 17.6 µL of ddH<sub>2</sub>O. PCR was performed under the following procedure: initial denaturation at 96 °C for 5 min, 35 cycles of amplification (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s), and ended with a final extension of 72 °C for 10 min. The PCR products were electrophoresed in 1% agarose gels (0.5 × TBE buffer) and visualized by Goldview (Geneshun Biotech Ltd., Guangzhou, China) under UV illumination.

### 3. Results

PCR results on NJ5-T1 and NJ8-T2 were shown in Figure 1. Sample NJ5-T1 harbored only Type 1 phage, and NJ8-T2 harbored only Type 2 phage. None of the two strains harbored Type 3 phage. Type 3 phage was not the target of this study. HiSeq sequencing generated a total of 74,443,296 short reads (100 bp per read) with a total of 36,326 bp from sample NJ5-T1 (Table 2). Similarly, there was a total of 85,867,742 short reads (100 bp per read) with a total of 38,343 bp from sample NJ8-T2 (Table 2). All sequence reads had a Q value > 30.



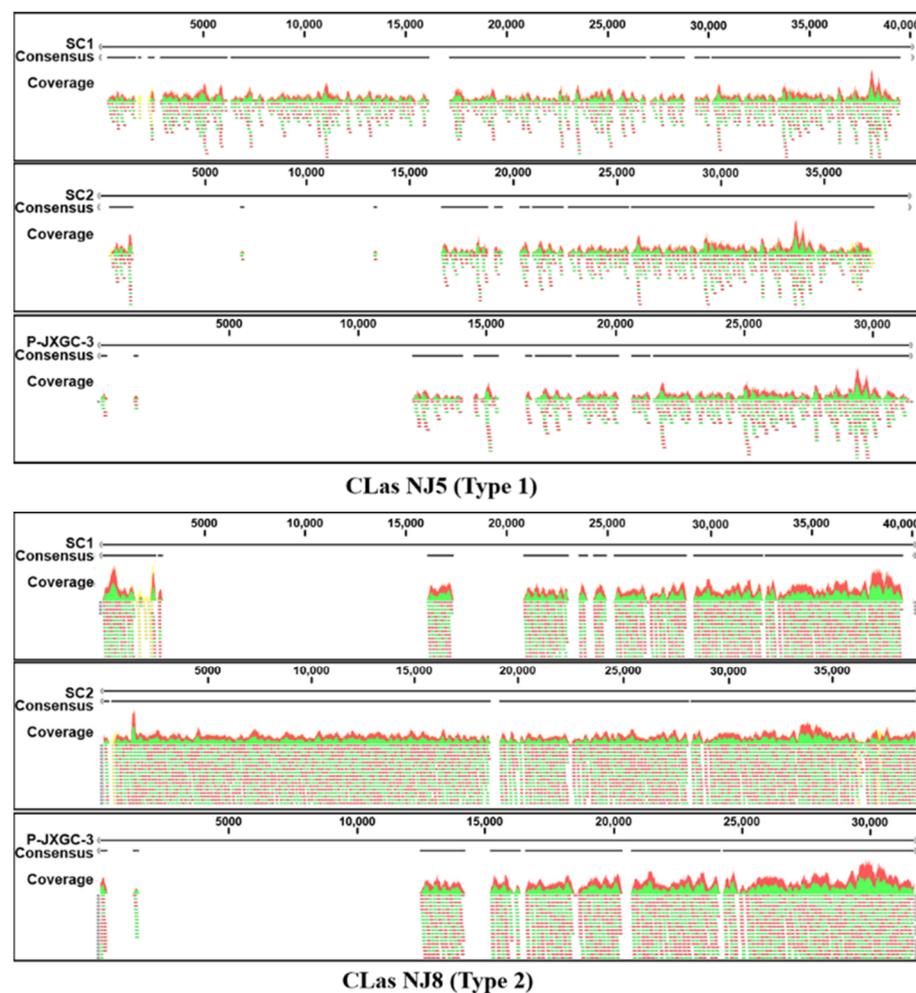
**Figure 1.** Detection of three phages in “*Candidatus Liberibacter asiaticus*” (CLas) strains using standard agarose gel PCR with phage-type-specific primer sets. Lane M, DNA ladder (top to bottom: 3000, 2000, 1550, 100, 750, 500 bp). Type 1 = specific primer sets: 1, T1-2F/T1-2R and 2, T1-3F/T1-3R; Type 2 = specific primer sets: 3, T2-2F/T2-2R and 4, T2-3F/T2-3R; Type 3 = specific primer sets: 5, 891-1F/891-1R and 6, 891-2F/891-2R. [8]. Ct values were based on primer set CLas4G/HLBr [17].

**Table 2.** Results of HiSeq read mapping to reference phage sequences of SC1 (Type 1, HQ377372), SC2 (Type 2, HQ377373), and P-JXGC-3 (Type 3, KY661963) of “*Candidatus Liberibacter asiaticus*” (CLas).

CLas Strain	Total Reads	SC1 (40,048 bp)	SC2 (38,997 bp)	P-JXGC-3 (31,449 bp)
NJ5-T1:				
Total (reads)	74,443,296	1130	641	550
Consensus (bp)		36,326	20,678	17,277
% coverage to reference		90.7	53.0	54.9
NJ8-T2:				
Total (reads)	85,867,742	4491	8273	3835
Consensus (bp)		21,392	38,343	17,937
% coverage to reference		53.5	98.3	57.0

As shown in Table 2, reference-mapping of HiSeq reads from sample NJ5-T1 identified 1130 reads matching SC1 with a coverage of 90.7%, as compared to that of 53.0% to SC2 (Type 2 phage) and that of 54.9% to P-JXGC-3 (Type 3 phage). This high percentage length coverage (90.7%) further confirmed that sample NJ5-T1 harbored a Type 1 phage based on the published analysis procedure [18]. Similarly, sample NJ8-T2 showed high coverage to SC2 (98.3%) and low coverages to SC1 (53.4%) and P-JXGC-3 (57.0%), confirming the sample harboring a Type 2 phage.

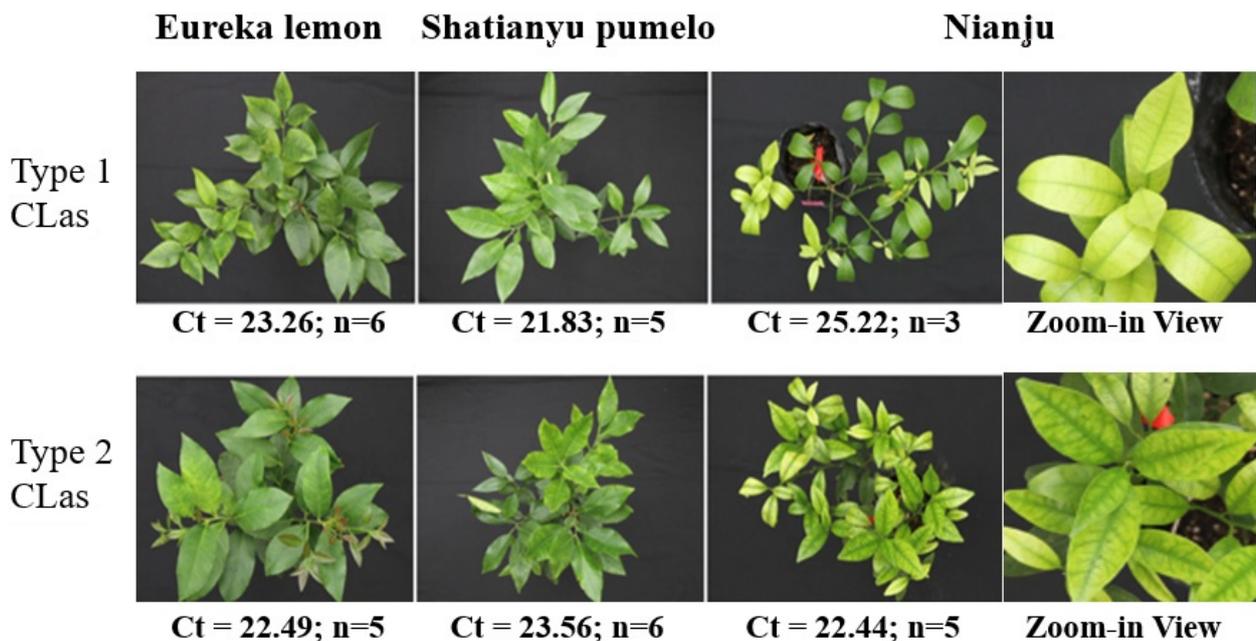
Figure 2 showed the overall distribution of matched HiSeq reads to the three reference CLas phage sequences. Again, reads from NJ5-T1 sample covered mostly the SC1 sequence. In contrast, a large mapping gap (from position 1575 bp to 16,474 bp) was evidenced in SC2 mapping. Similarly, a large gap (from position 251 bp to 12,141 bp) was present in the P-JXGC-3 mapping. All these validated that NJ5-T1 harbored only a Type 1 phage [18]. Similarly, read track data confirmed that sample NJ8-T2 only harbored a Type 2 phage.



**Figure 2.** Results of HiSeq read mapping of “*Candidatus Liberibacter asiaticus*” (CLas) strains NJ5-T1 and NJ8-T2 to the sequences of phage/prophage SC1 (Type 1, HQ377372), SC2 (Type 2, HQ377373) and P-JXGC-3 (Type 3, KY661963). Numbers at the top are nucleotide positions. On the left and under each prophage names, consensus = assembled contigs; coverage = read coverage. Note the almost complete coverage of SC1 by CLas NJ5-T1, indicating the presence of Type 1 phage, and the almost complete coverage of SC2 by NJ8-T2, indicating the presence of Type 2 phage.

As expected, not all grafted budwoods survived. In this experiment, the successful graft rate ranged from 50% (3/6) to 100% (6/6). Only plants with successful graft transmission were selected for symptom evaluation and comparison. Figure 3 showed the HLB symptoms from one representative plant in each cultivar six months after graft

transmission. Eureka lemon tended to show more obvious blotchy-mottling and water-soaked symptoms around the midrib. No significant difference was observed between Type 1 CLAs and Type 2 CLAs infections. For cultivar Shatianyu pomelo, relatively even yellowing of leaves was the trend, with no clear difference between Type 1 CLAs and Type 2 CLAs infections. Cultivar Nianju exhibited variable symptoms of yellowing, chlorophyll accumulation in main and lateral veins (Zn-deficiency-like), and defoliation. Infection from Type 1 CLAs appeared to produce more pronounced even yellowing and severe defoliation. Infection from Type 2 CLAs was more prone to show Zn-deficiency-like symptom, or a significant delay or no yellowing in leaf veins.



**Figure 3.** Representative symptoms of three citrus cultivars (Eureka lemon (*Citrus limonia* Osbeck), Shatianyu pomelo (*C. maxima* (Burm.) Merr.), and Nianju (*C. reticulata* Blanco)) six months after inoculation with “*Candidatus Liberibacter asiaticus*” (CLAs) strains carrying Type 1 phage (Type 1 CLAs) and Type 2 phage (Type 2 CLAs). Ct values were based on primer set CLas4G/HLBr (17); letter n represents number of replications. Ct values were means of all replicates.

#### 4. Discussion

This research is an application of the early reported information that single-phage (Type 1 or Type 2) CLAs strains are common in southern China [16]. Efforts were made to identify and characterize two single phage CLAs strains, NJ5-T1 and NJ8-T2. The use of single-phage CLAs strains will simplify and benefit future research in the biology of CLAs phages. Both NJ5-T1 and NJ8-T2 strains were from the same Eureka lemon orchard, suggesting that the chromosomal regions of the two strains were likely the same or highly similar. Current knowledge from whole-genome sequence analyses shows that all known CLAs strains have a highly conserved chromosomal region. It is the prophage region that shows the most genomic and genetic variations. This assures us that results from the comparison of single-phage CLAs strains can be considered as comparisons of CLAs phages. Furthermore, future experiments can be designed by the inoculation of both CLAs strains to create a mixed-phage infection scenario.

As shown in Table 2 and Figure 2, HiSeq reads from NJ5-T1 only covered 90.7% of SC1, suggesting that the NJ5 Type 1 phage was not the same as SC1. HiSeq reads from NJ8-T2 had a higher coverage (98.3%) to SC2. However, there was still a 1.7% of the ~40K bp (size of SC2) nucleotide difference. On the other hand, since NJ5-T1 and NJ8-T2 were from China, and SC1 and SC2 were from US, the phage sequence variations were good evidence that CLAs strains between the two geographical locations were different or had

different sources of origin, supporting previous observations on a chromosome-based tandem repeat locus [19] and sequence of *ter* gene [10].

Although generalizations can be made [1,14], HLB symptoms are in general highly variable depending on citrus cultivars, citrus culture environment, etc. With the controlled environmental conditions as in this greenhouse experiment, HLB symptoms among the three citrus cultivars/species were all noticeable. Within the same cultivar with different CLas phage types, differences in HLB symptoms in Eureka lemon and in Shatianyu pomelo were difficult to find. However, we were able to observe the HLB symptom variations from the two-phage-type CLas strains in cultivar Nianju (Figure 3), suggesting that cultivar Nianju was more sensitive in its interaction with different CLas strains or different CLas phages. It should be noted that only a limited number of citrus seedlings (three to six plants, Figure 3) were examined in this study, indicating the preliminary nature of the data in this study. However, such results could provide baseline information for future research on the roles of CLas phages in HLB symptom development.

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