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Seasonal Transcriptome Profiling of Susceptible and Tolerant Citrus Cultivars to Citrus Huanglongbing

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Abstract

Citrus huanglongbing (HLB) caused by '*Candidatus* Liberibacter asiaticus' (CLas) is the most devastating citrus disease worldwide. Most commercial citrus cultivars are susceptible to HLB, with a few more tolerant exceptions such as 'LB8-9' Sugar Belle mandarin. Transcriptomic analyses have been widely used to investigate the potential mechanisms for disease susceptibility, resistance, or tolerance. Previous transcriptomic studies related to HLB mostly focused on single time point data collection. We hypothesize that changes in day length and temperature throughout the seasons have profound effects on citrus−CLas interactions. Here, we conducted RNA-seq analyses on HLB-susceptible Valencia sweet orange and HLB-tolerant mandarin 'LB8-9' in winter, spring, summer, and fall. Significant variations in differentially expressed genes (DEGs) related to HLB were observed among the four seasons. For both cultivars, the highest number of DEGs were found in the spring. CLas infection stimulates the expression of immune-related genes such as NBS-LRR, RLK, RLCK, CDPK, MAPK pathway, reactive oxygen species (ROS), and PR genes in both cultivars, consistent with the model that HLB is a pathogen-triggered immune disease. HLB-positive mandarin 'LB8-9' trees contained higher concentrations of maltose and sucrose, which are known to scavenge ROS. In addition, mandarin 'LB8-9' showed higher expression of genes involved in phloem regeneration, which might contribute to its HLB tolerance. This study shed light on the pathogenicity mechanism of the HLB pathosystem and the tolerance mechanism against HLB, providing valuable insights into HLB management.

Keywords: antioxidants, '*Candidatus* Liberibacter asiaticus', citrus, greening, HLB, immune-mediated plant disease, phloem function, reactive oxygen species

Citrus huanglongbing (HLB, also known as citrus greening) is the most devastating citrus disease worldwide. It is caused by the phloem-colonizing α-proteobacteria '*Candidatus* Liberibacter asiaticus' (CLas), '*Ca*. L. africanus', and '*Ca*. L. americanus' (CLam), with CLas being the most widespread pathogen (Bové 2006; Garnier et al. 2000; Jagoueix et al. 1994, 1997; do Carmo Teixeira et al. 2005). CLas is transmitted by Asian citrus psyllids (*Diaphorina citri*) or African citrus psyllids (*Trioza erytreae*) (da Graça et al. 2022). CLas invades psyllids cells via endocytosis- and exocytosis-like mechanisms (Lin et al. 2022). Consistent with CLas being the most prevalent HLB pathogen, *D. citri* preferentially transmit CLas compared with CLam from infected plants (Gasparoto et al. 2022). Most commercial citrus cultivars are susceptible to HLB (Folimonova et al. 2009). Partly owing to the phloem localization of the causative agent, HLB management is notoriously challenging. For citrus-producing regions with low HLB incidence, citrus growers have relied on psyllid control, the removal of inoculum (HLB-diseased trees), and replantation with HLB-free trees (Alquezar et al. 2022; Wang 2019; Yuan et al. 2021b). For HLB endemic regions, the focus has been on improving tree performance using horticultural approaches (Alquezar et al. 2022; Li et al. 2019). Diverse approaches have been tested to manage HLB, including antimicrobials (Akula et al. 2011; Huang et al. 2021; Li et al. 2019, 2021; Zhang et al. 2014, 2021), plant defense inducers (Huang et al. 2021; Li et al. 2019), growth hormones (Canales et al. 2016; Tang and Vashisth 2020), heat treatment (Hoffman et al. 2013; Li et al. 2019; Thapa et al. 2021), microbiome manipulation (Riera et al. 2017; Wang et al. 2017), and enhanced nutrition programs (Gottwald et al. 2012; Stansly et al. 2014). However, none of the approaches has solved the HLB crisis. The development of HLB-resistant or HLB-tolerant citrus cultivars is deemed to be the most efficient, economic, and environmentally sustainable approach for HLB management.

It is critical to understand the pathogenicity mechanism of HLB to develop efficient disease management approaches and to breed HLB-resistant/tolerant citrus cultivars. CLas triggers phloem plugging due to callose deposition and phloem protein accumulation (Achor et al. 2010, 2020; Kim et al. 2009; Koh et al. 2012). Starch accumulation was suggested to contribute to blotchy mottle symptoms (Gonzalez et al. 2012). CLas infection results in root decay before HLB symptom development (Johnson et al. 2014; Vasconcelos et al. 2021). Vasconcelos et al. (2021) quantified the metabolic burden of CLas on surrounding plant cells. It was estimated that the ratio of citrus cells to CLas cells ranges from 39 to 3,760, which

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is much higher than most apoplastic bacterial pathogens. Multiple studies have suggested the involvement of Sec-dependent effectors and prophage genes in HLB disease development or suppressing plant defense (Clark et al. 2018; Jain 2015; Jain et al. 2018; Prasad et al. 2016; Pang et al. 2020; Pitino et al. 2018; Thapa et al. 2020). Our recent study demonstrates that CLas triggers systemic, chronic, and excessive reactive oxygen species (ROS) production that stimulates cell death in phloem tissues, which in turn is responsible for HLB symptoms, representing a pathogen-triggered immune disease (Ma et al. 2022).

'LB8-9' Sugar Belle (SB) is a relatively new commercial citrus cultivar that was released in 2006. Intriguingly, SB demonstrates superior growth, vigor, and fruit yield compared with most susceptible citrus cultivars, such as Valencia sweet orange, in the presence of HLB (Gmitter et al. 2010; Stover et al. 2016b). When infected by HLB, SB develops blotchy mottle symptoms and presents detectable bacterial titers similar to other susceptible cultivars. However, the HLB-positive SB seldom shows the thinned canopy phenotype of susceptible citrus cultivars (Deng et al. 2019; Stover et al. 2016a). Deng et al. (2019) showed that HLB causes similar phloem necrosis, phloem plugging, and starch accumulation as susceptible citrus cultivars but suggested that lower levels of phloem disruption and greater phloem regeneration capacity are responsible for the HLB tolerance of SB.

Transcriptomic analyses of host response have been widely used to investigate the potential mechanism for disease susceptibility, resistance, or tolerance. Multiple studies were conducted to investigate the pathogenicity mechanism of CLas, or CLam, in diverse citrus cultivars and the tolerance mechanisms of citrus cultivars (such as US-897 [*Citrus reticulata* Blanco × *Poncirus trifoliata* L. Raf.], rough lemon [*Citrus jambhiri*], and 'Jackson' grapefruit-like hybrid) in response to HLB (Wang et al. 2016; Yu et al. 2017). However, transcriptomic analyses of SB mandarin infected with CLas have not yet been reported. Multiple transcriptomic analyses of *C. sinensis* have been conducted in the past (Albrecht and Bowman 2008; Balan et al. 2018; Chin et al. 2020, 2021; Fu et al. 2016; Kim et al. 2009; Martinelli and Dandekar 2017; Martinelli et al. 2013; Ramsey et al. 2020; Zheng and Zhao 2013). Previous studies mostly focused on single time point data collection in either the greenhouse or the grove. Due to the complex environmental conditions, single time point data collection might not represent a comprehensive picture of the transcriptome. Changes in day length and temperature have profound effects on plant growth (Nagano et al. 2019a) and plant pathogen responses (Nagano et al. 2019a), which might affect how citrus responds to CLas. Temperature is known to affect CLas growth (Lopes et al. 2017; Thapa et al. 2021), thus affecting bacterial titers and probably the host response. Here, we evaluated the seasonal transcriptomic responses of HLB-tolerant mandarin and HLB-susceptible Valencia sweet orange to CLas infection between symptomatic and asymptomatic leaf samples in the winter, spring, summer, and fall.

Materials and Methods

Overall experimental design

Citrus leaf samples were collected on the 20th day of January (winter), April (spring), July (summer), and October (fall) of 2019. Each biological replicate of leaf samples consisted of four pooled leaves collected from different sections of one plant. The pooled leaf samples were used for DNA and RNA extraction and leaf starch and sugar analysis.

Plant material

Samples were collected in a citrus grove located in Avon Park, Florida (location coordinates: 27°35′15.5″N 81°34′17.0″W). Fully expanded mature leaf samples (developmental stage V6) (Cifuentes-Arenas et al. 2018; Ribeiro et al. 2021) were collected from Valencia sweet orange (*Citrus sinensis*); Pummelo (*Citrus maxima*) × (type-2 early admixture mandarins *Citrus reticulata*) (Wu et al. 2018), grafted to a rootstock X639 [Cleopatra mandarin (*Citrus reshni*) × Flying Dragon (*Poncirus trifoliata*)] and 'LB8-9' SB mandarin ('Clementine' mandarin (*Citrus clementina*) × 'Minneola' tangelo ('Duncan grapefruit' {*C. paradisi*} × 'Dancy tangerine {*C. reticulata*})) (Stover et al. 2016a) grafted to a US-942 rootstock (Sunki (*Citrus reticulata*) × Flying Dragon (*Poncirus trifoliata*)). The trees were planted in 2016. Samples were collected in the morning between 9:30 a.m. and 11:30 a.m. (EST). Samples were collected from symptomatic and asymptomatic trees by visual observation of blotchy mottle symptoms followed by qPCR confirmation using CLas-specific primers/probes (Wang et al. 2006). Six biological replicates from symptomatic and asymptomatic plants were collected for each time point, with one tree as one replicate (Supplementary Table S1). Each biological replicate of leaf sample consisted of four leaves collected from different sections of one plant. Samples were frozen in nitrogen and transported to the lab for further processing in dry ice.

Detection of CLas

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method. Extracted DNA samples were treated with the One-Step PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, U.S.A.). For quantitative real-time PCR (qPCR), 100 ng of total genomic DNA was mixed with Quantitec Probe PCR master mix (Qiagen, Hilden, Germany) and the primers/probe CQULA04F-CQULAP10-CQULA04R (10 μM) (Kim and Wang 2009). All qPCR assays were performed using a QuantStudio 3-96-Well 0.1 ml instrument (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Three biological replicates from each tissue and symptomatic condition were selected for each time point (winter, spring, summer, and fall). For asymptomatic samples, we selected samples with higher Ct values indicating lower CLas titers. For symptomatic samples, we selected samples with lower Ct values indicating higher CLas titers (Supplementary Table S1). In total, 48 samples from 96 samples (Supplementary Table S1) were selected for further experiments based on Ct values.

RNA extraction, RNA sequencing, and data analyses

Total RNA was extracted using an RNeasy Plant Kit (Qiagen, Valencia, CA, U.S.A.), followed by treatment with RQ1 RNase-Free DNase (Promega, Madison, WI, U.S.A.). RNA concentration and quality were measured by a Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). Samples meeting the following requirements (concentration $\geq$ 20 ng/μl, OD260/280 $>$ 2.0) were sent to Novogene (Davis, CA, U.S.A.) for cDNA library construction and RNA-seq analyses. Libraries were constructed with the NEBNext Ultra II RNA Library Prep Kit from Illumina (San Diego, CA, U.S.A.). Samples were sequenced to generate 100-bp paired-end reads using the Illumina NovaSeq 6000 platform (Illumina). Data were filtered to remove low-quality reads and adapters by Novogene (Supplementary Table S2). The biosamples are PRJNA739184 for Valencia and PRJNA739186 for SB mandarin. Clean reads were then aligned to the Citrus sinensis v2 genome from Huazhong Agricultural University (Xu et al. 2012) with HISAT2 (Kim et al. 2019) and SAMtools (Li et al. 2009) to select good-quality alignments (Supplementary Table S2). Raw counts were quantified using HTSeq-count (Anders et al. 2015). Based on the normalized gene expression data using the DESeqVS and log2 scale methods (Love et al. 2014), samples were clustered based on Euclidean distance. We removed the outlier samples identified by hierarchical clustering using normalized gene expression data. These outlier samples probably resulted from technical issues (Supplementary Figs. S1 and S2) (Brechtmann et al. 2018; Kremer et al. 2017). Differentially expressed gene (DEG) analysis was performed using DESeq2 packages in R (Love et al. 2014). Genes were considered significantly expressed with an adjusted $P < 0.05$ (FDR method) (Bourgon et al. 2010). Gene Ontology (GO) term enrich-

ment of DEGs was analyzed using AgriGO v2.0: a GO analysis toolkit for the agricultural community (Tian et al. 2017) using the singular enrichment analysis tool. Heatmap plots of gene expression data were drawn using the gplots package in the R program (Warnes et al. 2020).

Starch and sugar quantification

From the same samples selected for qPCR, total starch was extracted with an aqueous ethanol solution (80% vol/vol) from 100 mg of frozen ground tissue as described previously (Macrae et al. 1974). Starch was quantified using the total starch assay procedure (Megazyme, Bray, Ireland), following the manufacturer's instructions, which combines amyloglucosidase and α-amylase in a colorimetric method (McCleary et al. 2019). Soluble sugars were extracted from 100 mg of frozen ground tissue with deionized water, vortexed, homogenized, incubated at 65°C, and centrifuged three times. The final extract was boiled at 100°C for 30 min to denature all enzymes, as described elsewhere (Maness 2010). Maltose, sucrose, D-fructose, and D-glucose (Megazyme) were then quantified following the manufacturer's protocol.

Results

Seasonal CLas titers in Valencia sweet orange and SB mandarin

There were no significant differences in CLas titers between Valencia sweet orange and SB mandarin in symptomatic or asymptomatic mature leaf samples in all four seasons (Fig. 1). CLas titers were higher in symptomatic leaves than asymptomatic leaves (Fig. 1), which is consistent with previous studies (Pandey et al. 2022; Trivedi et al. 2009). Significant differences were observed between symptomatic and asymptomatic samples for Valencia in all four seasons, whereas a significant difference was observed for Sugar Belle only in winter. Nevertheless, among the leaf samples selected for RNA-seq, CLas titers were significantly higher in the symptomatic samples than in the asymptomatic ones in both cultivars among four seasons (Supplementary Table S1).

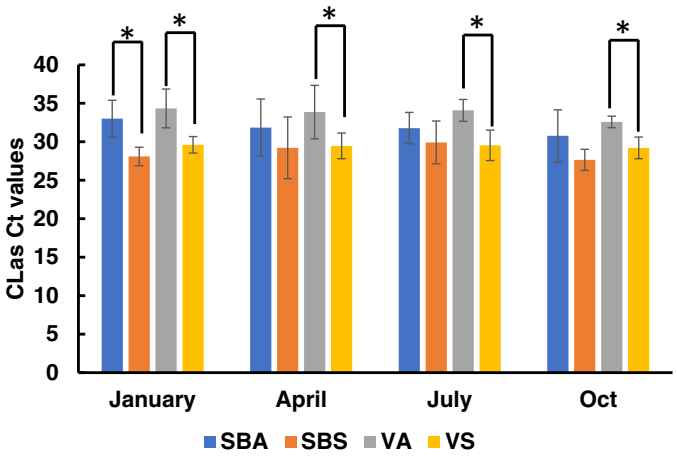

Fig. 1. 'Candidatus Liberibacter asiaticus' (CLas) titers in Valencia sweet orange (V) and Sugar Belle mandarin (SB) in different seasons quantified by quantitative PCR. Citrus leaf samples were collected in January (winter), April (spring), July (summer), and October (fall) of 2019. Each biological replicate of leaf samples consisted of four pooled leaves collected from different sections of one plant. Each experiment included six biological replicates. *, $P < 0.05$.

Overview of RNA-seq analyses

We conducted RNA-seq analyses on HLB-susceptible Valencia sweet orange and HLB-tolerant SB mandarin at four time points in 2019, representing four seasons: winter (January), spring (April), summer (July), and fall (October). Mature leaf samples were collected from asymptomatic and symptomatic trees. The Ct values for asymptomatic samples ranged from 32 to 38 and 31 to 37, whereas the Ct values for symptomatic trees ranged from 26 to 30 and 25 to 28 for Valencia sweet orange and SB mandarin, respectively (Supplementary Table S1). Ct values of asymptomatic samples indicate that the asymptomatic trees were infected, but with lower CLas titers than symptomatic trees.

Three biological replicates from each sample were sequenced by the NovaSeq 6000 platform, and approximately 60 million reads of raw data/sample were obtained (Supplementary Table S2). After filtering, approximately 59 million clean reads were obtained for each leaf sample (Supplementary Table S2). On average, 91.45% of the reads were mapped to the *C. sinensis* genome (Supplementary Table S2). In addition to filtering low-quality reads, the reference genome used was a di-haploid of a heterozygous diploid rather than a complete genome; thus, some reads could not be mapped. The samples were clustered based on Euclidean distance calculated using gene expression data. PCA analyses demonstrated that the overall expression profiles of Valencia sweet orange and SB mandarin showed similar segregation. PCA analyses suggested that season is the primary factor that affects citrus expression in response to CLas ($P < 0.01$), which is consistent with our hypothesis. On the other hand, symptomatic/asymptomatic status had no significant effect, indicating the overall robustness of our sampling approach. According to the sample clustering, JanVAL3, JulVAL2, and OctVSL2 of Valencia (Supplementary Fig. S1) and JulSBAL3 and JulSBSL2 of SB mandarin (Supplementary Fig. S2) were considered outliers and not included in downstream analyses. The variations in data might result from the CLas infection stages/disease progress of different leaf samples. Removing outliers is a commonly used strategy in RNA-seq analyses (Brechtmann et al. 2018; Kremer et al. 2017).

Pairwise comparisons were performed between symptomatic and asymptomatic samples for each time point (Fig. 2). For Valencia leaves, the number of DEGs was 172, 6,670, 677, and 2,655 in

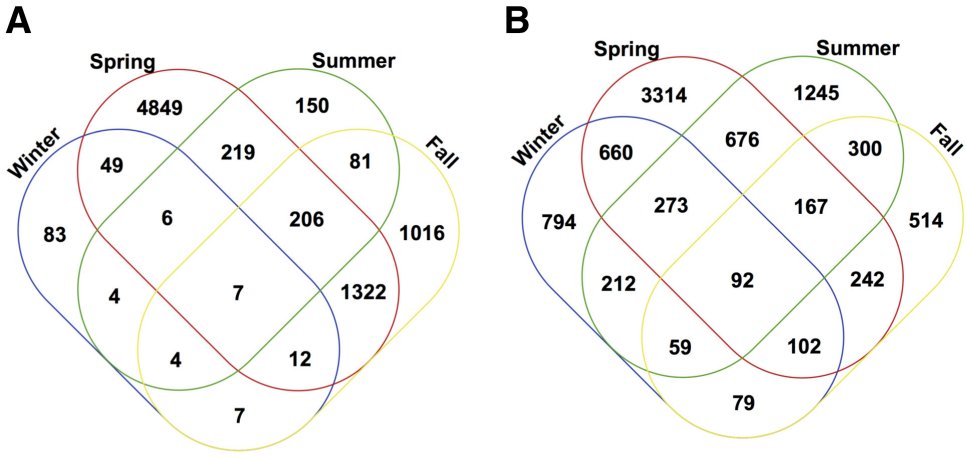

Fig. 2. Venn diagram depicting the number of differentially expressed genes (DEGs) across four seasons for **A,** Valencia sweet orange and **B,** Sugar Belle (SB) mandarin. DEGs were defined as genes with a *P* value less than 0.05.

the winter, spring, summer, and fall, respectively (Supplementary Dataset S1). For SB mandarin leaves, the number of DEGs was 2,271, 5,526, 3,024, and 1,555 in the winter, spring, summer, and fall, respectively (Supplementary Dataset S1). Large variation was observed between DEGs in different seasons for both SB mandarin and Valencia (Fig. 2), and the numbers of upregulated and downregulated genes differed between cultivars in different seasons (Supplementary Fig. S3). For Valencia, there were 0, 71, 27, and 88 enriched GO terms in the winter, spring, summer, and fall, respectively. For SB mandarin, there were 78, 95, 125, and 86 enriched GO terms in the winter, spring, summer, and fall, respectively (Supplementary Dataset S2).

Transcriptional profiling of genes related to the plant immune response to HLB

Here, we analyzed the DEGs related to the plant immune response to HLB. CLas triggered both upregulation and downregulation of PR genes in Valencia and SB mandarin. For Valencia, a total of 39 upregulated features (with a feature defined as one DEG at a certain time point) and 17 downregulated features were identified, whereas 36 upregulated features and 17 downregulated features were identified for SB mandarin, indicating an overall induction of PR genes by CLas in both Valencia and mandarin (Fig. 3). Mitogen-activated protein kinase (MAPK), MAPKK, and MAPKKK genes were mostly upregulated in both Valencia and SB mandarin (Supplementary Fig. S4). NBS-LRR genes showed a more complex regulation pattern. A total of 99 upregulated features and 110 downregulated features were identified for Valencia. On the other hand, 112 upregulated features and 59 downregulated features were identified for SB mandarin (Supplementary Fig. S5). Genes encoding receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs) were induced by CLas for both Valencia (283 upregulated features and 134 downregulated features) and SB mandarin (281 upregulated features and 144 downregulated features) in the four seasons (Supplementary Figs. S6 to S8). In addition, Ca^{2+}-dependent protein kinase (CDPK) DEGs were mostly upregulated by CLas (Supplementary Fig. S9). Significant variations were observed for immune response-related genes among seasons and between Valencia and mandarin (Fig. 3, Supplementary Figs. S4 to S8).

CLas significantly affects ROS pathways and antioxidant genes in both Valencia and SB mandarin

CLas was reported to activate excessive ROS production in sweet orange (Ma et al. 2022). CLas stimulated the expression of *RbohB*, *RbohC*, *RbohD*, and *RbohF* in both Valencia and SB mandarin (Fig. 4). *RbohB*, *RbohD*, and *RbohF* are known to be responsible for ROS production against pathogen infection and pathogen-associated molecular patterns (PAMPs) (Torres et al. 2002; Yoshioka et al. 2003). It is important to note that *RbohB*, *RbohC*, *RbohD*, and *RbohF* were not induced in all four seasons in both Valencia and SB mandarin, probably due to environmental effects.

DEGs encoding antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase, thioredoxins, and glutaredoxins were mostly downregulated by CLas (Fig. 5; Supplementary Fig. S10), whereas other antioxidant enzyme genes, such as glutathione peroxidase (GPX) genes, showed more complex expression patterns (Supplementary Fig. S11).

DEGs involved in the biosynthesis of nonenzymatic antioxidants, such as ascorbic acid, tocopherols, glutathione, carotenoids, and phenolic compounds, were mostly upregulated in both Valencia and SB mandarin (Supplementary Fig. S12).

SB mandarin showed higher expression of DEGs involved in phloem specification and differentiation than Valencia

It was reported that SB mandarin has a more robust phloem regeneration capacity than Valencia in response to CLas infection (Deng et al. 2019). CLas stimulated an overall induction of DEGs involved in phloem specification and differentiation (Fig. 6; Supplementary Dataset S3) in both Valencia and SB mandarin; however, SB mandarin demonstrated a higher expression level than Valencia, especially in the summer (Fig. 6; Supplementary Dataset S3). Similar patterns were observed for DEGs related to the phloem cell wall and cytoskeleton (CWC), subcellular trafficking, and cell polarity (STCP) (Fig. 6; Supplementary Dataset S3).

Transcriptional profiling of hormone genes in response to HLB

Cytokinin-related DEGs were mostly downregulated by CLas in both Valencia and SB mandarin (Fig. 7). In contrast, ethylene-related DEGs were upregulated by CLas (Fig. 7). DEGs related to auxin, salicylic acids, abscisic acid (ABA), gibberellic acids, and jasmonic acids showed mixed expression patterns (Fig. 7). Significant variation was also observed for different hormone-related genes in different seasons (Fig. 7).

Transcriptional profiling of callose synthase and phloem protein genes in response to HLB

Phloem blockage related to callose deposition and phloem protein accumulation is commonly observed in HLB-positive leaf tissues

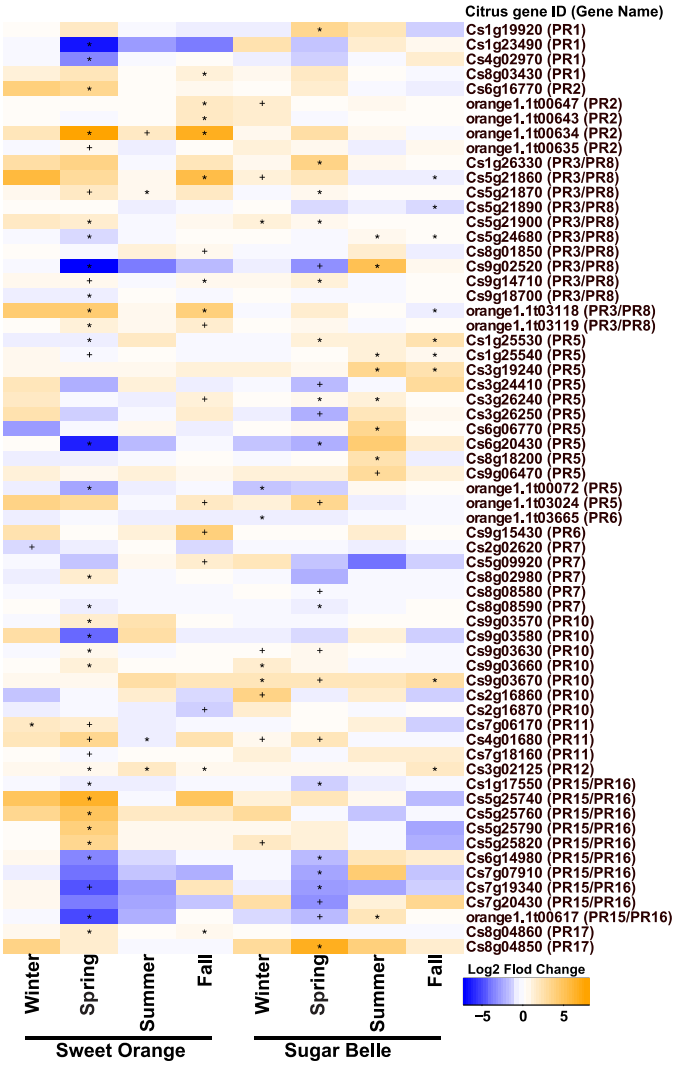

Fig. 3. Expression profiling of PR genes between huanglongbing (HLB) symptomatic and asymptomatic samples of Valencia sweet orange and Sugar Belle (SB) mandarin. The affiliation of each gene is indicated in brackets. Orange denotes higher expression in symptomatic samples than asymptomatic samples, whereas blue denotes higher expression in asymptomatic samples than symptomatic samples. The asterisk denotes a P value < 0.01; the plus sign denotes a P value < 0.05.

(Maness 2010). Intriguingly, callose synthase DEGs showed an overall downregulation in Valencia but were mostly upregulated in SB mandarin in response to CLas infection (Supplementary Fig. S13). DEGs related to phloem proteins were mostly upregulated and found in the spring, with few identified in other seasons, for both Valencia and SB mandarin (Supplementary Fig. S13).

Seasonal starch metabolism response to HLB

Starch accumulation in CLas-positive leaves is one of the most prominent characteristics of HLB. For both Valencia and SB mandarin, most starch synthesis- and degradation-related DEGs were upregulated, except for *glgC*, which encodes glucose-1-phosphate adenylyltransferase and participates in starch synthesis (Supplementary Fig. S13). More upregulated *glgC* DEG features were identified in SB mandarin than in Valencia (Supplementary Fig. S13). Significant variations were observed for starch metabolism genes in different seasons (Supplementary Fig. S13).

Next, we measured starch, sucrose, glucose, and maltose contents and correlated them with the observed expression patterns. Starch significantly accumulated in symptomatic leaves compared with asymptomatic leaves in the winter, spring, and fall but not in the summer for SB mandarin, whereas in Valencia, starch accumulation in symptomatic leaves was observed only in the spring and summer in comparison with asymptomatic leaves (Fig. 8). The sucrose concentration in symptomatic leaves was significantly higher than that of asymptomatic leaves in the spring and summer, but it was similar across the other two seasons in SB mandarin (Fig. 8A). Conversely, the sucrose concentration in symptomatic leaves was significantly decreased in the spring but was similar in the other seasons for Valencia (Fig. 8B). The glucose content was significantly higher in symptomatic leaves than in asymptomatic leaves of SB mandarin in all seasons, but no significant changes were observed in Valencia leaves. The maltose content was significantly higher in the symptomatic leaves than in the asymptomatic leaves in the winter and fall for SB mandarin, whereas it was decreased in Valencia in the winter, spring, and summer.

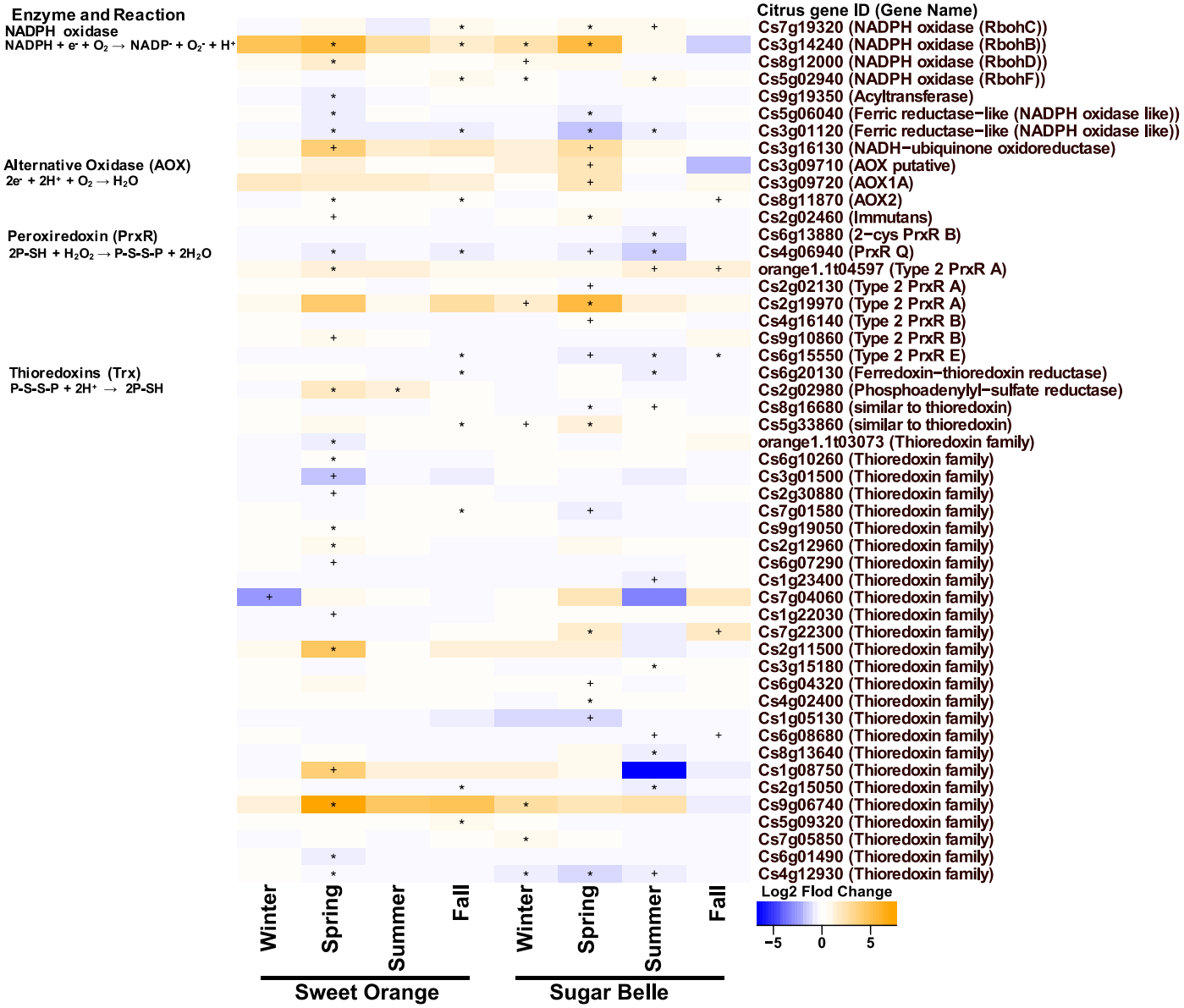

Fig. 4. Expression profiling of reactive oxygen species (ROS) genes encoding NADPH oxidase, alternative oxidase, peroxiredoxin, and thioredoxins between huanglongbing (HLB) symptomatic and asymptomatic samples of Valencia sweet orange and Sugar Belle (SB) mandarin. The affiliation of each gene is indicated in brackets. Orange denotes higher expression in symptomatic samples than asymptomatic samples, whereas blue denotes higher expression in asymptomatic samples than symptomatic samples. The asterisk denotes a P value < 0.01; the plus sign denotes a P value < 0.05.

Discussion

The citrus response to CLas infection is affected by seasonal changes

In this study, we evaluated two different cultivars planted in the same location with contrasting responses (susceptible and tolerant) to HLB in winter, spring, summer, and fall. Significant variations were observed among the four seasons. Seasonal transcriptomic variability in field plants has been previously reported (Aikawa et al. 2010; Burow and Halkier 2017; Chin et al. 2020, 2021; Honjo et al. 2020; Kudoh 2019; Nagano et al. 2012, 2019b; Plessis et al. 2015; Ramsey et al. 2020). This probably reflects the effect of temperature and day length on the citrus transcriptome and adaptation to seasonal environments (Nagano et al. 2019b). Temperature affects plant development and HLB disease progression (Lopes et al. 2017; Nagano et al. 2019b; Thapa et al. 2021). In addition, because we selected trees for sampling in different seasons, the transcriptomic variations might partially result from the effect of HLB disease progression over time. Transcriptome profiling of citrus to CLas in four seasons enables a more comprehensive view, which is missing when analyzing single time point data. The significant seasonal effect on HLB-induced host transcriptome calls for caution in relying on a single time point for transcriptional profiling of HLB and probably other pathosystems. It is noteworthy that the transcriptome data are between asymptomatic and symptomatic samples rather than healthy versus CLas infected samples because almost all trees were infected in the groves in Florida.

The relationship between HLB susceptibility and tolerance and CLas titers

Most commercial citrus varieties are susceptible to HLB with few exceptions, such as SB (Deng et al. 2019; Wu et al. 2018). Multiple citrus relatives, such as *Microcitrus australis*, *M. warbur-*

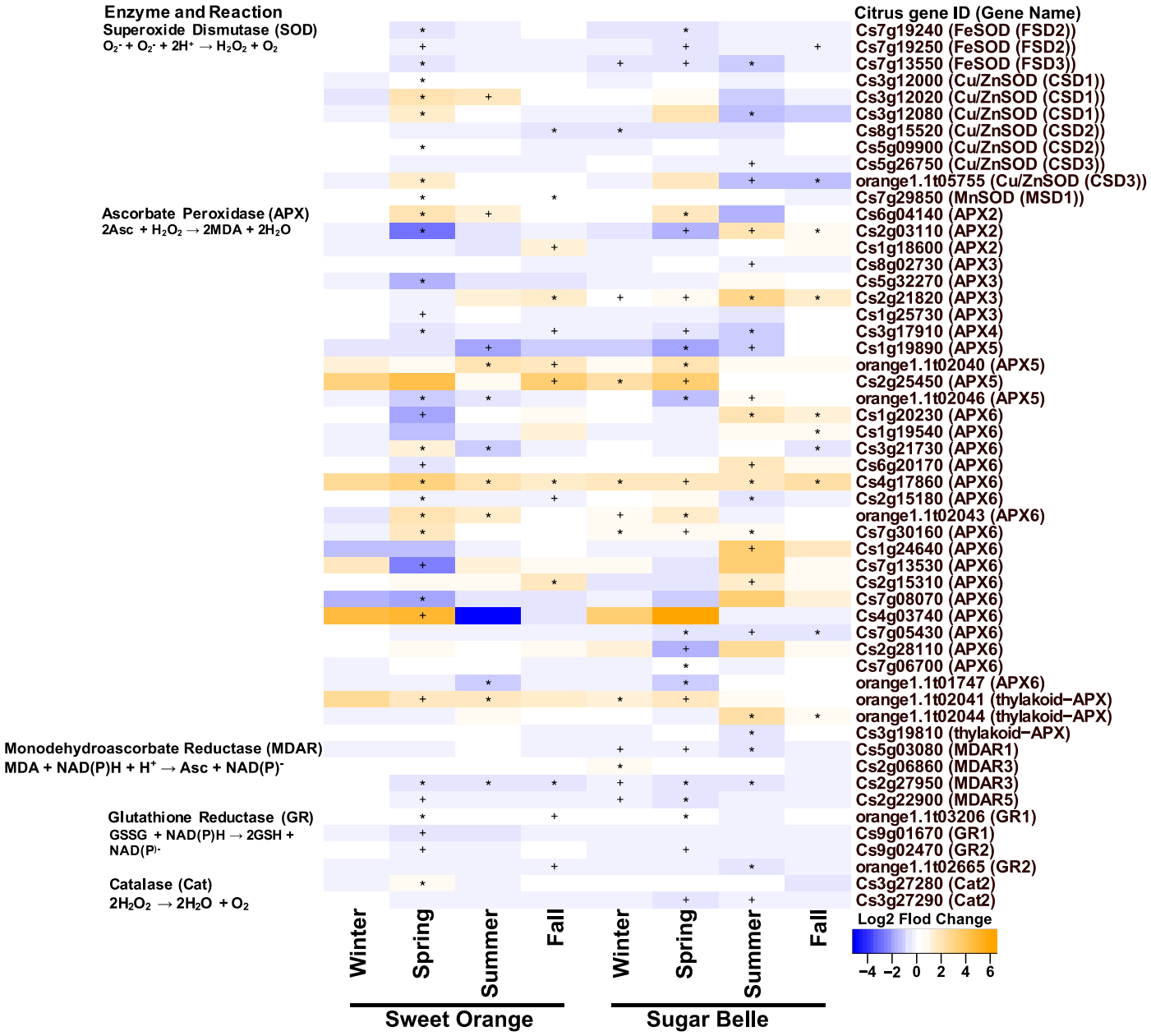

Fig. 5. Expression profiling of reactive oxygen species (ROS)-related genes encoding superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, glutathione reductase, and catalase between huanglongbing (HLB) symptomatic and asymptomatic samples of Valencia sweet orange and Sugar Belle (SB) mandarin. The affiliation of each gene is indicated in brackets. Orange denotes higher expression in symptomatic samples than asymptomatic samples, whereas blue denotes higher expression in asymptomatic samples than symptomatic samples. The asterisk denotes a P value < 0.01; the plus sign denotes a P value < 0.05.

giana, *M. papuana*, *Eremocitrus glauca*, and *Swinglea glutinosa*, have shown tolerance/resistance against HLB (Alves et al. 2020; Cifuentes-Arenas et al. 2018). Pathogen titers have been commonly used as an indicator for disease resistance (Pagán and García-Arenal 2018). A strong correlation between HLB rating and CLas titer was previously reported for multiple citrus cultivars (Stover and McCollum 2011), consistent with the observation that CLas titers are higher in symptomatic leaves than asymptomatic leaves (Pandey et al. 2021). A novel class of stable antimicrobial peptides (SAMPs) was identified from Australian finger lime and relatives, which might contribute to the lower titers of CLas in those genotypes (Alves et al. 2020; Cifuentes-Arenas et al. 2018). However, similar CLas titers were observed in Valencia sweet orange and SB mandarin, indicating that SB mandarin's tolerance against HLB is not dependent on the antimicrobial properties of the host.

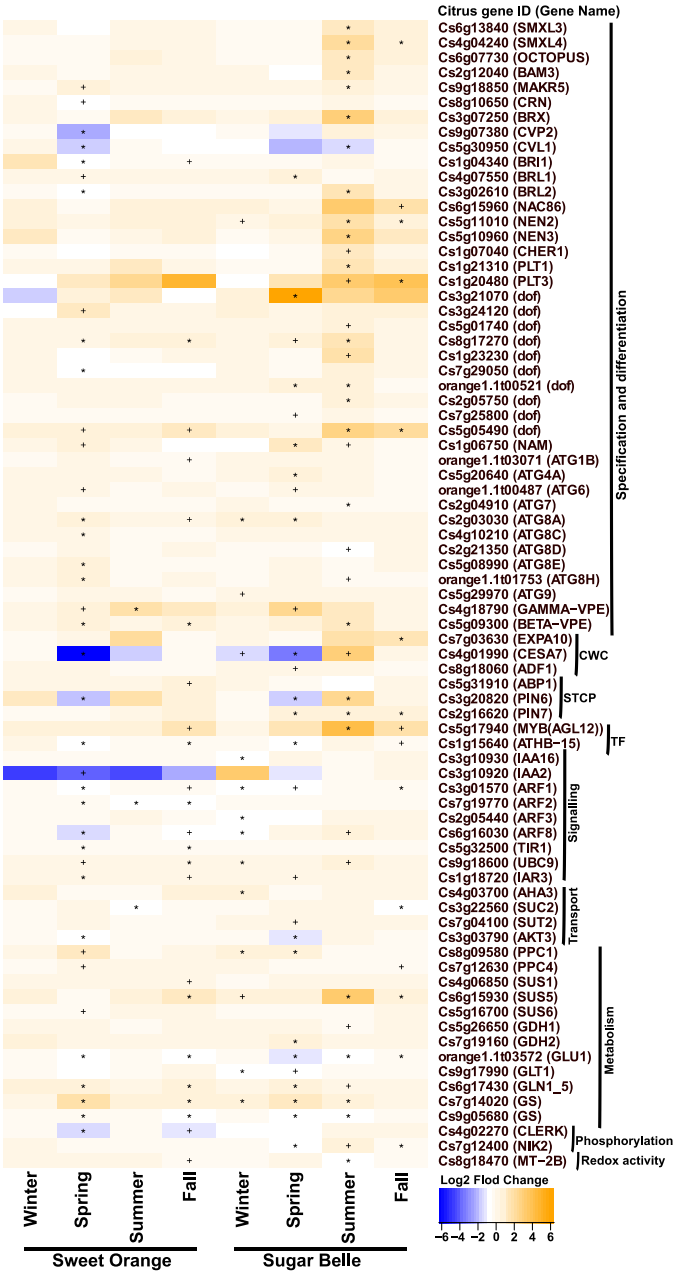

Fig. 6. Expression profiling of phloem-specific genes, such as phloem specification and differentiation, phloem cell wall and cytoskeleton (CWC), subcellular trafficking and cell polarity (STCP), transcription factors (TFs), transport, metabolism, phosphorylation, and redox activity, between huanglongbing (HLB) symptomatic and asymptomatic samples of Valencia sweet orange and Sugar Belle (SB) mandarin. The affiliation of each gene is indicated in brackets. Orange denotes higher expression in symptomatic samples than asymptomatic samples, whereas blue denotes higher expression in asymptomatic samples than symptomatic samples. The asterisk denotes a P value < 0.01; the plus sign denotes a P value < 0.05.

CLas induces immune responses in both HLB-susceptible Valencia sweet orange and HLB-tolerant SB mandarin

CLas infection stimulates the expression of NBS-LRR, RLK, RLCK, CDPK, MAPK pathway, ROS, and PR genes in both Valencia and SB mandarin. Induction of NBS-LRR, RLK, RLCK, CDPK, MAPK pathway, ROS, and PR genes is characteristic of a PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Boller and Felix 2009; Segonzac and Zipfel 2011). It was reported that CLas infection of phloem tissues triggers systemic and chronic immune responses that stimulate excessive ROS production, which causes the death of sieve elements and companion cells in susceptible sweet orange. Cell death in phloem tissues subsequently leads to HLB symptoms (Ma et al. 2022). The similar expression patterns of immune-related genes between Valencia and SB mandarin suggest that the HLB-tolerant mandarin does not have a superior immune system compared with Valencia. Instead, the HLB symptoms of SB mandarin probably result from a similar immune response to CLas as that in Valencia. This is consistent with the observation that CLas infections trigger similar phloem cell death and callose depositions in SB mandarin as in Valencia (Deng et al. 2019), which is further corroborated by similar ROS production in both cultivars. Both cultivars resulted from complex introgression hybridization of mandarin and pummelo, although with different proportions of the ancestral species in their background despite their different paths in hybridization. In addition, similar immune responses, including ROS and callose deposition, were observed in both HLB-tolerant Persian lime (*C. latifolia*) and HLB-susceptible Mexican lime (*C. aurantiifolia*) (Sivager et al. 2021).

ROS production is primarily dependent on RBOHD in response to PAMP activation or pathogen attacks in Arabidopsis, tobacco, and rice (Kobayashi et al. 2007; Nühse et al. 2007; Simon-Plas et al. 2002; Torres et al. 2002; Wong et al. 2007; Yoshioka et al. 2003; Zhang et al. 2007). In addition, RBOHF is also involved in ROS production upon PAMP activation or pathogen infection in Arabidopsis (Torres et al. 2002). RBOHB plays similar roles in potato (Kobayashi et al. 2007). RBOH genes responsible for ROS production are induced by pathogen infection and immune recognition (Yuan et al. 2021a). CLas infection triggers the expression of four NADPH oxidase genes, *RBOHB*, *RBOHC*, *RBOHD*, and *RBOHF*. Expression of the *RBOHB* gene in *Nicotiana benthamiana*, the ortholog of *RBOHD* of Arabidopsis, is induced by a MAPK cascade (Yoshioka et al. 2003). The MAPK pathway was also induced by CLas.

CLas infection induces the expression of NBS-LRR genes in both Valencia and SB mandarin. However, large variations were observed between the induced NBS-LRR genes. Among the 99 upregulated NBS-LRR features for Valencia and 112 upregulated NBS-LRR features for SB mandarin, only 25 overlapped. NBS-LRR-encoding genes are constitutively expressed at low levels in asymptomatic and unchallenged tissues and are induced in response to PAMP activation or pathogen infection (Chakraborty et al. 2016; Lai and Eulgem 2018). The expression levels of NBS-LRR genes vary considerably in susceptible and resistant cultivars of *Vitis vinifera* in response to *Erysiphe necator* infection (Goyal et al. 2020). Higher expression of NBS-LRR genes was observed in roots of coconut genotypes resistant to root wilt disease caused by phytoplasma than in susceptible genotypes (Rachana et al. 2016). How CLas induces NBS-LRRs and how their induction affects the immune response remain to be characterized.

CLas infection causes oxidative stress in both Valencia and mandarin

Oxidative stress results from the overproduction and accumulation of ROS. One potential reason for such excessive ROS produc-

tion is an imbalance between ROS generation and detoxification (Dumanović et al. 2021). CLas induces overexpression of RBOH genes but suppresses the expression of many genes encoding antioxidant enzymes, such as SOD, APX, catalase, thioredoxins, and glutaredoxins. It is possible that this disparity in RBOH and antioxidant enzyme genes and enzyme activities lead to ROS accumulation, thus causing oxidative stress. Damage to lipids, proteins, and DNA caused by oxidative stress can cause cell death. Similar expression patterns were observed for both enzymatic and nonenzymatic antioxidants (such as ascorbic acid, tocopherols, glutathione, carotenoids, and phenolic compounds) between HLB-susceptible Valencia and HLB-tolerant SB mandarin. However, SB mandarin and Valencia showed similar trends in glutathione reductase but different trends in guaiacol peroxidase, catalase, APX, and SOD in response to CLas infection. It is not expected that this difference plays a critical role in the oxidative stress in these two cultivars because CLas infection causes significant ROS accumulation in both SB mandarin and Valencia (Ma et al. 2022).

SB mandarin is known to contain high concentrations of phenolic compounds (Killiny et al. 2017). Mono- and disaccharides have been suggested to act as ROS scavengers (Couée et al. 2006), and their ability to scavenge •OH is as follows: maltose > sucrose > fructose > glucose > deoxyribose > sorbitol (Morelli et al. 2003). HLB-positive SB mandarin trees contained higher concentrations of maltose and sucrose in winter, spring, or summer than Valencia trees, which probably contributed to the superior HLB tolerance of mandarin trees. Biochemical analyses revealed better detoxification capacity in Persian limes than in Mexican limes (Sivager et al. 2021), suggesting that ROS tolerance is critical for HLB-tolerant cultivars, and they might utilize diverse approaches to manipulate oxidative stress.

Differential carbon metabolism modulation in response to HLB

Similar to multiple previous studies (Albrecht and Bowman 2008; Fan et al. 2011, 2013; Kim et al. 2009), both Valencia and SB mandarin demonstrated a mixed expression pattern for the starch synthesis gene ADP-Glc pyrophosphorylase, starch synthases, starch branching enzymes, and starch debranching enzymes. In addition, both cultivars showed an overall upregulation of DEGs involved in starch degradation after CLas infection, as previously reported (Albrecht and Bowman 2008; Martinelli et al. 2013). Transitory starch usually accumulates in leaves during the day as part of the photosynthetic products to be utilized during the night to aid in energy for respiration processes and growth (Smith and Zeeman 2020; Stitt et al. 2012). This process is highly regulated by the circadian rhythm (Flis et al. 2019; Lu et al. 2005; Mugford et al. 2014; Seki et al. 2017) to provide enough energy throughout the day. When plants are stressed, this relationship can be impaired by activating starch degradation enzymes (Doyle et al. 2007; Ribeiro et al. 2022). In Valencia, leaf starch accumulates in warmer seasons; however, in SB mandarin, significant starch accumulation occurs in winter, spring, and fall. Intriguingly, blotchy mottle symptoms in sweet orange have been reported to be less apparent when temperatures are lower (Bové 2006; McClean and Schwarz 1970). This is consistent with the suggestion that starch accumulation contributes to the blotchy mottle symptoms of HLB (Aritua et al. 2013; Koh et al. 2012; Martinelli et al. 2013). On the other hand, it is unknown how different developmental cycles of both cultivars affect starch accumulation in response to HLB, as Valencia fruit ripens between February and June, but SB mandarin fruit ripens from November to January in Florida (Albrecht et al. 2020). Additionally, it was previously reported that increased sugar concentration in infected samples can trigger photosynthetic protein downregulation, probably caused by feedback inhibition (Chin et al. 2021). Consequently, sugar content might also impact vector feeding preferences and dispersal throughout the seasons (Lewis-Rosenblum et al. 2015; Lopes et al. 2017; Pelz-Stelinski and Killiny 2016).

Callose deposition in response to CLas infection

Phloem plugging due to callose deposition and accumulation of phloem proteins has been suggested to contribute to the dysfunction of phloem transportation and HLB disease development (Pandey et al. 2022; Wang et al. 2017). Surprisingly, callose synthase DEGs showed an overall downregulation in Valencia in the spring but were

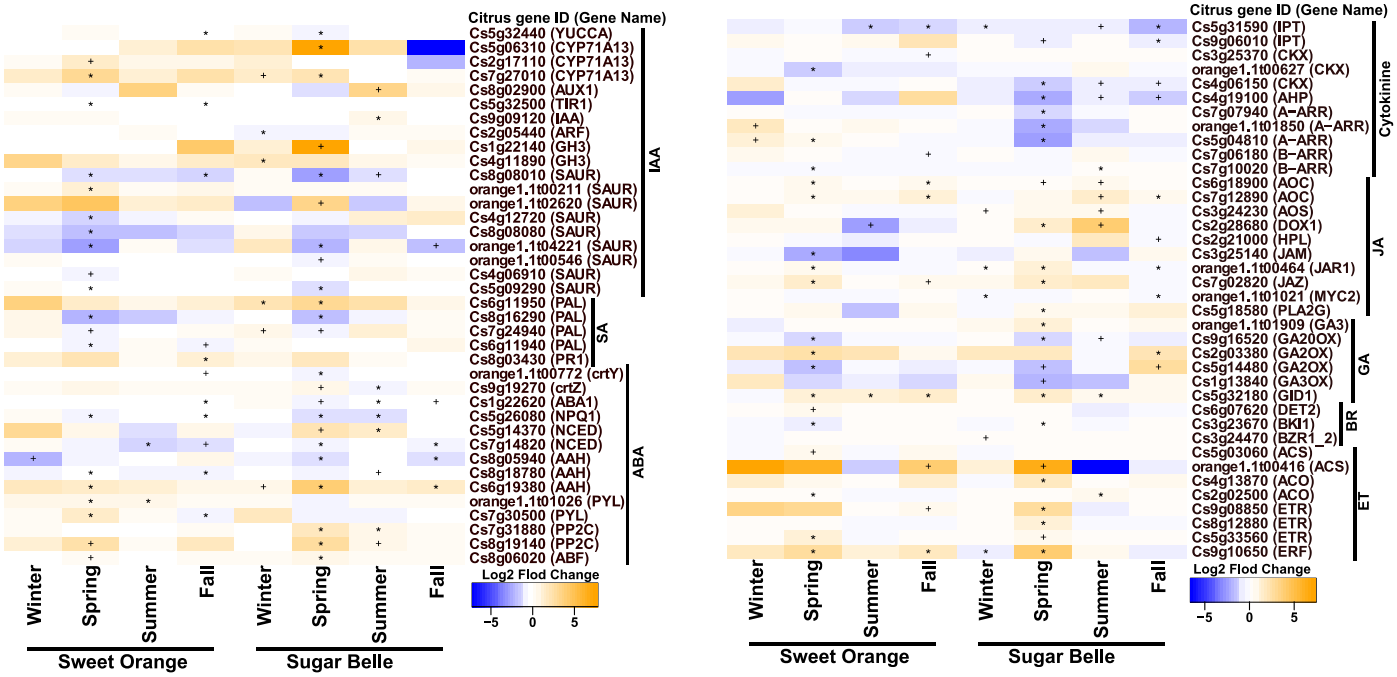

Fig. 7. Expression profiling of genes involved in the synthesis and signaling of cytokinin, jasmonic acid (JA), gibberellin (GA), brassinosteroid (BR), and ethylene (ET) between huanglongbing (HLB) symptomatic and asymptomatic samples of Valencia sweet orange and Sugar Belle (SB) mandarin. The affiliation of each gene is indicated in brackets. Orange denotes higher expression in symptomatic samples than asymptomatic samples, whereas blue denotes higher expression in asymptomatic samples than symptomatic samples. The asterisk denotes a P value < 0.01; the plus sign denotes a P value < 0.05.

mostly upregulated in SB mandarin in response to CLas infection. Specifically, *CalS7* is the callose synthase gene responsible for callose deposition in the phloem (Xie et al. 2011). *CalS7* expression was largely unchanged in HLB-susceptible cultivars in response to CLas in previous studies (Albrecht and Bowman 2008; Fan et al. 2011; Kim et al. 2009; Li et al. 2012). This expression pattern contradicts the observation of callose deposition under a microscope (Kim et al. 2009; Koh et al. 2012). One probable explanation is that CLas infection induces callose synthase genes in the early stage of CLas infection and that significant callose deposition in the late stage inhibits the expression of the *CalS7* gene.

Differential expression of phloem specific genes in Valencia sweet orange and SB mandarin

Deng et al. (2019) reported that SB mandarin has superior phloem regeneration capacity compared with susceptible citrus varieties such as Valencia sweet orange. Consistently, we have observed that multiple genes involved in phloem formation were induced by CLas in SB mandarin including SUPPRESSOR OF MAX2 1-LIKE3 (SMXL3, Cs6g13840) and SMXL4 (Cs4g04240) (Wallner et al. 2017), BRX (Cs3g07250, a novel regulator of cell proliferation and elongation in the root) (Mouchel et al. 2004), OCTOPUS (OPS, a potentiator of phloem differentiation) (Truernit et al. 2012), PLT1 (Cs1g21310) and PLT3 (Cs1g20480) that maintain the meristematic competence of cells (Mizukami and Fischer 2000), NEN2 (Cs5g11010) and NEN3 (Cs5g10960) that drive formation of functional protophloem (Cho et al. 2018), CHER1 (Cs1g07040, a choline transporter-like protein required for sieve plate development) (Dettmer et al. 2014), and DOF (DNA-binding with One Finger) transcription factors (Cs3g21070, Cs5g01740, Cs1g23230, orange1.1t00521, and Cs2g05750) that are involved in plant growth, hormone response, cell cycle regulation, cambium formation, and vascular tissue development (Gabriele et al. 2010; Guo et al. 2009; Kang et al. 2003; Skirycz et al. 2008; Ward et al. 2005). In addition, CLas downregulated BRI1 (Cs1g04340) and BRL2 (Cs3g02610, BRI1 like 2) that regulate plant development in response to brassinosteroid (Wang et al. 2008) in Valencia sweet orange. Protophloem sieve element differentiation is impaired in *bri1 brl1 brl3* mutants of Arabidopsis (Kang et al. 2017). CLas upregulated ADF1 (Cs8g18060, actin-depolymerizing factor 1) that is known to be involved in plant growth and development, and various abiotic and biotic stress responses (Inada 2017). CLas regulated multiple genes related to auxin, which is known to be involved in plant growth, including PIN7 (Cs2g16620, auxin efflux carrier component 7 required for a broad range of developmental and tropic processes; Adamowski and Friml 2015), IAA16 (Cs3g10930), IAA2 (Cs3g10920), ARF2 (Cs7g19770), and ARF3 (Cs2g05440), suggesting that auxin might be involved in the differential responses of SB mandarin and Valencia sweet orange to CLas infection. CLas induced SUT2 (Cs7g04100, sucrose transporter 2) in SB mandarin but not in Valencia, indicating that phloem functions more normally in CLas-infected SB mandarin than Valencia. Moreover, SUT2 is known to contribute to plant growth (Leach et al. 2017).

In summary, seasonal comparison between the transcriptomes of HLB-tolerant SB mandarin and HLB-susceptible Valencia sweet orange in response to CLas provides a dynamic picture of the host response to HLB. CLas infection triggers a similar immune-mediated plant disease in both SB mandarin and Valencia, which results from excessive ROS production and cell death in phloem tissues. SB mandarin has a superior capacity for fine-tuning the expression of genes involved in phloem regeneration and scavenging ROS, which might contribute to its tolerance to HLB. This study shed light on the pathogenicity mechanism of the HLB pathosystem and tolerance mechanism against HLB, and it provides valuable insights into HLB management, such as via genome editing (Huang et al. 2022; Jia and Wang 2020; Jia et al. 2022).

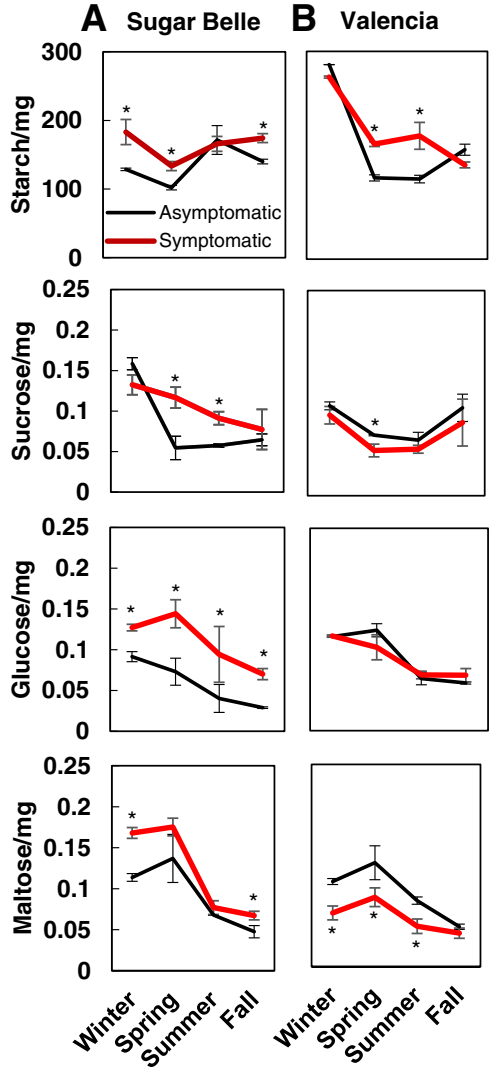

Fig. 8. Seasonal starch and sugar changes in response to huanglongbing (HLB). Starch, sucrose, glucose, and maltose were quantified (per milligram of tissue). **A,** Sugar Belle (SB) mandarin leaf samples; **B,** Valencia leaf samples. Black lines represent the concentrations of starch and sugars in asymptomatic samples, and red lines are the concentrations of starch and sugars in symptomatic samples across the four seasons. Error bars: standard deviation of three biological replicates. *Student's *t* test ($P < 0.05$) (symptomatic versus asymptomatic).

Accession numbers

Citrus sinensis (Valencia orange) v2.0 genome assembly (HZAU) (http://citrus.hzau.edu.cn/orange/). The clean filtered data were uploaded to NCBI and deposited in the sequence read archive (SRA). The biosamples are PRJNA739184 for Valencia and PRJNA739186 for SB mandarin.

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