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Mutations in the *SWEET15* Sugar Transporter Gene Affect Response of Citrus to Huanglongbing Disease and Citrus Canker

Archana Khadgi[1] | Omar Zayed[2] | Cintia H. D. Sagawa[1] | Fei Zhang[1] | Danelle K. Seymour[2] | Vivian F. Irish[1,3]

[1]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut, USA | [2]Botany and Plant Sciences Department, University of California, Riverside, Riverside, California, USA | [3]Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut, USA

Correspondence: Vivian F. Irish (vivian.irish@yale.edu)

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ABSTRACT

Bacterial diseases like huanglongbing (HLB) and citrus canker severely impact citrus production. HLB, caused by "*Candidatus* Liberibacter asiaticus" (*C*Las), leads to tree decline, while citrus canker, caused by *Xanthomonas citri* pv. *citri* (Xcc) causes necrotic lesions on leaves and fruit. Many bacterial pathogens secrete effector proteins that suppress host plant immunity and promote pathogenesis through the upregulation of host-encoded susceptibility genes. Xcc uses the type III secretion system to introduce effector proteins such as the transcription factor-like (TAL) effector PthA4 that can directly activate host susceptibility gene expression. In contrast, *C*Las lacks most bacterial secretion systems and relies predominantly on the Sec secretion system for pathogenesis. While some Sec-secreted proteins have been identified in *C*Las, their direct role in causing HLB symptoms remains unproven. Several *Sugars Will Eventually be Exported Transporter* (*SWEET*) genes, encoding sucrose transporters, are candidate susceptibility genes. Here we investigate the roles of the citrus *SWEET10, SWEET12* and *SWEET15* genes and show that mutations of *SWEET15* resulted in reduced susceptibility to citrus canker in three different citrus cultivars: Carrizo citrange (*Citrus sinensis* 'Washington' sweet orange × *Poncirus trifoliata*), 'Limoneria 8A' Lisbon lemon (*Citrus limon*) and 'Pineapple' sweet orange (*C. sinensis*). Furthermore, Lisbon lemon plants mutated for *SWEET15* also showed reduced *C*Las titre in infected plants. These results suggest that *SWEET15* may act as a broad-spectrum susceptibility gene, and disruption of *SWEET15* gene activity could be a viable approach to mitigating bacterial diseases such as citrus canker and HLB in a variety of citrus cultivars.

1 | Introduction

Citrus, including orange, tangerine, lemon, grapefruit, lime and citron, is a major fruit crop, cultivated in over 140 countries (FAO 2022). However, global citrus production is facing unprecedented challenges from the spread of bacterial diseases, including citrus canker (caused by *Xanthomonas citri* pv. *citri*, Xcc), citrus variegated chlorosis (*Xylella fastidiosa*) and huanglongbing (HLB) disease (caused by "*Candidatus* Liberibacter asiaticus", *C*Las) (Almeida et al. 2019; Ference et al. 2018; Gottwald 2010). In particular, phloem-limited *C*Las is one of the most destructive bacterial species affecting citrus, and as of yet there are no reports of natural resistance to *C*Las infection in any commercial citrus scion or rootstock cultivars (da Graça et al. 2016; Wang et al. 2017). Furthermore, crop losses due to disease are likely to be significantly exacerbated in the near future by climate warming, with yield losses estimated to be between 10% and 25% for each degree Celsius

rise in temperature (Deutsch et al. 2018; Laine 2023). As such, generating citrus with reduced susceptibility to these bacterial diseases is a key priority of many research programmes, using either traditional breeding or genome editing approaches (Jia et al. 2017).

Unlike many bacterial plant pathogens that use the type III secretion system (T3SS) to deliver effectors directly into host cells, *C*Las lacks T3SS machinery and instead relies on the general Sec secretion system for protein export (Hu et al. 2025; Sugio et al. 2011). While several Sec-delivered effectors have been identified in *C*Las (Prasad et al. 2016; Hu et al. 2025), their exact roles in HLB symptom development remain unclear, and no individual Sec-dependent effector has been conclusively demonstrated to directly cause HLB symptoms. However, parallels can be drawn from other insect-transmitted, phloem-colonising bacteria such as phytoplasmas, where Sec-dependent effectors are known to manipulate host cellular functions and induce disease-like phenotypes in model plants (MacLean et al. 2011; Hoshi et al. 2009). Furthermore, at least 86 *C*Las-encoded proteins contain signal peptides, suggesting their potential involvement in pathogen colonisation and/or disease progression (Prasad et al. 2016).

Susceptibility (S) genes encode non-immunity-related functions of the host that facilitate infection and/or proliferation of pathogens (Lapin and Van den Ackerveken 2013; van Schie and Takken 2014). S genes have been grouped into three categories: those that facilitate host recognition and pathogen penetration; those that encode negative regulators of host immune signalling pathways; and those that alter host metabolic or structural features to facilitate pathogen proliferation (van Schie and Takken 2014). In contrast to resistance (R) genes, which mainly encode dominant resistance traits that are often race-specific, S genes are generally recessive and homozygous loss-of-function mutations in an S gene can confer host resistance to the pathogen as its normal function is essential to the survival of an invading pathogen. Furthermore, S gene mutations, especially those that influence post-penetration processes, can provide broad-spectrum resistance against a variety of pathogens (Lapin and Van den Ackerveken 2013; van Schie and Takken 2014; Zaidi et al. 2018).

The *SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER* (*SWEET*) genes encode seven-transmembrane spanning sugar efflux transporters that can be grouped into four clades. *SWEET* genes in clades I and II encode proteins that transport hexose sugars, while clade III gene products preferentially transport sucrose and clade IV gene products participate in vacuolar transport of fructose (Chen et al. 2010, 2012). Several members of clade III have been shown to act as S genes because they encode proteins that transport sucrose across the plasma membrane into the apoplast, and this pathway can be hijacked to sustain pathogen proliferation (Chen et al. 2010, 2012). For instance, a number of bacterial *Xanthomonas* species encode transcription activator-like (TAL) effectors that bind to the promoters of, and directly induce the expression of, host S genes and so enhance pathogenesis (Hogenhout et al. 2009; van Schie and Takken 2014). In rice, five CLADE III *SWEET* genes in rice have been shown to be activated by TAL effectors to support *Xanthomonas* growth, resulting in bacterial blight disease (Chen et al. 2010, 2012; Eom et al. 2015; Oliva et al. 2019; Streubel et al. 2013). Furthermore, gene editing to produce loss-of-function mutations of the clade III *OsSWEET13* or *OsSWEET14* genes in rice results in broad-spectrum resistance to multiple *Xanthomonas* strains as well as attenuating brown planthopper attack (Oliva et al. 2019; Yu et al. 2024). Loss-of-function mutations in clade III *SWEET* genes have also been shown to reduce susceptibility to *Xanthomonas* virulence in cassava (Elliott et al. 2024) and in cotton (Cox et al. 2017).

Clade III *SWEET* gene upregulation has been observed in a number of host species in response to pathogen attack. For instance, infection of *Arabidopsis thaliana* with *Pseudomonas syringae* pv. *tomato*, the protist *Plasmodiophora brassicae*, or the powdery mildew pathogen *Golovinomyces cichoracearum*, all result in the induction of clade III *SWEET* gene expression (Chen et al. 2010; Walerowski et al. 2018). Similarly, the infection of grapes or tomato with the necrotrophic fungus *Botrytis cinerea* (Asai et al. 2016; Chong et al. 2014) results in upregulation of clade III *SWEET* genes. In the sweet orange cultivar Valencia, at least 11 *SWEET* genes appear to be transcriptionally upregulated in response to *C*Las infection (Yao et al. 2021). Together, these observations suggest that clade III *SWEET* genes may act as broad-spectrum host susceptibility factors for many types of pathogens.

Here, we explicitly investigate the role of three clade III *SWEET* genes as potential disease susceptibility genes in citrus. Using CRISPR/Cas9 genome editing approaches optimised for citrus (Khadgi et al. 2024; LeBlanc et al. 2018; Zhang et al. 2020), we mutated *SWEET10*, *SWEET12* and *SWEET15* individually and in combination in three cultivars, Carrizo citrange (*Citrus sinensis* 'Washington' sweet orange × *Poncirus trifoliata*), 'Limoneria 8A' Lisbon lemon (*Citrus limon*) and 'Pineapple' sweet orange (*C. sinensis*) to assess the effects of these mutations on plant responses to both *C*Las and Xcc infection. We show that *SWEET15* mutations can reduce citrus canker disease symptoms and affect the *C*Las bacterial titre of HLB-affected plants.

2 | Results

2.1 | Identification of Candidate *SWEET* Susceptibility Genes in Citrus

We identified 15 *SWEET* genes in the *C. sinensis* 'Valencia' sweet orange DVS_A1.0 genome assembly, and a neighbour-joining phylogenetic tree was constructed based on the predicted amino acid sequences of *SWEET* genes from 15 plant species, including *C. sinensis* (Figure 1). This phylogeny exhibited the same topological structure as previously described (Chen et al. 2010). Clade III possessed four *C. sinensis* members: *CsSWEET9, CsSWEET10, CsSWEET12* and *CsSWEET15*. Because *CsSWEET10*, *12* and *15* are all upregulated in response to *C*Las (Yao et al. 2021), we focused on these three genes as candidate S genes. We assessed the relative expression levels of *CsSWEET10*, *12* and *15* in roots, leaves and stems using reverse transcription-quantitative PCR (RT-qPCR), with all three showing highest expression in the leaves (Figure S1).

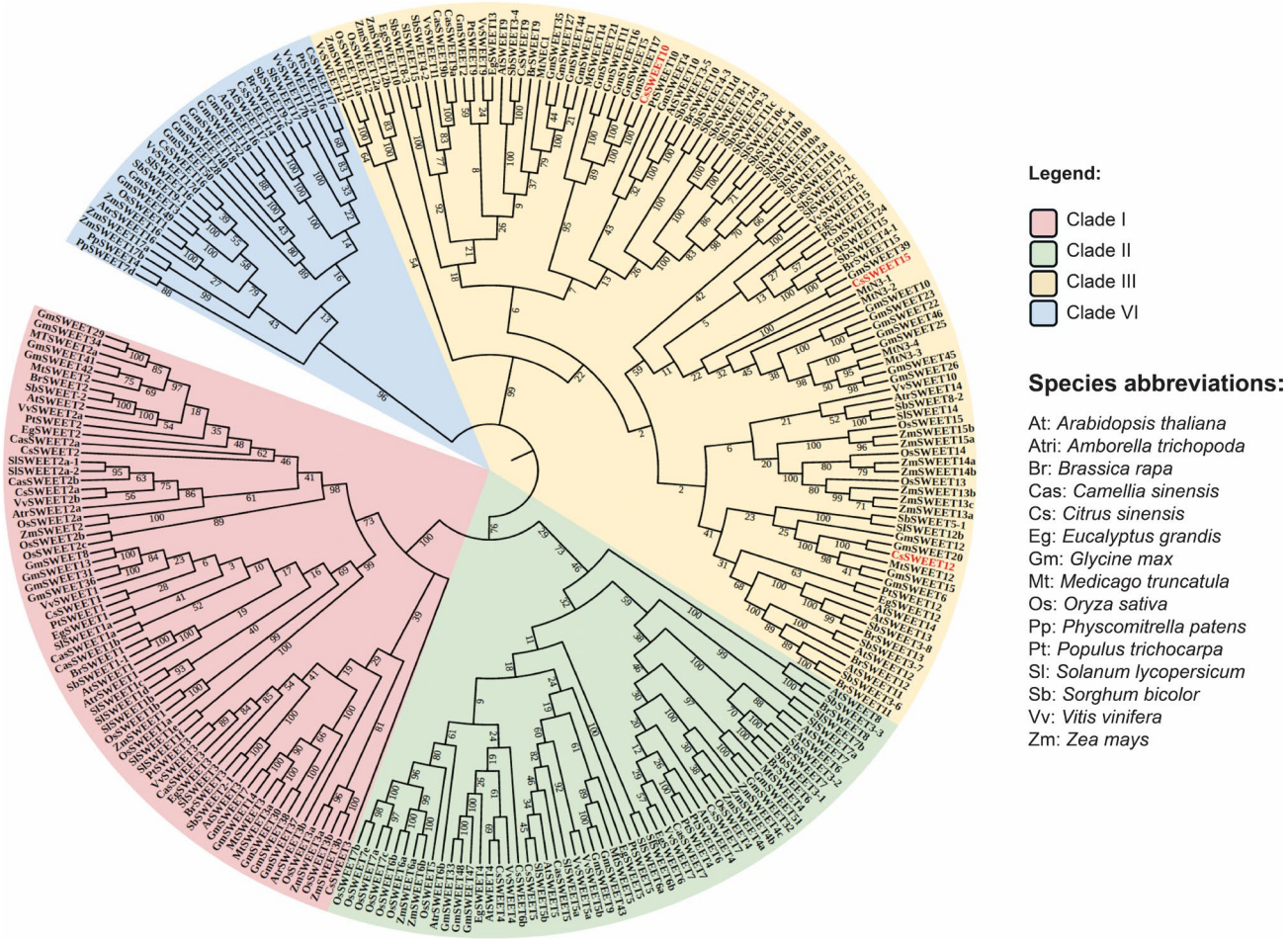


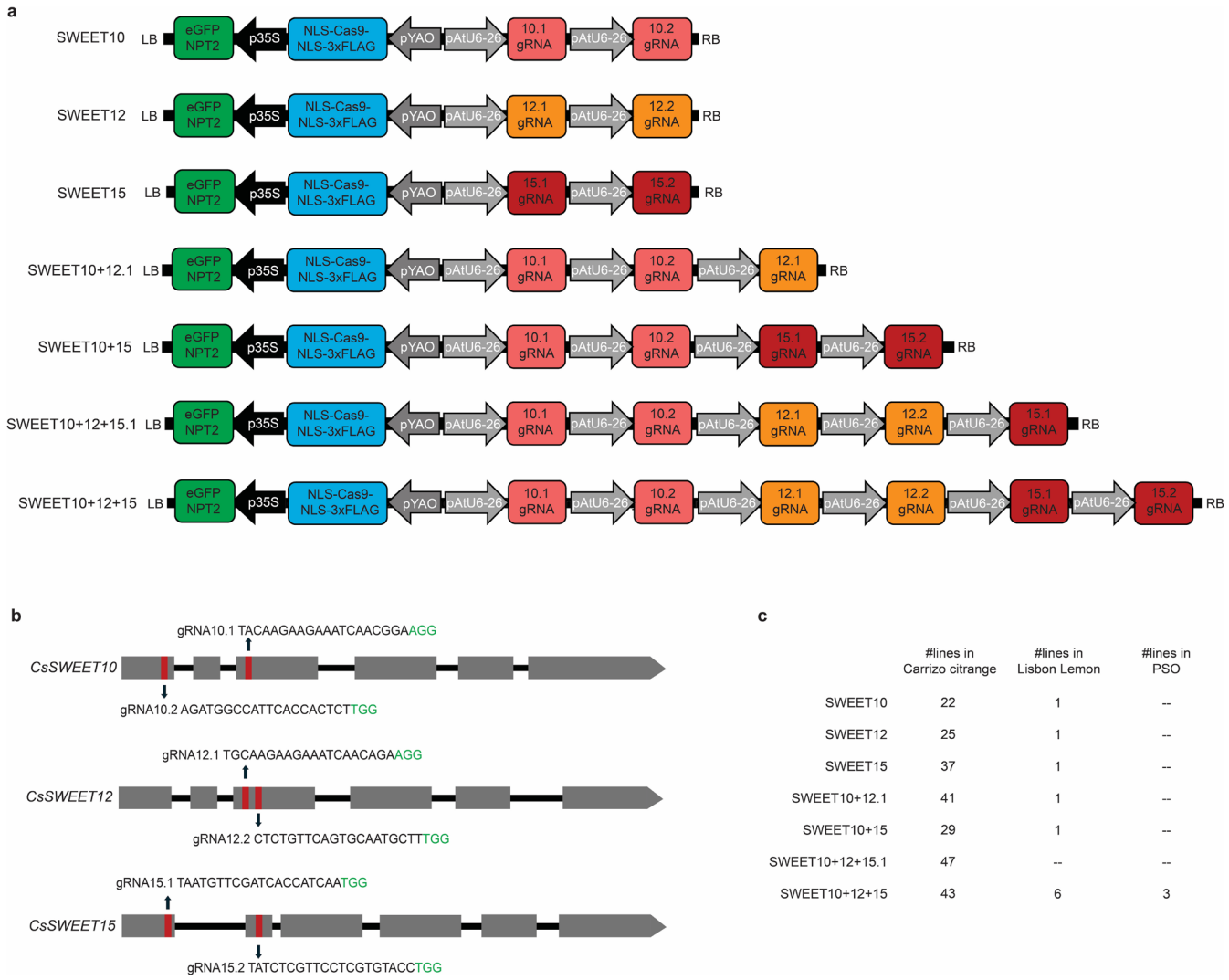
FIGURE 1 | Neighbour-joining analysis of predicted SWEET proteins from 15 different species. The phylogenetic tree was constructed using the neighbour-joining (NJ) method and with 1000 bootstrap replications. The numbers at the nodes represent bootstrap percentage values. *CsSWEETs* belonging to clade III are coloured in red. The *SWEET* genes from *Physcomitrella patens* (Pp) were selected as the outgroups.

2.2 | Citrus *SWEET* Gene Mutants Affect Phloem Loading

To understand the roles of *SWEET10*, *12* and *15*, we generated CRISPR/Cas9 genome-editing constructs targeting one, two, or all three genes, with one or two gRNAs targeting each of the genes (Figure 2a,b). We introduced these constructs into Carrizo citrange, Limoneira 8A Lisbon lemon (hereafter Lisbon lemon) and 'Pineapple' sweet orange, using *Agrobacterium*-mediated transformation (Khadgi et al. 2024; LeBlanc et al. 2018; Zhang et al. 2020, 2017). We successfully recovered a total of 258 transgenic lines across Carrizo citrange, Lisbon lemon and 'Pineapple' sweet orange (Figure 2c). Upon sequence analysis, we identified 49 gene-edited homozygous loss-of-function mutant lines in Carrizo citrange, 11 in Lisbon lemon and three in 'Pineapple' sweet orange (Figure S2). We further analysed five homozygous mutant lines for each construct in Carrizo citrange, and all of the mutant lines that were generated in Lisbon lemon and 'Pineapple' sweet orange. All of the lines selected for further analyses showed a decrease in transcript levels, consistent with nonsense-mediated decay and indicating that all of these mutations represent loss-of-function lesions (Figure S3). We further demonstrated that the loss of function of one *SWEET* gene did not affect the expression of other *SWEET* genes, indicating that the disruption of one *SWEET* gene did not cause a compensatory transcriptional effect (Figure S4).

Single mutant lines for *SWEET10*, *SWEET12*, or *SWEET15* showed no obvious visible phenotypes in either Carrizo citrange or Lisbon lemon 7-month-old plants (Figure 3a,b). However, by 24 months, the Lisbon lemon *SWEET15* mutant showed some chlorosis in comparison to wild type (WT). All of the double mutant combinations in both Carrizo citrange and Lisbon lemon showed somewhat slower and stunted growth. In all three cultivars, plants triply mutant for *SWEET10, 12* and *15* were stunted, were somewhat weaker than WT, and by 24 months developed chlorotic leaves, consistent with the symptoms expected for impaired phloem loading (Figure 3a–c).

To directly examine the effects of these mutations on phloem loading, we quantified soluble sugar levels in the phloem-rich midrib of the leaf in comparison to the whole leaf minus the midrib. All of the mutant lines tested showed a statistically significant reduction in soluble sugar content in the midrib as compared to the rest of the leaf, with the triple mutants showing a drastic reduction of soluble sugar content in the midrib as compared to the rest of the leaf (Figures 3d–f, S5 and S6). These results indicate that the citrus *SWEET10, 12* and *15* genes are



c

	#lines in Carrizo citrange	#lines in Lisbon Lemon	#lines in PSO
SWEET10	22	1	--
SWEET12	25	1	--
SWEET15	37	1	--
SWEET10+12.1	41	1	--
SWEET10+15	29	1	--
SWEET10+12+15.1	47	--	--
SWEET10+12+15	43	6	3

FIGURE 2 | Schematic representations of the CRISPR/Cas9 gene-editing construct, *CsSWEET10*, *CsSWEET12* and *CsSWEET15* genes, location of the gRNAs, and number of transgenic lines for different constructs in Carrizo citrange, Lisbon lemon and 'Pineapple' sweet orange (PSO). (a) Two single guide RNAs (sgRNAs) were designed to target each of the *SWEET10*, *SWEET12* or *SWEET15* genes and each sgRNA was driven by an AtU6-26 promoter. The constructs include an eGFP-NPTII marker (eGFP, encoding enhanced green fluorescent protein; NPTII, encoding neomycin phosphotransferase) driven by the CaMV 35S promoter, encoding a fusion protein. Cas9 was driven by the YAO promoter. (b) Schematic representation of *CsSWEET10*, *CsSWEET12* and *CsSWEET15* target regions and location of the gRNAs. Exons are shown as grey square frames, and surrounding introns appear as black lines. Protospacer adjacent motif (PAM) is highlighted in green. (c) Number of transgenic citrus plants generated in each cultivar for different constructs.

functionally required for sugar transport from the mesophyll to the phloem, and that they appear to have an additive role in this process.

2.3 | *SWEET15* Mutants Are Resistant to Citrus Canker

We assessed whether the *SWEET* Carrizo citrange mutants showed resistance to citrus canker by infecting detached leaves with Xcc (Figure 4a). Disease progression was recorded 4, 7 and 11 days post-inoculation (dpi). The *SWEET10*, *SWEET12* and *SWEET10+12.1* mutant lines, as well as the WT, displayed large spongy white pustules that eventually progressed to water-soaked lesions, characteristic of canker symptoms (Brunings and Gabriel 2003). The development of canker symptoms in the mutant *SWEET15*, *SWEET10+15*, *SWEET10+12+15.1* and *SWEET10+12+15* lines was reduced as compared to the WT. These findings were corroborated by the quantification of Xcc titres at 11 dpi (Figure 4b) where all of the single, double and triple mutant lines containing a *SWEET15* mutation showed significantly reduced Xcc titres in comparison to the WT, indicating that *SWEET15* acts as an S gene for citrus canker.

Similarly, *SWEET15* mutant lines generated in Lisbon lemon and 'Pineapple' sweet orange showed decreased susceptibility to citrus canker in comparison to WT. These mutant lines developed fewer and smaller necrotic lesions as compared to the WT plants that produced larger pustules (Figure 5a,c). Quantification of Xcc titres showed that these mutants had reduced titres as compared to the WT, further indicating that *SWEET15* acts as an S gene for Xcc in multiple cultivars (Figure 5b,d).

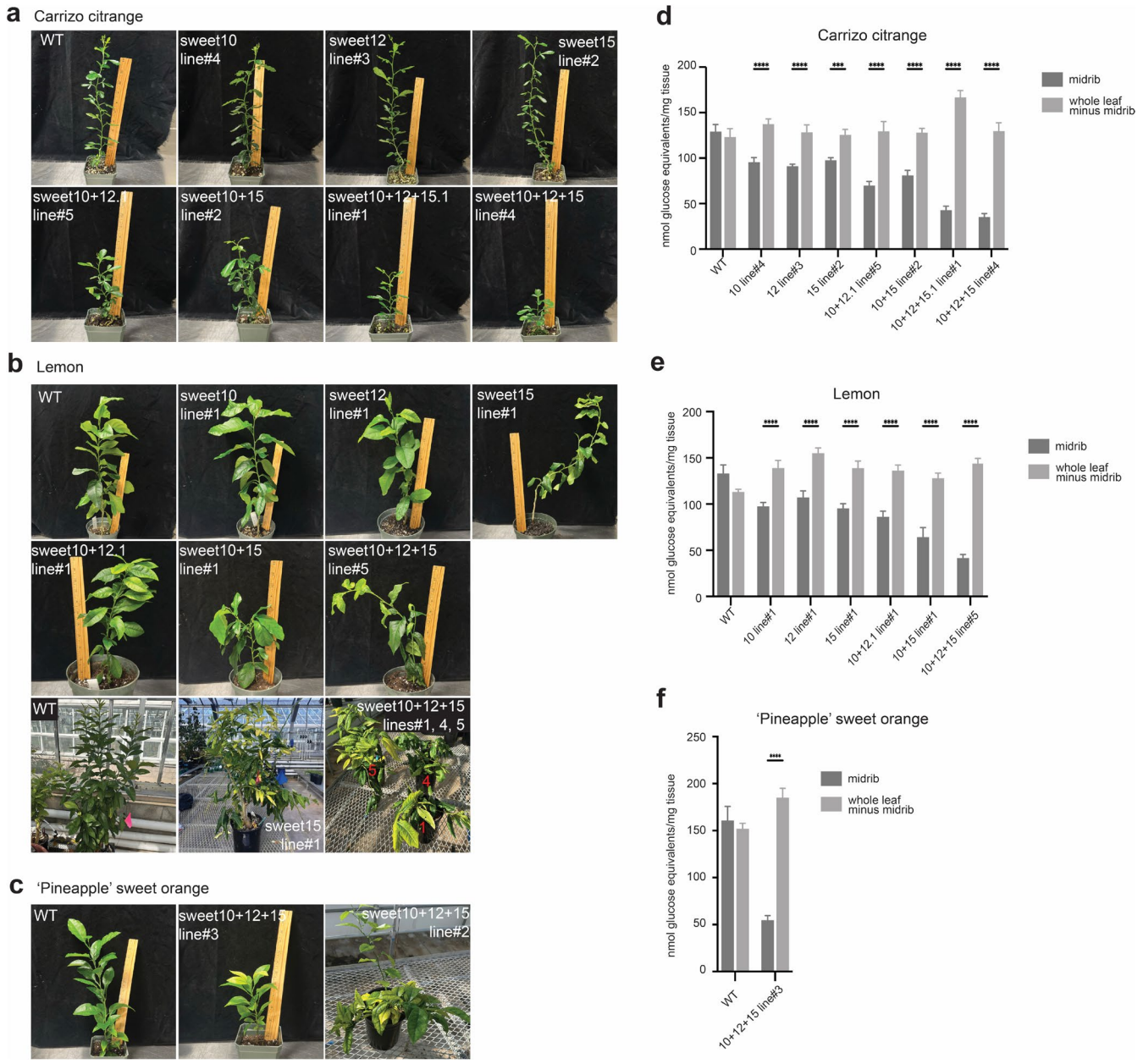


FIGURE 3 | Phenotypes and quantification of glucose equivalents in CRISPR/Cas9-generated *SWEET* mutants of Carrizo citrange, Lisbon lemon and 'Pineapple' sweet orange. (a) Examples of 8-month-old Carrizo citrange wild type (WT) and CRISPR/Cas9 mutant plants. The double and triple mutant lines show stunted growth as compared to the WT. (b) Examples of 7-month-old (top two panels) and 24-month-old (bottom panel) lemon WT and CRISPR/Cas9 mutant plants. Double and triple mutant lines, including *SWEET15* mutants, display weaker and stunted growth with chlorotic leaves. (c) Left to right, 7-month-old WT, 7-month-old triple mutant and 22-month-old triple *SWEET10 + 12 + 15* mutant 'Pineapple' sweet orange lines. The triple mutant line shows stunted growth, with shorter and narrower leaves that turn chlorotic as the plant matures. (d) Quantification of glucose equivalents using anthrone colourimetry in WT Carrizo citrange and mutant lines. (e) Quantification of glucose equivalents using anthrone colourimetry in WT Lisbon lemon and mutant lines. (f) Quantification of glucose equivalents using anthrone colourimetry in WT 'Pineapple' sweet orange and mutant lines. (d–f) Phloem-rich midribs and whole leaves without the midribs were collected, lyophilised and then used for total soluble sugar quantification. Values are the means of three replicates per genotype ± SD. Data were analysed for normality using the Shapiro–Wilk test. Normal data were then analysed by two-way analysis of variance (ANOVA), and significance levels were calculated by Šídák's multiple comparisons test, with $p < 0.05$ considered significant. ***$p < 0.001$, ****$p < 0.0001$.

2.4 | *SWEET15* Lisbon Lemon Mutants Show Reduced *C*Las Titres

To test for HLB susceptibility, we grafted individual WT, or *SWEET15*, *SWEET10 + 12.1*, *SWEET10 + 15*, mutant Lisbon lemon buds onto *C*Las-infected and noninfected citron rootstock and used quantitative PCR (qPCR) to assess *C*Las titres beginning after 8 weeks of infection. Higher C_t values reflect lower titres of *C*Las. The WT and three mutants could all be infected with *C*Las, which is reflected in the higher titre of *C*Las (lower C_t values) relative to uninfected grafts of each genotype. We observed differences in *C*Las titre between infected genotypes

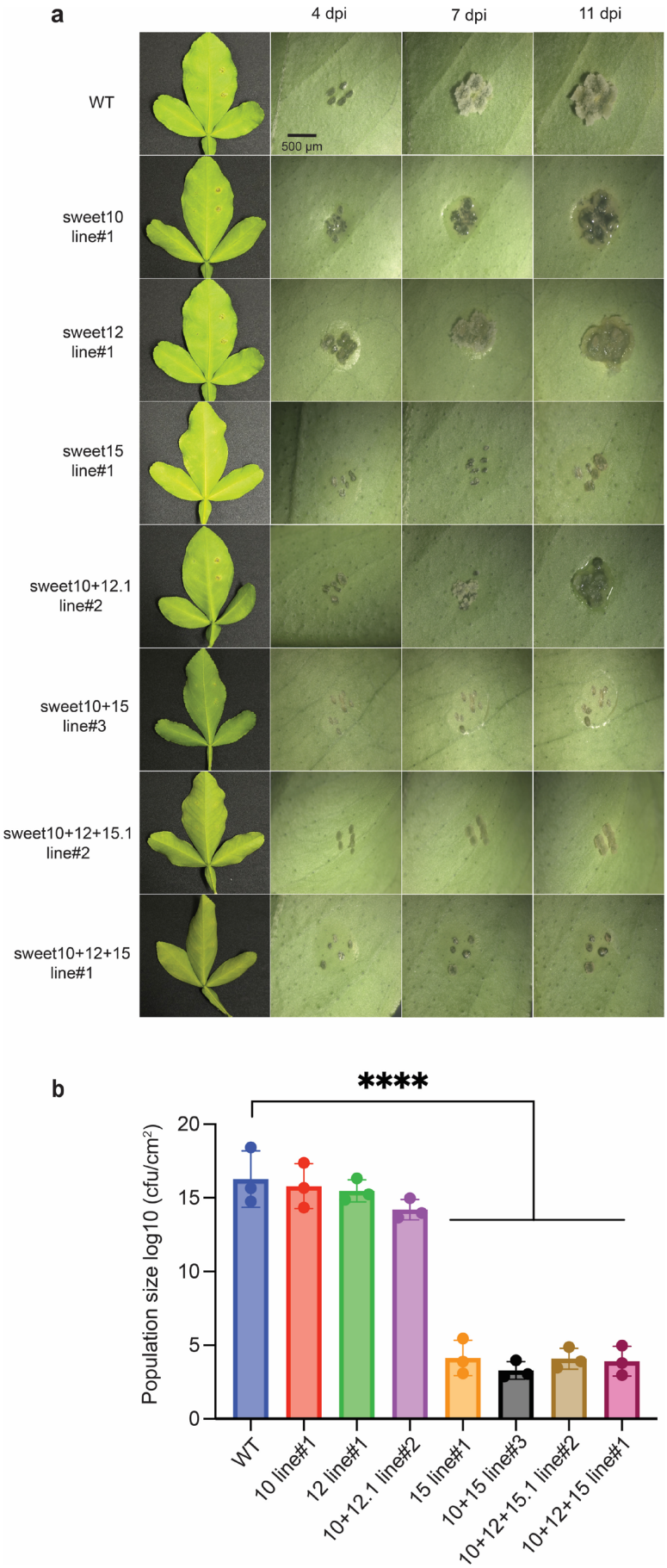


FIGURE 4 | Legend on next page.

FIGURE 4 | Assessment of citrus canker disease resistance of Carrizo citrange mutant lines. (a) Symptoms of citrus canker on wild type (WT) and mutant lines in Carrizo citrange. Young fully expanded leaves were inoculated with *Xanthomonas citri* pv. *citri* (Xcc) at a concentration of 10^7 CFU/mL on the abaxial side of the leaf. Five wounds per inoculation site were made for infiltration. Left panel, whole leaf images at 11 days post-inoculation (dpi), and right panels, close-ups at 4, 7 and 11 dpi. (b) Assessment of Xcc titres in WT and mutant Carrizo citrange leaves at 11 dpi. Three biological replicates were used, and the mean values ± SD ($n = 3$) are displayed. The experiments were conducted three times with similar results. Statistical analysis was conducted between wild type and each mutant line separately using Student's *t* test. Significant differences are indicated by asterisks, ****$p < 0.0001$.

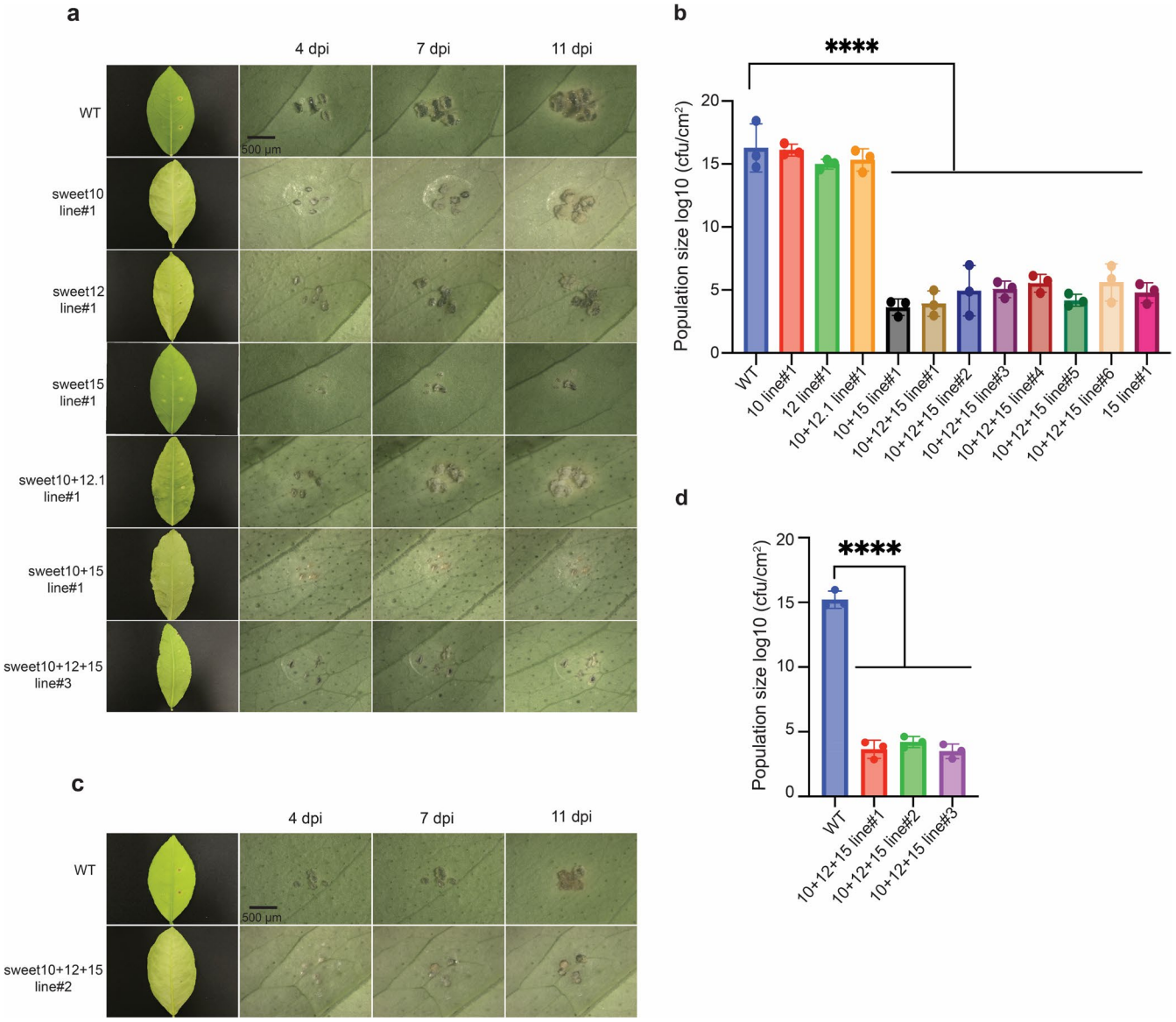


FIGURE 5 | Assessment of citrus canker disease resistance of Lisbon lemon and 'Pineapple' sweet orange mutant lines. (a) Symptoms of citrus canker on wild type (WT) and mutant lines in lemon. Young fully expanded leaves were inoculated with *Xanthomonas citri* pv. *citri* (Xcc) at a concentration of 10^7 CFU/mL on the abaxial side of the leaf. Five wounds per inoculation site were made for infiltration. Left panel, whole leaf images at 11 days post-inoculation (dpi); right panels, close-ups at 4, 7 and 11 dpi. (b) Assessment of Xcc titres in WT and mutant lemon leaves at 11 dpi. (c) Symptoms of citrus canker on WT and mutant lines in 'Pineapple' sweet orange, assays performed as in (a). (d) Assessment of Xcc titre in 'Pineapple' sweet orange leaves at 11 dpi. (b, d) Three biological replicates were used, and the mean values ± SD ($n = 3$) are displayed. The experiments were conducted three times with similar results. Statistical analysis was conducted between WT and each mutant line separately using Student's *t* test. Significant differences are indicated by asterisks, ****$p < 0.0001$.

(Figure 6). Similar to patterns of Xcc susceptibility, we observed reduced titres of *C*Las in single and double mutants of *SWEET15* grafted onto the *C*Las-infected rootstock as compared to the grafted WT buds (Figure 6). Moreover, the double mutant *SWEET10 + 12.1* line also showed a slight reduction in *C*Las titres as compared to controls.

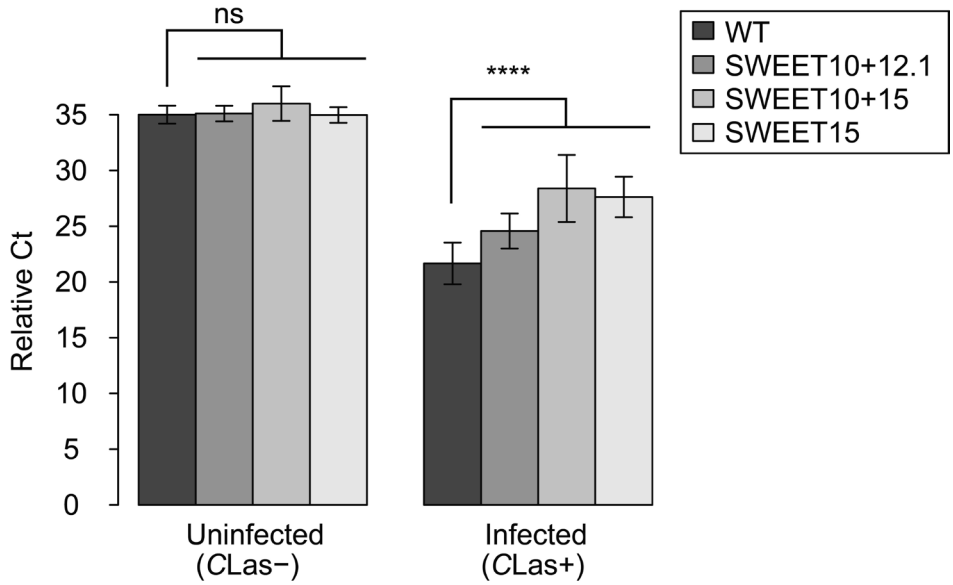


FIGURE 6 | Huanglongbing susceptibility test. Abundance of "*Candidatus* Liberibacter asiaticus" (*C*Las) in wild-type (WT) lemon and *SWEET* mutants. WT lemon and three mutant lines (*SWEET15*, *SWEET10+12.1* and *SWEET10+15*) were T-grafted onto both *C*Las-infected and non-infected citron rootstock (*Citrus medica*). The total number of grafts per treatment were as follows: *C*Las-uninfected rootstocks—*SWEET15* ($n=3$), *SWEET10+12.1* ($n=4$), *SWEET10+15* ($n=3$), WT ($n=4$); *C*Las-infected rootstocks—*SWEET15* ($n=4$), *SWEET10+12.1* ($n=6$), *SWEET10+15* ($n=7$), WT ($n=10$). For each graft, three leaf petioles were sampled for quantification of *C*Las titre by quantitative PCR, and these values were averaged. C_t values are normalised to the reference gene *COX*. Data were analysed by two-way analysis of variance (ANOVA), and significance levels were calculated by Tukey's test, honest significant difference (HSD) test. ****$p<0.0001$. Plotted values are the group means ± SD.

3 | Discussion

With the advent of CRISPR/Cas9 technology, as well as improvements in plant transformation approaches, it is becoming feasible to assess the roles of potential S genes in pathogenicity. By mutating S genes, homozygous or biallelic recessive alleles of these genes can be deployed for developing crop cultivars with resistance to a wide array of diseases (Chen et al. 2010; Feng and Frommer 2015). Here, we have investigated whether gene editing of three clade III *SWEET* genes in citrus would cause reduced susceptibility to citrus canker or HLB. We found that disruption of the *SWEET15* gene can reduce susceptibility to citrus canker in three citrus varieties, as well as reduce the titre of *C*Las in infected Lisbon lemon. These results suggest that modifying the *SWEET15* gene in citrus can not only lead to citrus canker resistance but may be a potential path towards reducing susceptibility to HLB as well.

SWEET15 gene products in other species have been shown to reside in the plasma membrane and function in the transport of sucrose from maternal to embryonic tissues during seed filling (Chen et al. 2015; Sosso et al. 2015; Wang et al. 2019; Yang et al. 2018). In rice, artificial transcriptional activation of *OsSWEET11, OsSWEET12, OsSWEET13, OsSWEET14* and *OsSWEET15* led to a gain of virulence phenotype in response to *Xanthomonas oryzae* pv. *oryzae* infection, suggesting that these genes may function as S genes (Streubel et al. 2013). Gene-edited loss-of-function lines of *OsSWEET15* have been shown to be affected seed filling and seed set (Hu et al. 2023; Yang et al. 2018), but to our knowledge, *SWEET15* has not been previously shown to cause reduced disease susceptibility when mutated in rice or in other species.

While mutation of just *SWEET15* can have an effect on citrus disease susceptibility, the results that we see with the *SWEET10+12.1* mutant in response to *C*Las infection suggest that an even stronger tolerance to disease could be obtained by mutating multiple S genes. Similarly, in rice, overexpression of multiple *SWEET* genes can confer heightened susceptibility to *X. oryzae* pv. *oryzae*, suggesting that alterations in multiple *SWEET* sugar transporters can have a cumulative effect in response to pathogen attack (Chen et al. 2015; Wang et al. 2019; Yang et al. 2006).

Not surprisingly, we observed that loss-of-function mutations of *SWEET15*, particularly when combined with mutations in other *SWEET* genes, resulted in less vigorous plant growth, presumably due to compromised phloem loading. When sucrose transport is inhibited, it can lead to stunted growth, chlorosis and inhibition of photosynthesis (Riesmeier et al. 1994). Comparisons of the partitioning of soluble sugars in different tissues in our lines are consistent with these symptoms of impaired phloem loading. Furthermore, not only is sucrose a key metabolic resource, but it functions in regulating signal transduction pathways required for plant growth and development (Hanson and Smeekens 2009), suggesting that by altering soluble sugar partitioning, pathogens may be indirectly altering host plant physiology.

In rice, mutations in, or natural variation of, TAL effector binding sites in the promoters of *SWEET* genes can result in the disruption of responsiveness to *Xanthomonas* spp. but do not appear to affect the normal gene function of the gene (Hutin et al. 2015; Oliva et al. 2019; Zhou et al. 2015). Similarly, mutations in the TAL effector binding site of the

CsLOB1 susceptibility gene in citrus resulted in resistance to Xcc (Huang et al. 2022). A gene-editing approach could also be taken with *SWEET15* to assess whether such effector binding site mutations would alleviate the plant growth defects we observe with loss-of-function mutations without compromising citrus canker disease resistance. Furthermore, field trials of plants containing such effector-binding site mutations will be needed to demonstrate a reduction in susceptibility under field conditions, while at the same time maintaining robust growth and fruit yield.

It is unclear, though, if such promoter mutations would have a similar effect on susceptibility to HLB. It is not known what *C*Las effectors are acting to upregulate *SWEET* gene expression in citrus, or the mechanism by which such effectors are acting. Several *C*Las effectors have been identified, and in at least one case, appear to act through binding to an immune-related cysteine protease (Clark et al. 2018). It may be that *C*Las effectors are acting indirectly through other factors to modulate *SWEET* gene transcription. Developing citrus varieties with effector binding site or other promoter mutations in *SWEET15* and potentially other *SWEET* genes could provide a new paradigm for managing diseases such as HLB and citrus canker in commercially valuable citrus varieties.

4 | Experimental Procedures

4.1 | Plant Materials

Seeds of citrus rootstock cultivar Carrizo citrange (*C. sinensis* 'Washington' sweet orange × *P. trifoliata*, accession number PI 150916) were germinated in vitro (MS with vitamins, 30 g/L sucrose, 2.5 g/L Phytagel, pH 5.8) in the dark at 24°C for 4–6 weeks to promote etiolation, followed by 7–10 days under 16-h light/8-h dark photoperiod (40 μmol/m^2/s) at 28°C. Seed coats of 'Limoneira 8A' Lisbon lemon and 'Pineapple' sweet orange (University of California, Riverside) were peeled, and 25–30 seeds were germinated in Promix soil in a 5-inch round pot. The pots were covered with Saran wrap and placed in the dark at 28°C for 7–10 days. The soil was initially well-watered and subsequently watered every 3–5 days. After germination, the seedlings were exposed to light at 28°C and grown for 2–3 weeks.

4.2 | Phylogenetic Analysis

The full-length amino acid sequences of SWEETs of *Arabidopsis thaliana* (At), *Amborella trichopoda* (Atri), *Brassica rapa* (Br), *Camellia sinensis* (Cas), *Citrus sinensis* (Cs), *Eucalyptus grandis* (Eg), *Glycine max* (Gm), *Medicago truncatula* (Mt) and *Populus trichocarpa* (Pt) were identified through BLAST on NCBI, and the full-length amino acid sequences of SWEETs for *Oryza sativa* (Os), *Solanum lycopersicum* (Sl), *Vitis vinifera* (Vv) and *Zea mays* (Zm) were obtained from the Aramemnon database (http://aramemnon.botanik.uni-koeln.de/). Amino acid sequences for *Sorghum bicolor* (Sb) were downloaded from SorghumBase. An alignment was generated using the MUSCLE algorithm, and the neighbour-joining phylogenetic tree was constructed using MEGA X software with a bootstrap of 1000 replicates (www.megasoftware.net) (Kumar et al. 2018). The *SWEET* genes from *Physcomitrella patens* (Pp) were selected as the outgroups. Then, the phylogenetic tree (Figure 1) was visualised using iTOL v. 6 (https://itol.embl.de/) (Letunic and Bork 2019).

4.3 | *Agrobacterium* and Vector Materials

CRISPR/Cas9 genome editing constructs were created using a binary vector that expresses Cas9 under the *Arabidopsis* YAO promoter, as detailed by Zhang et al. (2017). In summary, primers for different sgRNAs were synthesised, annealed and ligated into an AtU6-26-sgRNA-SK vector (Table S1). This AtU6-26 promoter with gRNA was then transferred to the proYAO-Cas9-NOS binary vector. For multiplex genome editing, additional AtU6-26-gRNA cassettes were added to the final vector at the SpeI site. Figure 2 illustrates the constructs used in this study. Constructs targeting *SWEET10*, *SWEET12* and *SWEET15* each contain two sgRNA targets. The double mutant construct *SWEET10 + 12.1* includes two sgRNAs for *SWEET10* and one for *SWEET12*, while *SWEET10 + 15* has two sgRNAs for *SWEET10* and two for *SWEET15*. The triple mutant construct *SWEET10 + 12 + 15.1* consists of two sgRNAs for *SWEET10*, two for *SWEET12* and one for *SWEET15*, whereas *SWEET10 + 12 + 15* has two sgRNAs for each of the three genes: *SWEET10*, *SWEET12* and *SWEET15*. Transgenic plants were selected using kanamycin NPTII—neomycin phosphotransferase II and GFP signal as a visual marker.

4.4 | Transformation of Carrizo Citrange Plants

The epicotyls of etiolated seedlings were used in transformation as described previously (Orbović and Grosser 2015; Zhang et al. 2020) with a few modifications. In brief, 75–100 explants were incubated with *Agrobacterium tumefaciens* EHA105 harbouring the testing construct in co-cultivation culture (MS basal medium with vitamins, 30 mM MES, 1% sucrose, pH 7) for 15 min under agitation at room temperature. After draining excess medium, dried explants were transferred to plates containing co-cultivation agar medium (MS with vitamins, 30 g/L sucrose, 3 mg/L BA, 0.1 mg/L NAA, 2.5 g/L Phytagel, pH 5.8) for a 3-day co-culture in the dark at 24°C. The explants were then transferred to the regeneration medium (MS with 30 g/L sucrose, 1 mg/L BA, 0.1 mg/L NAA, 250 mg/L cefotaxime, 50 mg/L kanamycin, 2.5 g/L Phytagel, pH 5.8) in the dark at 28°C. Transgenic shoots were identified by screening for GFP signal using a NIGHTSEA system with royal blue LED light. GFP-positive shoots were transferred to elongation medium (MS with vitamins, 30 g/L sucrose, 1 mg/L BA, 0.1 mg/L NAA, 250 mg/L cefotaxime, 100 mg/L timertin, 50 mg/L kanamycin, 2.5 g/L Phytagel, pH 5.8) and incubated under the same previously described light conditions. Once elongated, transgenic shoots were cut from the explant and rooted on rooting medium (MS with vitamins, 30 g/L sucrose, 0.5 mg/L NAA, 2 mg/L IBA, 2.5 g/L Phytagel, pH 5.8). Well-rooted plants were then transferred to MS medium for 2–4 weeks and then transferred to soil (Promix) under a plastic dome under a 16-h light/8-h dark photoperiod (195 μmol/m^2/s) at 28°C with a relative humidity of 40%–60%.

4.5 | Transformation of Lisbon Lemon and 'Pineapple' Sweet Orange

SWEET mutants for Lisbon lemon and 'Pineapple' sweet orange were obtained from our previous in planta transformation experiment (Khadgi et al. 2024). Briefly, 11 transgenic Lisbon lemon lines and three transgenic 'Pineapple' sweet orange lines were generated using AGWC (apical bud incision with axillary meristems grown for 3–5 days and fresh microwounds made, followed by cotton inoculation) and blunt cut with tip inoculation methods of inoculations.

4.6 | Heat Stress and Confirmation of Transgene Integration

GPF-positive shoots grown for 1.5–2 months were exposed to 14 cycles of heat stress treatments consisting of 72 h at 37°C and recovery for 24 h at 25°C. After heat treatment, genomic DNA was isolated from regenerated leaves as described previously with minor modifications (LeBlanc et al. 2018). Briefly, 50 mg of leaf tissue was ground in 500 μL of extraction buffer (200 mM Tris–HCl pH 8, 250 mM NaCl, 25 mM EDTA, 1% SDS). The tubes were vortexed, followed by centrifugation for 10 min at 3200 *g*. The supernatant was transferred to a new tube, and 70 μL isopropanol was added, followed by centrifugation for 10 min at 3200 *g*. The supernatant was removed, DNA pellets were washed twice with 70% ethanol, and resuspended in 100 μL water. PCR was performed to confirm the editing events in the transformed Lisbon lemon plants using the primers specific for the gRNAs. Sequences obtained from Sanger sequencing were analysed using Benchling for confirmation of gene editing. Knockout scores were calculated using Synthego ICE (v. 3.0) analysis tool.

4.7 | Anthrone Colourimetry for Soluble Sugar Quantification

Soluble sugar concentrations were determined by anthrone colourimetry as described previously, with some modifications (Updegraff 1969). Five different lines per construct, each with three replicates, were used for the assay. Midrib and whole leaf without the midrib from the same leaf were collected in two different tubes. Leaf tissues were frozen in liquid nitrogen, lyophilised for 72 h, and then homogenised using a Qiagen Tissue Lyser and 5 mm steel balls. Next, 50 mg lyophilised samples were weighed into 1.5 mL screw-cap tubes. To each tube, 500 μL of 70% ethanol was added and was heated at 60°C for 1 h with occasional vortexing. The process was repeated three times, and following each extraction, supernatants were combined. Twenty microlitres of the supernatant was used for further analysis, and a blank tube was also included. After evaporation, 100 μL of 67% H_2SO_4 was added to the tube and vortexed. To this, 400 μL of double-distilled water was added, mixed well, and the tubes were put in ice immediately. One millilitre of freshly prepared anthrone solution (100 mg anthrone in 10 mL H_2SO_4) was added to the solution and mixed well. The tubes were then placed on a heat block at 60°C for 5 min. After 5 min, the tubes were transferred back to the ice, and soluble sugar concentration was measured using a spectrophotometer (Jenway Genova Life Science Analyser) at 620 nm. A glucose standard curve was prepared using 0, 50, 100, 200, 300 and 400 nmol from a stock 10 mM glucose solution.

4.8 | Expression Analysis

Total RNA was extracted from samples of tissues using a Direct-zol RNA MiniPrep kit (ZYMO Research) with DNase I treatment to remove any contaminating DNA. One microgram of RNA from each sample was used to generate cDNA using an iScript cDNA synthesis kit (Bio-Rad). qPCR analyses were carried out with a CFX96 Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green supermix (Bio-Rad). *Actin* was used as a reference gene, and relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Fold change and standard error were calculated from three biological replicates (Table S2).

4.9 | Detached Leaf Xcc Assay in Citrus

Xcc strain FL1656, obtained from Nian Wang from the University of Florida; FL1656 is genetically identical to 306 and equivalently pathogenic (Xu et al. 2023). Xcc single colonies grown in nutrient agar at 28°C for 48 h were seeded into the nutrient broth and grown to log phase for 24 h at 28°C and 250 rpm. The bacterial solution, after being centrifuged at 10,000 *g* for 20 min, was then resuspended in sterile phosphate buffer solution (PBS; 40 mM Na_2HPO_4, 25 mM KH_2PO_4) and adjusted to an optical density (OD) of 0.1 at 620 nm, or 10^7 CFU/mL for the detached leaf inoculation (Francis et al. 2010). Each mutant plant was cultivated in a greenhouse, and three fully young expanded leaf samples were taken from each plant in the morning. These samples were kept in sealable plastic bags and then pressed inside a book to make them flat. These leaves were sterilised for 10 s with 10% sodium hypochlorite in a flow hood. Then, sterile distilled water was used to rinse the leaves twice. Disinfected leaves were handled by the petiole end and dried with Kimwipes. The leaves were then placed onto the 0.5% agar plate with the abaxial side facing up. The left side of the leaf was inoculated with mock solution and the right side with bacterial suspension. Inoculation was performed by adding two drops (10 μL each) of bacterial suspension to two different spots. Five wounds per drop were made immediately using the 1 mL needle. All the mock treatments were completed first and before infiltration with bacterial solution. The plates were then incubated in light at 28°C. The leaves were inspected every day and photographed on 4 days post-inoculation (dpi), 7 dpi and 11 dpi to record disease progression. Experiments were repeated independently three times.

The bacterial population in the inoculated areas was estimated at 11 dpi. Leaf discs (5 mm diameter) circumscribing each inoculated area were excised and ground with 0.2 mL of sterile water. To facilitate the assessment of bacterial concentration, serial dilutions (ranging from 10^{-1} to 10^{-6}) of the suspensions, each amounting to 100 μL, were spread across nutrient agar plates. Total bacterial colonies per inoculation site were counted after 48 h, and the number of CFU per cm^2 of leaf disc was calculated and presented with Prism GraphPad software.

4.10 | Assessment of *C*Las Titre via Graft Transmission and Quantification by qPCR

Grafting tests on Lisbon lemon mutant lines generated from an in planta transformation protocol and wild-type Lisbon lemon were carried out at the University of California, Riverside (UCR). Budwoods from WT plants and three mutant lines (*SWEET15*, *SWEET10+12.1* and *SWEET10+15*) were sent to UCR, which were then T-grafted onto both *C*Las-infected and noninfected citron rootstock (*C. medica*). To enhance graft success, especially for double mutants, the new grafts were treated with 5 ppm gibberellic acid (GA_3) and benzylaminopurine (BAP) 1 week after grafting. This treatment was applied once per week for 3 weeks. For each mutant line, at least one successful graft was generated on multiple independent *C*Las-infected rootstocks: *SWEET15* ($n=3$), *SWEET10+12.1* ($n=4$) and *SWEET10+15* ($n=3$). In some cases, there were multiple grafts per mutant per infected rootstock. A WT budwood and a budwood from a mutant line grafted onto the same rootstock were considered an independent replicate. For each graft, three leaf petioles were sampled for DNA extraction and quantification of *C*Las titre, and these values were averaged. At least three replicates were used to quantify the titre of *C*Las in this study. In some cases, there were multiple grafts to a rootstock for a given genotype. The total number of grafts per treatment were as follows: *C*Las-uninfected rootstocks—*SWEET15* ($n=3$), *SWEET10+12.1* ($n=4$), *SWEET10+15* ($n=3$), WT ($n=4$); *C*las-infected rootstocks—*SWEET15* ($n=4$), *SWEET10+12.1* ($n=6$), *SWEET10+15* ($n=7$), WT ($n=10$). Because graft success was variable, multiple rounds of grafting for WT and mutants were required to reach appropriate levels of replication. For consistency, established grafts were trimmed and sampled for qPCR-based estimation of *C*Las titre 8 weeks post-trimming.

DNA was extracted from petiole tissue using a CTAB extraction protocol (Inglis et al. 2018) with some modifications, including a sorbitol prewash. The sorbitol wash buffer was prepared with 100 mM Tris–HCl (pH 8.0), 0.35 M sorbitol, 5 mM EDTA (pH 8.0) and 1% (wt/vol) polyvinylpyrrolidone (PVP-40), and 1% 2-mercaptoethanol was added. Tissue samples were freeze-dried and ground. Each sample was washed twice with 900 μL of sorbitol wash buffer, and 450 μL of CTAB lysis buffer was added to each sample and mixed, then incubated at 65°C for 30 min. An equal volume (450 μL) of chloroform:isoamyl alcohol (24:1) was added, mixed and centrifuged; the upper aqueous phase was transferred to a new plate. Then, 0.1 volumes of 3 M sodium acetate (pH 5.2) and 0.7 volumes of cold isopropanol were added to the aqueous phase, mixed and incubated at −20°C for 1 h. The samples were centrifuged, and the supernatant was discarded. The DNA pellets were washed with 70% ethanol; the DNA pellets were resuspended in water containing 0.1 mg/mL RNase.

*C*Las titre was quantified by qPCR using a CFX96 real-time PCR system (Bio-Rad). First, each 20 μL reaction mixture contained 40 ng DNA, 2× SSO Advanced Universal Supermix with reference dye Rox (Bio-Rad), and primers for 16S rDNA and a reference gene (*COX*). Inclusion of the reference gene was used to normalise the concentration of each sample, ensuring all samples had the same C_t value for the *COX* gene (18 ± 0.2). After normalisation, a second qPCR was performed to estimate *C*Las titre using primers for 16S rDNA (Li et al. 2006) (Table S3). The C_t values for *COX*-normalised samples were analysed to determine if *C*Las titre differed between WT and mutant plants. C_t values for the three petioles assayed per graft were averaged. Two-way analysis of variance (ANOVA) was used to determine if there were significant effects of treatment, genotype and treatment × genotype interaction. Levene's test was used to confirm homogeneity of variances between groups. QQ plots were used to evaluate the normality of residuals. Both main effects and the interaction effect were significant at $p<0.0001$. Group-level comparisons were then performed using Tukey HSD at a confidence level of 0.95 (Table S4).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available on request from the authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.