Molecular Plant Pathology

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ORIGINAL ARTICLE OPEN ACCESS

"*Candidatus* Liberibacter asiaticus" Infection Induces Citric Acid Accumulation and Immune Responses Mediated by the Transcription Factor CitPH4

Bin Hu[1] | Tao Yuan[1] | Zhihao Lu[1] | Rongyan Huang[1] | Jiaxian He[2] | Kun Yang[1] | Qinchun Wu[1] | Wanqi Ai[1] | Wang Zhang[1] | Weikang Zheng[1] | Xiaoxiao Wu[3] | Xia Wang[1] | Yuantao Xu[1] | Xiuxin Deng[1] | Qiang Xu[1]

[1]National Key Laboratory for Germplasm Innovation & Utilization of Horticultural Crops, Joint International Research Laboratory of Germplasm Innovation & Utilization of Horticultural Crops, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan, China | [2]College of Horticulture, Sichuan Agricultural University, Chengdu, China | [3]Guangxi Key Laboratory of Citrus Biology, Guangxi Academy of Specialty Crops, Guilin, China

Correspondence: Qiang Xu (xuqiang@mail.hzau.edu.cn)

Received: 12 August 2024 | **Revised:** 6 December 2024 | **Accepted:** 26 January 2025

Funding: This work was supported by National Natural Science Foundation of China (grants 31925034 and U23A20198); Key S&T Projects in Nanning city (grant 20232078); Key Project of Hubei Provincial Natural Science Foundation (grant 2021CFA017); Hubei Provincial Natural Science Foundation of China (grant 2024AFB138), Postdoctor Project of Hubei Province (grant 2024HBBHCXA044); Fundamental Research Funds for the Central Universities (grant 2662023PY003) and the Postdoctoral Fellowship Program of China Postdoctoral Science Foundation (CPSF) (grant GZC20230913).

Keywords: citric acid | citrus | defence response | fruit quality | huanglongbing | salicylic acid

ABSTRACT

Citrus huanglongbing (HLB), caused by "*Candidatus* Liberibacter" spp., is one of the most disastrous citrus diseases worldwide. HLB-affected citrus fruits are significantly more acidic than healthy fruits. However, the molecular mechanism behind this phenomenon remains to be elucidated. Here, we report that HLB-affected fruits have higher levels of citric acid (CA) than healthy fruits. Moreover, *Citrus PH4* (*CitPH4*), which encodes a MYB transcription factor that functions as a key regulator of CA accumulation, was upregulated in HLB-affected fruits relative to healthy fruits. Heterologous overexpression of *CitPH4* in tobacco (*Nicotiana tabacum*) plants enhanced tolerance to HLB. Subsequently, overexpression and gene-editing experiments indicated that CitPH4 can affect the salicylic acid (SA) pathway, which directly binds to and activates the promoter of *CsPBS3*, a key gene of SA biosynthesis. HLB-affected fruits had higher SA levels than healthy fruits. Furthermore, application of SA activated CA biosynthesis and application of CA activated SA biosynthesis and signalling in citrus fruits and decreased "*Candidatus* Liberibacter asiaticus" (*C*Las) titres in infected leaves. This work suggests that CitPH4 is a key node between CA and SA, thus revealing crosstalk between defence responses and fruit quality in citrus.

1 | Introduction

The citrus industry is facing an unprecedented challenge worldwide from huanglongbing (HLB) disease, which has been documented in approximately 53 out of 140 citrus-cultivating countries around the world, resulting in billions of dollars of annual economic losses in fruit yield, quality, tree damage and management costs (Gottwald 2010; Wang 2020; Wang et al. 2017; Hu et al. 2021). HLB is caused by fastidious phloem-limited α-proteobacteria "*Candidatus* Liberibacter" spp., including "*Ca*. Liberibacter asiaticus" (*C*Las), "*Ca*. Liberibacter africanus" and "*Ca*. Liberibacter americanus" (Bové 2006; Gottwald 2010;

Bin Hu, Tao Yuan, Zhihao Lu and Rongyan Huang contributed equally to this work.

Wang and Trivedi 2013). *C*Las is the most prevalent species associated with HLB and cannot be cultured in vitro (Bové 2006; Zheng et al. 2024). All commercially cultivated citrus varieties are susceptible to *C*Las, and there is no known cure for this disease once citrus plants are infected (Bové 2006; Folimonova et al. 2009; Ramadugu et al. 2016; Folimonova and Achor 2010).

After *C*Las infection, visible symptoms appear in roots, young shoots, leaves and fruits; these are followed by twig dieback and decreased productivity (da Graça et al. 2016; Hu et al. 2021; Bové 2006; Johnson et al. 2013). HLB affects citrus fruit quality (da Graça et al. 2016; Stokstad 2006); typical HLB-affected citrus fruits are smaller and misshapen and often contain aborted seeds compared to healthy fruits (Rosales and Burns 2011; Liao and Burns 2012). Young *C*Las-infected fruits tend to drop prematurely, and mature fruits from *C*Las-infected citrus trees often fail to ripen properly, retaining a green colour (Bové 2006; da Graça et al. 2016). Indeed, HLB is also known as citrus greening. *C*Las-infected citrus fruits produce poor-quality juice that tastes bitter (McClean and Oberholzer 1965; McClean and Schwarz 1970; Yao et al. 2019) and has higher acidity than the juice of healthy fruits (Massenti et al. 2016; Dagulo et al. 2010; Sajid et al. 2022; Zhang et al. 2022; Plotto et al. 2010). However, the reasons for the increased acidity in citrus fruits infected with *C*Las are still poorly understood.

Organic acids are natural antimicrobial agents commonly used in the food industry, and the regulatory mechanism of citric acid (CA) accumulation is well understood (Nicolau-Lapeña et al. 2019; Lepaus et al. 2020; Meireles et al. 2016; Sisson et al. 2024). An increasing number of studies have demonstrated that CA is the predominant organic acid in citrus fruits (Erner et al. 1975; Grewal and Kalra 1995; Wang, He et al. 2018; He et al. 2022; Zhang et al. 2022). *CitPH4* was previously reported as a key transcription factor gene in the regulation of CA accumulation in citrus fruits (Huang et al. 2023). The highest antimicrobial activity of CA has been demonstrated at low pH values (Young and Foegeding 1993; Buchanan and Golden 1994; Kundukad et al. 2020). Similarly, high-acidity citrus fruits exhibit higher disease resistance than low-acidity fruits (Wang, He et al. 2018; Rao et al. 2021). However, an antimicrobial effect of CA on *C*Las has not yet been reported.

The phytohormone salicylic acid (SA) plays a central role in regulating plant defence response (Jones and Dangl 2006; An and Mou 2011; Vlot et al. 2009) and often accumulates in *C*Las-infected tissues (Lu et al. 2013; Li, Zhang et al. 2021; Hu et al. 2021). Application of SA is sufficient to activate plant immunity and increase tolerance to HLB (Hu et al. 2017; Nehela and Killiny 2020; Li et al. 2017). Nevertheless, the relationship between the increased acidity of citrus fruits and citrus defence responses induced by *C*Las remains unexplored.

In the current work, we confirmed that *C*Las-infected citrus fruits have higher acidity compared to healthy fruits. *CitPH4*, the key regulator of CA accumulation, was significantly upregulated in *C*Las-infected fruits. Heterologous overexpression of *CitPH4* enhanced the tolerance to *C*Las in tobacco (*Nicotiana tabacum*) plants. The expression levels of SA-related genes were positively affected in *CitPH4*-overexpressing tobacco plants and *CitPH4*-knockout citrus fruits. Biochemical assays revealed that CitPH4 regulates the level of SA by directly binding to the promoter of the SA biosynthesis gene *AVRPPHB SUSCEPTIBLE 3* (*PBS3*) and activating its expression. In addition, application of CA activated SA biosynthesis and signalling and application of SA activated CA biosynthesis in citrus fruits; both treatments decreased *C*Las titres in *C*Las-infected citrus leaves. Therefore, this study suggests that the increased abundance of CA in *C*Las-infected citrus fruits may be involved in the defence response to *C*Las infection.

2 | Results

2.1 | CA Content Increases in *C*Las-Infected Fruits

To test whether the acid content of *C*Las-infected fruits increases relative to that of healthy fruits, we measured the titratable acid content of *C*Las-infected fruits from three citrus varieties. The titratable acid content of *C*Las-infected fruits in the three mandarin varieties tested here significantly increased compared to their respective healthy fruits (Figure 1A). More specifically, *C*Las-infected fruits accumulated significantly higher levels of CA compared to healthy fruits (Figure 1B). Reverse transcription-quantitative PCR (RT-qPCR) analysis showed that *CitPH4* expression was higher in *C*Las-infected fruits than in healthy fruits (Figure 1C), and the CA content was noticeably elevated in *CitPH4*-overexpressing tobacco plants compared to control plants (Figure 1D). These data suggest that the CA content of citrus fruits increased after infection by *C*Las.

2.2 | Heterologous Overexpression of *CitPH4* in Tobacco Enhances Tolerance to *C*Las

To investigate whether *CitPH4* is involved in the regulation of citrus defence responses to *C*Las infection, we inoculated *CitPH4*-overexpressing tobacco plants with *C*Las through approach grafting using *C*Las-infected citrus plants (Figure 1E), with wild-type (WT) tobacco plants serving as the control. After grafting inoculation, we determined the titres of *C*Las in the leaves of these tobacco plants every 2 weeks. The *C*Las titres of control tobacco plants were significantly higher than those in transgenic tobacco plants overexpressing *CitPH4* from 4 to 12 weeks after grafting (Figure 1F). These results demonstrate that *CitPH4*-overexpressing tobacco plants exhibit more tolerance to *C*Las infection compared to the WT plants.

2.3 | CitPH4 Positively Regulates SA Biosynthesis

To explore the mechanism by which *CitPH4* overexpression enhances tolerance to *C*Las in tobacco, we performed gene expression analysis to investigate the pathway(s) influenced by *CitPH4* in plants. In our previous transcriptome analysis, Gene Ontology term analysis of upregulated differentially expressed genes revealed that defence response-related genes were significantly enriched in *Arabidopsis thaliana* (Arabidopsis) plants heterologously overexpressing *CitPH4*. The expression levels of SA-related genes were dramatically elevated in the transgenic Arabidopsis plants compared to in WT plants (Figure S1). We confirmed the above results by RT-qPCR analysis, which revealed the upregulation of the SA biosynthesis genes *ISOCHORISMATE SYNTHASE 1* (*CsICS1*) and *CsPBS3* and the SA signal transduction

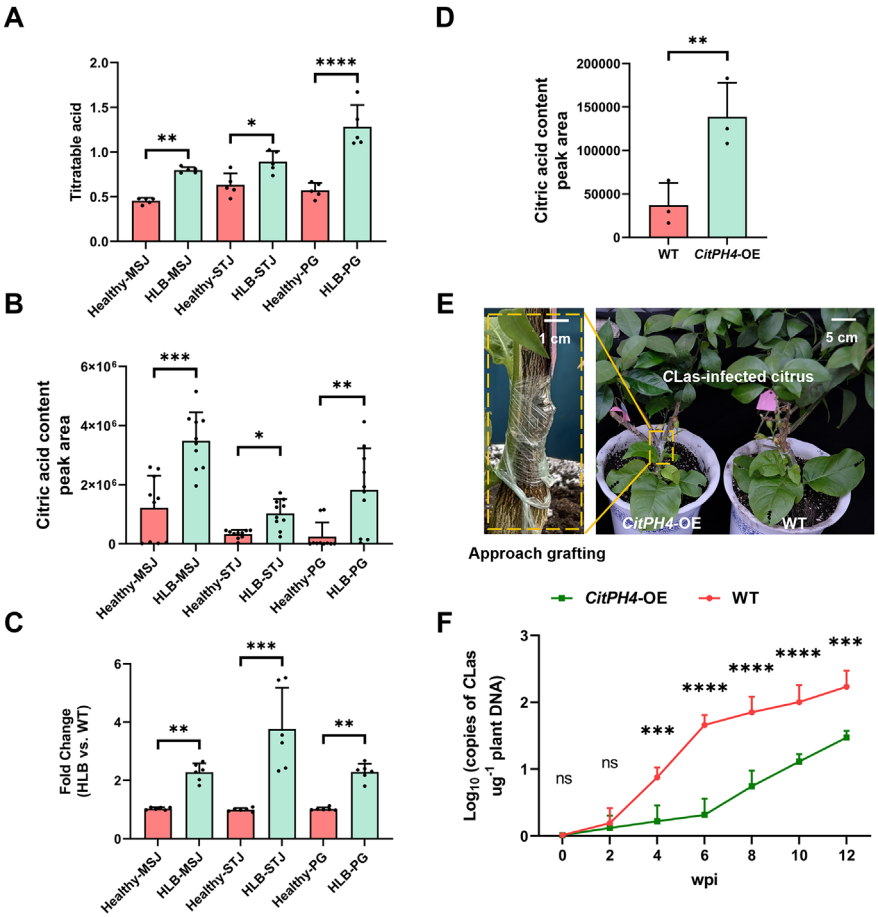

FIGURE 1 | Citric acid increased in "*Candidatus* Liberibacter asiaticus" (*C*Las)-infected fruits, and CitPH4 was involved in huanglongbing-tolerance. (A and B) The content of titratable acid and citric acid (CA) in *C*Las-infected (HLB) and healthy fruits of three mandarin varieties. (C) The expression level of *CitPH4* in *C*Las-infected and healthy fruits from the three mandarin varieties. (D) The content of citric acid in wild type (WT) and *CitPH4*-OE (overexpression) tobacco. (E) Inoculation of *C*Las in *CitPH4*-OE tobacco using *C*Las-infected citrus by approach grafting. (F) Quantitative analysis of dynamic changes of *C*Las titres in WT and *CitPH4*-OE tobacco. MSJ, Mashui mandarin, STJ, Shatang mandarin, PG, Pokan mandarin. Data are means ± standard error ($n \geq 3$ biologically independent replicates). Asterisks indicate significant differences based on Student's *t* test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

pathway-related genes *CALMODULIN-BINDING PROTEIN 60* (*CsCBP60G*), *PHYTOALEXIN DEFICIENT 4* (*CsPAD4*), *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*CsNPR1*) and *PATHOGENESIS-RELATED GENE 1* (*CsPR1*) in tobacco plants overexpressing *CitPH4* compared to their controls (Figure 2A). Conversely, we noticed a marked downregulation in the expression levels of these genes in *CitPH4*-knockout citrus fruits (Figure 2B). In addition, SA levels were significantly higher in *CitPH4*-overexpressing tobacco plants than in control plants (Figure 2C) and dramatically lower in *CitPH4*-knockout citrus fruits than in the WT (Figure 2D).

To investigate whether the induction of SA accumulation is more pronounced in transgenic plants than in controls upon *C*Las infection, we quantified the SA levels in WT and *CitPH4*-overexpressing plants upon *C*Las infection using liquid chromatography–tandem mass spectrometry (LC–MS/MS). We detected significantly higher SA contents in *CitPH4*-overexpressing tobacco plants than in their controls following infection with *C*Las. In addition, the SA content in *CitPH4*-overexpressing plants infected with *C*Las was significantly higher than that in healthy *CitPH4*-overexpressing plants. The SA content in WT plants infected with *C*Las appeared to be higher than that of their healthy counterparts, although this difference did not reach significance (Figure 2C). These results indicate that *C*Las infection significantly increases the SA level of *C*Las-infected *CitPH4*-overexpressing tobacco plants compared to those of healthy *CitPH4*-overexpressing plants and *C*Las-infected WT plants.

The increased expression level of *CitPH4* in *C*Las-infected citrus fruits and its role in regulating SA biosynthesis prompted us to investigate whether SA accumulates in these infected fruits. For this purpose, we measured the expression levels of the SA marker gene *CsPR1* and of SA biosynthesis-related genes by RT-qPCR. We observed that *CsPR1*, *CsPBS3* and *CsICS1* were highly expressed in *C*Las-infected fruits compared to healthy fruits (Figure 2E). SA content was also significantly increased in *C*Las-infected fruits compared to healthy fruits (Figure 2F), which is in agreement with previous reports of SA accumulation in *C*Las-infected tissues (Hu et al. 2021).

To explore how CitPH4 regulates the expression of SA-related genes, we conducted a dual-luciferase (*LUC*) assay in *Nicotiana benthamiana* leaves. To this end, we placed the

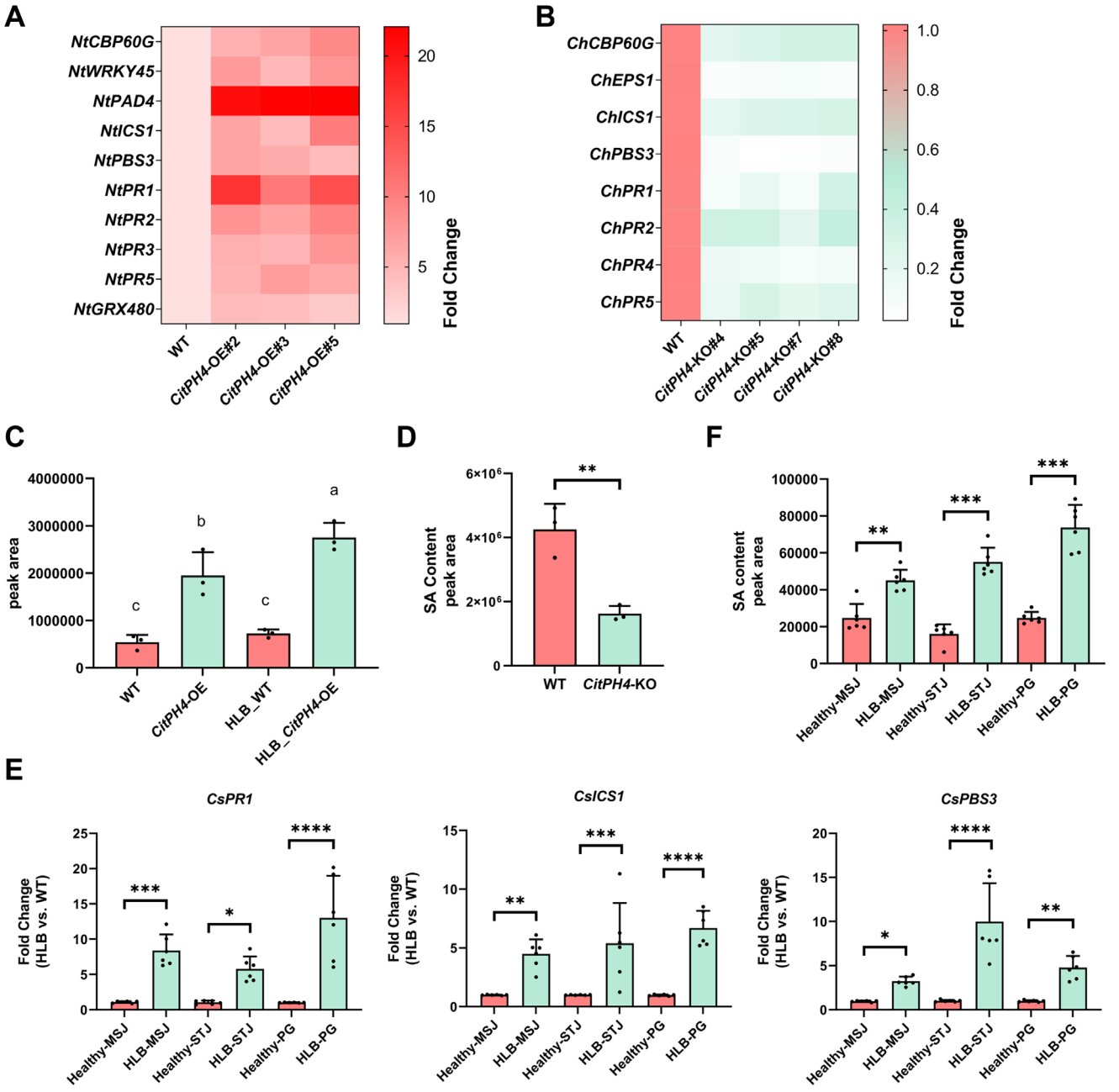

FIGURE 2 | CitPH4 is involved in salicylic acid (SA) pathway. (A and B) The expression levels of SA pathway-related genes in *CitPH4*-overexpressing (OE) tobacco and *CitPH4*-knockout (KO) citrus fruits, respectively. (C) The content of SA in *CitPH4*-OE and wild-type (WT) tobacco under healthy and "*Candidatus* Liberibacter asiaticus" (*C*Las)-infected (HLB) conditions. *CitPH4*-OE: *CitPH4* overexpressing tobacco. HLB_WT: *C*Las-infected wild type. HLB_*CitPH4*-OE: *C*Las-infected *CitPH4*-overexpressing tobacco. Error bars indicate *SE* ($n = 3$). Lowercase letters represent significant differences in different types of samples by one-way analysis of variance (ANOVA) followed by LSD post hoc test ($p < 0.05$). (D) The content of SA in *CitPH4*-knockout and WT citrus fruits. (E) The expression levels of SA pathway-related genes (*CsPR1*, *CsPBS3* and *CsICS1*) in *C*Las-infected citrus fruits from three mandarin varieties. (F) The content of SA in *C*Las-infected citrus fruits from three mandarin varieties. Data are mean ± *SE* ($n \geq 3$ biologically independent replicates). Asterisks indicate significant differences based on Student's *t* test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

firefly *LUC* reporter gene under the control of the *CsICS1* or *CsPBS3* promoter, using a *35S:CitPH4* construct as effector. Co-infiltration of the effector construct with each reporter construct indicated that CitPH4 can independently, or synergistically with CitAN1, activate the transcription of *CsPBS3*, one of the key genes involved in SA biosynthesis (Figure 3A–C). An electrophoretic mobility shift assay with recombinant purified CitPH4 fused to maltose-binding protein confirmed that CitPH4 directly binds to the *CsPBS3* promoter (Figure 3D). These data illustrate how CitPH4 positively regulates SA biosynthesis.

2.4 | SA and CA Induce Defence Response and Decrease *C*Las Titres in Citrus

To investigate whether SA and CA are involved in HLB disease resistance, we injected SA or CA into the leaves of *C*Las-infected

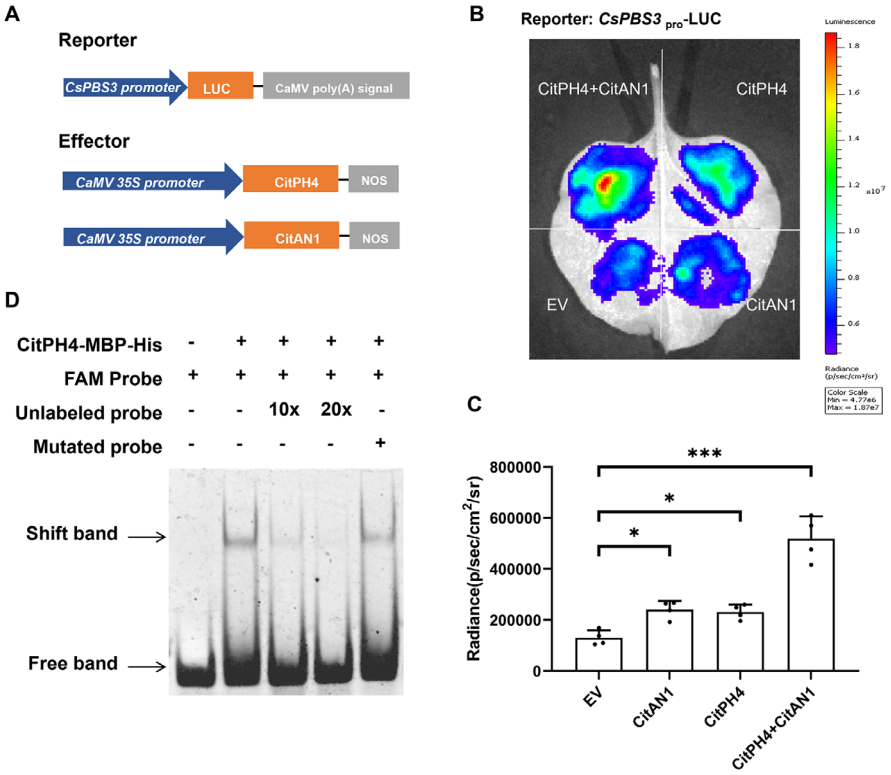

FIGURE 3 | CitPH4 regulates a key gene of salicyclic acid (SA) synthesis. (A) Schematic illustration of the vectors used in luciferase (*LUC*) assays. (B and C) CitPH4 activates the expression of *CsPBS3*, a key gene of SA synthesis, independently, or synergistically with *CitAN1*. CitAN1 (Citrus ANTHOCYANIN 1) is a basic helix–loop–helix transcription factor, which can form a transcription activation complex with CitPH4 to activate the expression of target genes. (D) Electrophoresis mobility shift assay (EMSA) indicates that CitPH4 directly binds to the promoter of *CsPBS3*. The unlabelled and mutated probes were used as competitors. The upper bands refer to the protein-labelled probe complex, and the lower bands indicate the free probe. Data are mean ± standard error ($n \geq 3$ biologically independent replicates). Asterisks indicate significant differences based on Student's *t* test: *$p < 0.05$, ***$p < 0.001$.

citrus plants. *CsPR1* expression levels were significantly upregulated after SA application, and *C*Las titres were significantly lower compared to that of control plants injected with water only (Figure 4A,G). This result is consistent with previous reports that application of SA is sufficient to diminish the *C*Las titres of the leaves from *C*Las-infected citrus plants (Nehela and Killiny 2020; Li et al. 2017).

We also tested the effect of CA application by infiltrating the leaves of *C*Las-infected citrus trees with different concentrations of CA (1, 10 and 100 mM). RT-qPCR analysis of the defence-related genes *CsPR1*, *CsPR2*, *CsPR3*, *CsPR5* and *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*CsFRK1*) revealed that CA treatment significantly induced their expression (Figure 4B–F). Similarly, treatment with 10 or 100 mM CA significantly lowered the *C*Las titres in the leaves of *C*Las-infected citrus trees (Figure 4H). These data suggest that the acidification of citrus fruits after *C*Las infection may represent a self-protective mechanism.

2.5 | CA and SA Reciprocally Activate Their Biosynthetic and Signalling Pathways in Citrus Fruits

To investigate whether CA treatment activates the SA pathway or vice versa, we treated kumquat (*Citrus japonica*) fruits, an ideal material for transient expression assay in citrus, using 10 mM CA or 2.5 mM SA, or with water only as the control. RT-qPCR analysis revealed that the expression levels of SA-related genes were significantly higher in CA-treated fruits than in the controls (Figure 5A–G). In addition, SA content was significantly increased in CA-treated fruits compared to control fruits treated with water (Figure 5H). Similarly, the expression levels of CA-related genes (*CsPH1*, *CsPH4* and *CsPH5*) were significantly upregulated in SA-treated fruits compared to control fruits treated with water only (Figure 5I–K). *CsPH1* and *CsPH5* encode subunits of a vacuolar proton-pumping P-ATPase complex, which contributes to the hyperacidification of citrus fruits (Strazzer et al. 2019). The CA content was also significantly higher in SA-treated fruits than in the control fruits (Figure 5L). These data suggest that the application of CA initiates the SA signalling pathway in citrus, which in turn enhances citrus immunity.

3 | Discussion

CA, the predominant organic acid in citrus fruits, not only confers the characteristic pleasant flavour of these fruits but also exerts a protective effect against biotic and abiotic stresses. Research on the antibacterial benefits of CA primarily focuses on its practical applications. For instance, treating postharvest peach (*Prunus persica*) fruits with about 50 mM CA limited fruit rot and maintained fruit quality over a prolonged period (Yang et al. 2019). Similarly, the treatment of chilli pepper

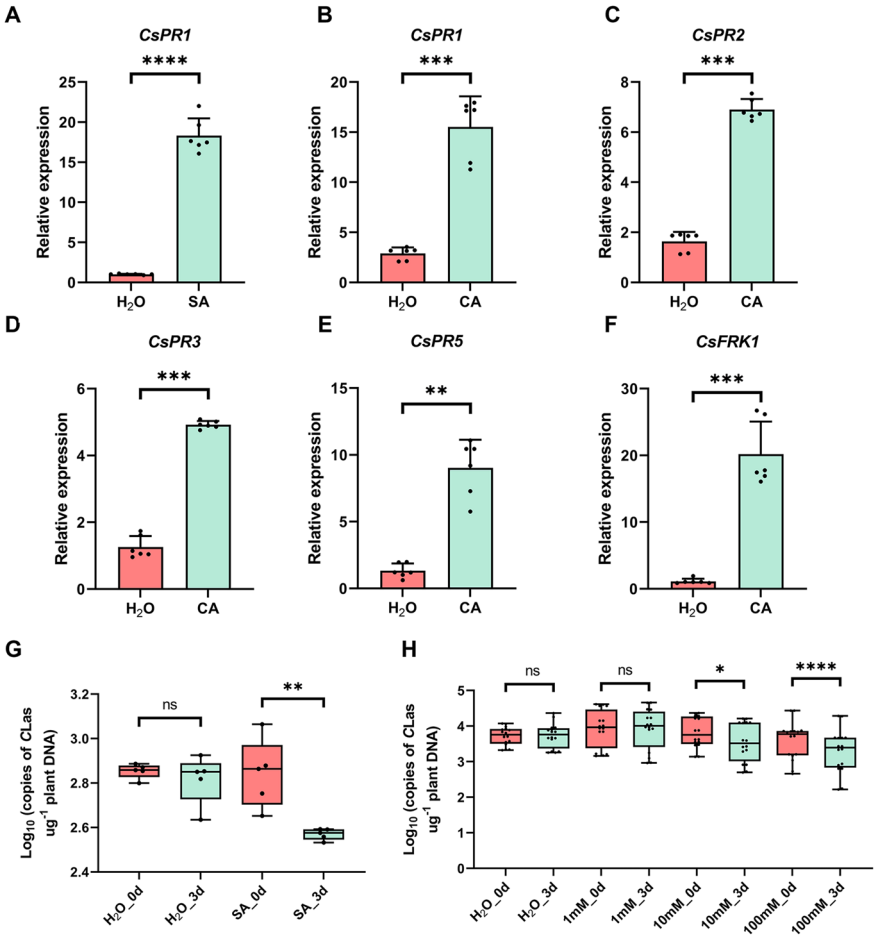

FIGURE 4 | The treatments by citric acid (CA) and salicylic acid (SA) reduces "*Candidatus* Liberibacter asiaticus" (*C*Las) titres. (A) The expression level of *CsPR1* gene after SA treatment. (B–F) The expression levels of *CsPR1*, *CsPR2*, *CsPR3*, *CsPR5* and *CsFRK1* genes after 10 mM CA treatment. (G) Changes in *C*Las content after 3 days of SA and water treatment in *C*Las-infected citrus leaves. (H) The effects of different concentrations of CA on the titre of *C*Las in *C*Las-infected citrus leaves. Water serves as control. Data are mean ± standard error ($n \geq 6$ biologically independent replicates). Asterisks indicate significant differences based on Student's *t* test: ns, no significance; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(*Capsicum annuum*) berries with 30 mM CA decreased the incidence of grey mould disease during storage, demonstrating the broad-spectrum antimicrobial potential of CA against pathogens (Mekawi et al. 2019). Previous studies have indicated that CA levels in healthy citrus fruits are typically in the 5–200 mM range (Huang et al. 2023), with CA representing over 90% of the organic acids in citrus fruits. In this study, we showed that CA treatment effectively led to lower *C*Las titres in the leaves of citrus plants infected with *C*Las, while also triggering the expression of plant immunity genes, supporting the idea that CA is involved in the defence against *C*Las infection. Considering that CitPH4 is a critical regulator of CA and that *CitPH4* expression is significantly induced upon *C*Las infection, CitPH4-mediated defence responses are probably one of the strategies deployed by citrus plants against *C*Las infection, concomitantly leading to the accumulation of CA in fruits.

SA is an important signalling molecule in plant immunity that is produced in response to pathogen infection, with a well-elucidated biosynthetic pathway (Rawat et al. 2023; Huang et al. 2020). Recent reports have revealed that SA plays a crucial role during both the infection of citrus plants by *C*Las and their resistance to the pathogen (Wang et al. 2017). When it first infects citrus plants, *C*Las expresses the SA hydroxylase gene *sahA*, encoding an enzyme that degrades SA in plant cells, thereby suppressing plant defences (Li et al. 2017). At a later stage, however, the accumulation of SA significantly increases in citrus plants after infection with *C*Las (Martinelli et al. 2012; Ibanez et al. 2019; Oliveira et al. 2019; Zou et al. 2019; Peng et al. 2021; Du et al. 2022; Ibanez et al. 2022; Liu et al. 2023), and the constitutive overexpression of SA-related genes in plants enhances their tolerance to *C*Las infection, such as *NPR1* (Dutt et al. 2015; Peng et al. 2021), *SALICYLIC ACID METHYLTRANSFERASE 1* (*SAMT1*) (Zou et al. 2021) and *SALICYLIC ACID BINDING PROTEIN 2* (*SABP2*) (Soares et al. 2022; Dong et al. 2024). The infiltration of SA through the trunk effectively combats *C*Las infection, with an application of 0.25 g per tree being the most cost-effective concentration (Hu et al. 2018; Li et al. 2016; Li, Kolbasov et al. 2021). Additionally, SA application significantly enhances the disease resistance of postharvest horticultural crops, prolonging their quality maintenance period (Adhikary et al. 2021; Jiang et al. 2022). However, the regulation of SA biosynthesis and accumulation in *C*Las-infected citrus plants remains largely unexplored. In this report, we describe how CitPH4, a key regulator of CA accumulation, regulates the expression of *CsPBS3*, an important gene involved in SA biosynthesis in citrus fruits.

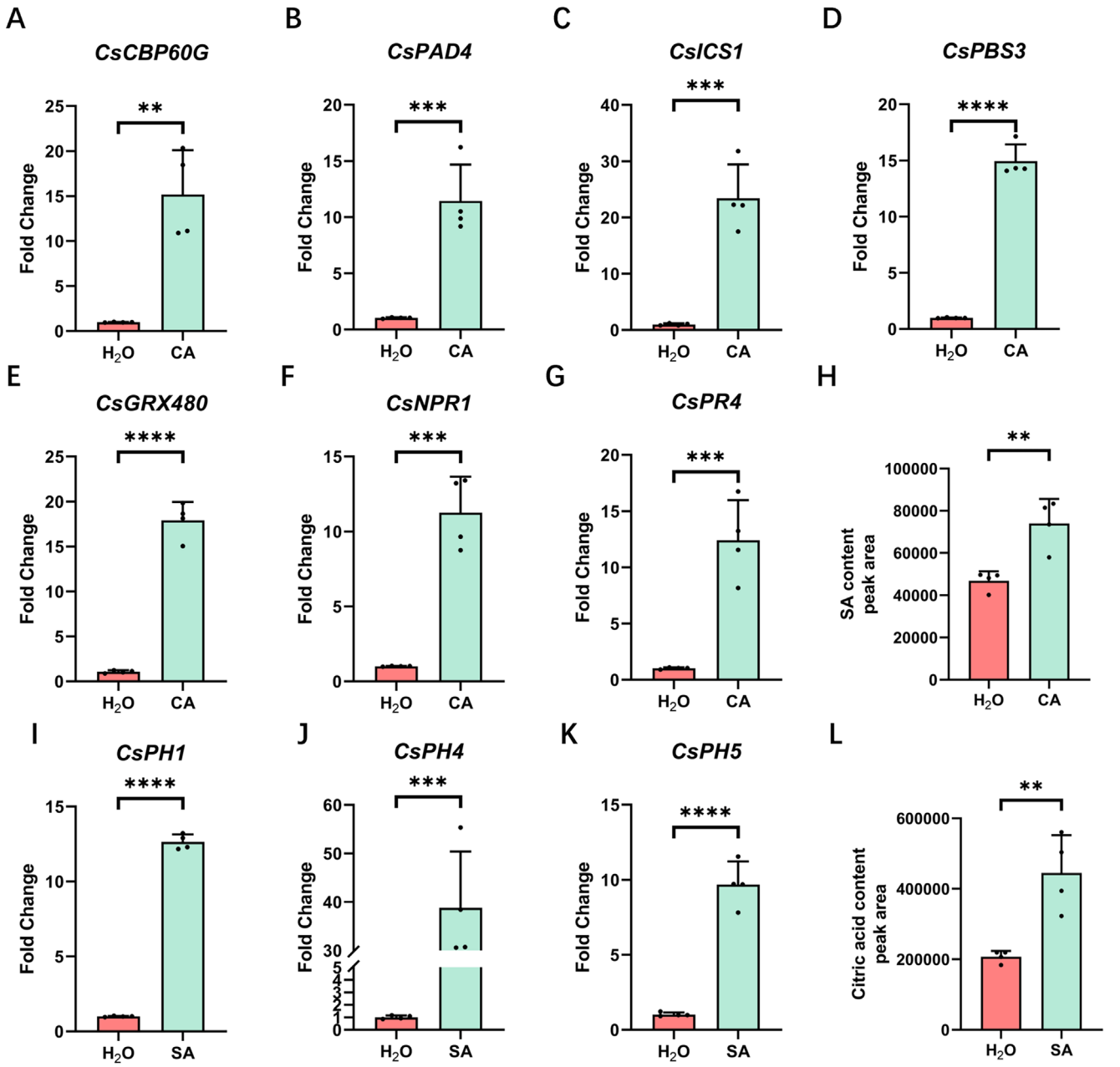

FIGURE 5 | The increased citric acid (CA) level can reciprocally activate the salicylic acid (SA) pathway in citrus fruits. (A–G) The expression levels of SA-related genes after treatment with water and CA. (H) The content of SA in water- and CA-treated fruits. (I–K) The expression levels of CA-related genes in water- and SA-treated fruits. (L) The content of CA in water- and SA-treated fruits. Asterisks represent significant differences based on Student's *t* test (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n \geq 3$).

Our findings further reveal that SA and CA activate plant immunity and decrease *C*Las levels, indicating crosstalk between defence response and fruit quality in citrus. However, because *C*Las cannot yet be cultured in vitro, further research is needed to determine whether CA and/or SA directly inhibit *C*Las proliferation or even kill *C*Las.

Gene pleiotropy plays a crucial role in plant resistance to stress and the formation of important quality traits (Wiesner-Hanks and Nelson 2016). In our study, CA was a determining factor for the acidity of citrus fruits. CitPH4 is a crucial transcription factor regulating CA formation. Our findings demonstrate that CitPH4 not only promotes CA accumulation but also raises SA levels. *C*Las pathogenicity assays indicated that overexpression of *CitPH4* enhanced tolerance to *C*Las in tobacco plants. Examples from other studies include the rice (*Oryza sativa*) gene *IDEAL PLANT ARCHITECTURE 1* (*IPA1*), which increases yield by enhancing the number of grains per panicle. During pathogen infection, IPA1 undergoes phosphorylation, enabling it to promote the function of disease resistance genes (Wang, Zhou et al. 2018). As another example, the zinc-finger transcription factor SENSITIVE TO PROTON RHIZOTOXICITY 1 from Arabidopsis regulates the transport of ions across the cell membrane and vacuolar membrane, enhancing the plant tolerance to metal stress and extreme pH stress conditions (Sadhukhan et al. 2021). For horticultural crops, developing high-quality,

disease-resistant varieties has been a long-standing breeding goal, although related research remains limited. Fine-tuning the expression of *CitPH4* may be important to maintain fruit quality while ensuring disease resistance.

This study is the first to report *C*Las inoculation in tobacco plants (scions) through approach grafting of *C*Las-infected citrus plants (stocks). Previous studies have shown that *C*Las can be transmitted to tobacco plants through the parasitic plant dodder (*Cuscuta australis*) (Wang and Trivedi 2013). However, dodder is a suboptimal method for *C*Las inoculation owing to its preferential parasitism and annual growth habits (Thakuria et al. 2023; Mishra 2009). Current grafting methods for *C*Las inoculation are time-consuming and labour-intensive, with grafting in citrus being subject to strict seasonal constraints (Ramsey et al. 2020; Stegemann and Bock 2009), making inoculations in autumn or winter particularly challenging. In this study, we transmitted *C*Las from citrus plants to tobacco plants via approach grafting, a method minimally influenced by environmental factors. Given the wide grafting compatibility of tobacco (Notaguchi et al. 2020), this method may be useful for preliminary screening assays in future HLB research.

This study investigated the molecular basis behind the increased fruit acidity in citrus plants infected by *C*Las. Our study indicates that CitPH4, a key regulator of CA accumulation, also induces the immune response to *C*Las infection by activating SA biosynthesis. Analysis of SA levels in the fruits of *CitPH4*-knockout plants confirmed that CitPH4 positively regulates the SA pathway. Treatment with SA or CA reciprocally activated the biosynthetic and signalling pathways in citrus fruits and decreased the *C*Las titres in the leaves of *C*Las-infected citrus plants, highlighting their roles in the defence responses to HLB. The elucidation of CitPH4 pleiotropic functions lays a foundation for its future applications in citrus breeding.

4 | Experimental Procedures

4.1 | Plant Materials

*C*Las-infected sweet oranges (*Citrus sinensis*) were obtained in the field from the Science Research Institute of Ganzhou, Jiangxi province, China. *C*Las-infected mandarin fruits were sampled in the field from the Guangxi Key Laboratory of Germplasm Innovation and Utilisation of Specialty Commercial Crops in North Guangxi, Guangxi Academy of Specialty Crops, Guangxi province, China. *Nicotiana tabacum* and *Nicotiana benthamiana* plants were cultivated in a growth chamber with a photoperiod of 14 h of light and 10 h of darkness, maintaining a temperature of 25°C.

4.2 | Gene Expression Analysis

The *CitPH4*-knockout early-flowering citrus (*Citrus hindsii*) plants were generated from our previous studies (Huang et al. 2023). Total RNA was extracted from mature *CitPH4*-knockout citrus fruits and *C*Las-infected mandarin fruits using TRIzol iso plus (Takara). Total RNA was reverse-transcribed to cDNA using HiScript II Reverse Transcriptase Kit (Vazyme Biotech). Quantitative PCR was performed with Hieff qPCR SYBR Green Master Mix (YEASEN) and Light Cycler 480 (Roche). Equal amounts of cDNA from three independent biological replicates were analysed. Three technical replicates were performed for each biological replicate. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table S1.

The transcriptome used in this study for the analysis of gene expression induced by *CitPH4* is cited from previously published papers, accession number PRJNA1076338.

4.3 | Construction of the Expression Vectors and Genetic Transformation

To generate *CitPH4* overexpression vectors, the full-length coding sequence of *CitPH4* was amplified from sweet orange and inserted into the pK7WG2D Gateway vectors using BP and LR enzymes. *Agrobacterium tumefaciens* GV3101(pSoup) with the recombinant plasmid was used for tobacco transformation as described previously (Mayo et al. 2006). The primers used are listed in Table S1.

4.4 | *LUC* Reporter Assays

The promoter sequence of *PBS3* was amplified and fused into the pGreen0800 vectors as reporters. The full-length coding sequence of *CitPH4* and *CitAN1* was cloned into the pK7WG2D vector to generate overexpression vectors. *CitPH4* or *CitPH4* and *AN1*, as well as empty vector (EV), were used as effectors. All the vectors were introduced into the *A. tumefaciens* GV3101 (pSoup-p19) strain. The *Agrobacterium* culture was diluted to an $OD_{600}=0.8$. *LUC* reporter assays were conducted as described previously (Zheng et al. 2023). The primers used are listed in Table S1.

4.5 | Recombinant Protein Purification and Electrophoretic Mobility Shift Assay

The coding sequence of *CitPH4* was amplified and inserted into pMAL-C6T to generate maltose-binding protein (MBP)-tagged and His-tagged recombinant proteins. These constructs were transformed into *Escherichia coli* BL21 (DE3), and protein expression was performed as described previously (Tang et al. 2021).

For the electrophoretic mobility shift assay, the purified CitPH4-MBP-His recombinant protein was employed. Oligonucleotides containing a potential MYB-binding site and the adjacent 10-bp sequence were synthesised and labelled with 5′ 6-FAM (Sangon). The annealed probes were incubated with the CitPH4-MBP-His protein in the dark for 40 min, followed by electrophoresis at 100 V in the dark for 1 h. The primer details can be found in Table S1.

4.6 | Measurement of SA and CA Content

The samples of *C*Las-infected fruits, *CitPH4*-KO fruits and tobacco leaves were stored at −80°C until analysis. The SA and

CA content was quantified using a liquid chromatograph mass spectrometer (LC–MS, Thermo Fisher Scientific).

4.7 | Pathogen Inoculation

For the approach grafting assay, we planted the *CitPH4*-overexpressing tobacco (about 1-month-old) near the *C*Las-infected sweet orange (Dahong), trimmed part of the *CitPH4*-overexpressing tobacco stem and similarly trimmed part of the citrus. The trimmed sections of both tobacco and citrus plants were then tightly aligned together. For the *C*Las pathogenicity assay, *CitPH4*-overexpressing tobacco plants (about 1-month-old) were graft-inoculated with *C*Las-infected sweet orange (Dahong) using approach grafting in the greenhouse (Li et al. 2009), with WT tobacco plants used as the control. Midrib DNA was isolated from the graft-inoculated tobacco weekly after grafting. Isolated DNA was used to quantify *C*Las by TaqMan qPCR with primer/probe combination.

The standard curve was generated using the serial dilutions (10^1–10^7) of a DNA extract of a sweet orange plant infected with *C*Las in greenhouse as previously described (Li et al. 2006). The bacterial populations (*C*Las cells per 1 μg of citrus DNA) were quantified with a qPCR assay that was described by Huang et al. (2021). *C*Las quantification was carried out as follows: DNA was used for qPCR amplification using 16S rRNA primers HLBasf and HLBr, the probe HLBp, TaqMan PCR master mix, and SYBR Green PCR master mix. The qPCR assays were performed with Light Cycler 480 (Roche) using the SYBR Green PCR Master probe mix (YEASEN) in a 10-μL volume. The data were normalised to the expression of the citrus *mitochondrial cytochrome oxidase* gene (*COX*). The standard amplification protocol was 95°C for 10 min followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. The sequences of primer and probe combinations used for detection of *C*Las titres were obtained from Fujikawa and Iwanami (2012) and synthesised by Biotechnology Company (Tsingke, Beijing).

4.8 | Exogenous Application of SA and CA on citrus

For the assessment of the control effect of CA and SA treatment on HLB, six *C*Las-infected and six healthy 4-year-old sweet orange (Dahong) were used as experimental plants for exogenous application of SA and CA assays. Aqueous solutions of 1, 10 and 100 mM CA, 0.25 mM SA (Sigma-Aldrich) were injected into leaves, in addition to the control (double-distilled water). After the application, all treatments (10 biological replicates derived from 10 individual citrus leaves taken from three different citrus plants) were kept under greenhouse conditions as described above. Two equally sized punctures from symmetrical positions on both sides of the leaf veins of each leaf were collected before the treatment (time 0) and 3 days post-inoculation (dpi). Isolated DNA was used to quantify *C*Las as described above.

For the exogenous application of SA and CA on citrus fruits assay, uniform kumquat fruits during the colour-change period were treated using 10 mM CA or 2.5 mM SA, with water as the control. The fruits surrounding the injection site were sampled at 3 dpi for gene expression analysis and at 5 dpi for metabolite content quantification. These kumquat fruits used in this study were kindly provided by Xiaoxiao Wu from the Guangxi Key Laboratory of Citrus Biology, Guangxi Academy of Specialty Crops.

4.9 | Measurement of Titrating the Acid

One millilitre of citrus juice was collected from fresh fruits, and then the titratable acidity was determined using a citrus sugar-acid meter (ATAGO, PAL-BX/ACID 1, 7101).

Acknowledgements

This project was supported by the National Natural Science Foundation of China (31925034 and U23A20198), Key S&T Projects in Nanning city (20232078), Key Project of Hubei Provincial Natural Science Foundation (2021CFA017), the Fundamental Research Funds for the Central Universities (2662023PY003), the Postdoctoral Fellowship Program of CPSF (GZC20230913), Hubei Provincial Natural Science Foundation of China (2024AFB138) and Postdoctor Project of Hubei Province (2004HBBHCXA044).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Additional data can be found in Table S1.

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Supporting Information

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