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ORIGINAL ARTICLE

Overexpressing a *NPR1*-like gene from *Citrus paradisi* enhanced Huanglongbing resistance in *C. sinensis*

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Abstract

Key message **Overexpression of *CiNPR4* enhanced resistance of transgenic citrus plants to Huanglongbing by perceiving the salicylic acid and jasmonic acid signals and up-regulating the transcriptional activities of plant–pathogen interaction genes.**

Abstract Developing transgenic citrus plants with enhanced immunity is an efficient strategy to control citrus Huanglongbing (HLB). Here, a *nonexpressor of pathogenesis-related gene 1* (*NPR1*) like gene from HLB-tolerant 'Jackson' grapefruit (*Citrus paradisi* Macf.), *CiNPR4*, was introduced into 'Wanjincheng' orange (*Citrus sinensis* Obseck). *CiNPR4* expression was determined in transgenic citrus plants using quantitative real-time PCR analyses. The *Candidatus* Liberibacter asiaticus (*C*Las) pathogen of HLB was successfully transmitted to transgenic citrus plants by grafting infected buds. HLB symptoms developed in transgenic and wild-type (WT) plants by 9 months after inoculation. A *C*Las population analysis showed that 26.9% of transgenic lines exhibited significantly lower *C*Las titer levels compared with the *C*Las-infected WT plants at 21 months after inoculation. Lower starch contents and anatomical aberration levels in the phloem were observed in transgenic lines having enhanced resistance compared with *C*Las-infected WT plants. *CiNPR4* overexpression changed the jasmonic acid, but not salicylic acid, level. Additionally, the jasmonic acid and salicylic acid levels increased after *C*Las infection. Transcriptome analyses revealed that the enhanced resistance of transgenic plants to HLB resulted from the up-regulated transcriptional activities of plant–pathogen interaction-related genes.

Keywords Citrus · Huanglongbing · *CiNPR4* · Transcriptional activity · Defense response

Introduction

Citrus Huanglongbing (HLB), mainly caused by *Candidatus* Liberibacter asiaticus (*C*Las), is a destructive bacterial plant disease worldwide. *C*Las specifically resides in the phloem of citrus plants and is mainly transmitted by the sap-sucking Asian citrus psyllid (*Diaphorina citri*) or by grafting *C*Las-infected shoots or buds onto healthy citrus plants (Bové 2006; da Graça et al. 2016). The typical symptoms of a *C*Las infection are an asymmetric blotchy mottling of leaves and yellow shoots. The fruit produced by *C*Las-infected trees are small, green, underdeveloped and misshapen, with a bitter and sour taste. The whole plant dies a few years after being infected. Common strategies to control HLB focus mainly on removing diseased trees, controlling the Asian citrus psyllid and using disease-free nursery trees (Belasque et al. 2010). However, to date, there are no curative methods to control HLB in citrus. The use of HLB-resistant or -tolerant citrus trees would be a more effective control strategy than those currently employed, however, the lack of resistant citrus resources has limited the development of resistant citrus cultivars. Thus, genetically modifying citrus to establish resistance against HLB is an attractive strategy.

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Plants resist pathogens using many mechanisms, such as the hypersensitive response, systemic acquired resistance (SAR) and induced systemic resistance. SAR is an inducible defense response that is activated by pathogen challenge. It is of great interest because it provides long-lasting, broad-spectrum defense responses to resist a wide range of bacterial, fungal and viral diseases. After pathogen invasion, some hosts produce salicylic acid (SA) signaling molecules, which are transmitted from the local infection site to distal tissues to activate the SAR system (Sticher et al. 1997; An and Mou 2011). A comparative analysis of HLB-tolerant sour pomelo (*Citrus grandis* Osbeck) and HLB-susceptible 'Jincheng' orange (*Citrus sinensis* Osbeck) after *C*Las infection determined that sour pomelo displays a greater SAR through the transmission of methyl salicylate, a main form of SA in plants, in the phloem to produce defense responses to resist the invasion of *C*Las (Zou et al. 2019), revealing that HLB-tolerant citrus recruit SAR to improve their innate immunity to *C*Las. Therefore, employing plant SAR to resist *C*Las infections in citrus may be feasible.

In the SA signal transduction pathway, three *nonexpressor of pathogenesis-related* (*NPR*) genes, *NPR1*, *NPR3* and *NPR4*, are involved in the plant defense responses (Fu et al. 2012; Wu et al. 2012; Ding et al. 2018). Fu et al. (2012) discovered that NPR1 lacks SA-binding activity, whereas NPR3 and NPR4 are SA receptors. NPR3 or NPR4 binds with NPR1 and functions as an adaptor of CULLIN3 ubiquitin ligases to mediate NPR1 degradation. However, in another study, both NPR1 and NPR3/4 acted as SA receptors, but had opposite roles in the transcriptional regulation of plant defenses against pathogens. NPR1 and NPR3/4 regulate the activities of the TGA transcription factors to promote and repress, respectively, the expression levels of defense genes. However, NPR1 and NPR3/4 function independently to regulate SA-induced plant defenses (Ding et al. 2018). A low SA level initiates the NPR3/4 repression activity, which prevents plant autoimmunity. Increased SA levels inhibit the repressive functions of NPR3/4 and allow SA to bind with NPR1, which induces the expression of defense genes. Irrespective of the mechanism employed by NPR1 or NPR3/4 in regulating plant immunity, accumulating the appropriate levels of SA and the NPR1 protein is the key to initiating plant defense responses. Therefore, manipulating *NPR1*, *NPR3* or *NPR4* to change plant resistance through SA-mediated defense responses may be an effective strategy to produce genetically modified plants with increased disease resistance.

Arabidopsis thaliana NPR1 and its homologs have been widely used to enhance plant fungal and bacterial resistance levels (Makandar et al. 2006; Meur et al. 2008; Wally et al. 2009; Zhang et al. 2010; Chen et al. 2013). In addition, many *NPR1*-like genes have been identified in different plant species (Pilotti et al. 2008; Le Henanff et al. 2009; Peraza-Echeverria et al. 2012; Shao et al. 2013), and their functions in disease resistance or susceptibility have been analyzed. A *NPR1*-like gene from the oriental hybrid lily 'Sorbonne' (*Lilium* spp.) was overexpressed in *Arabidopsis*, resulting in transgenic plants with enhanced bacterial resistance (Wang et al. 2017). Some *NPR1*-like genes highly homologous to *AtNPR3/4* have been used in disease-resistance studies. Introducing *Theobroma cacao NPR3* into an *Arabidopsis* npr3 mutant partially restores the susceptibility to bacterial infection. Knocking *TcNPR3* out of cacao leaf tissues enhances resistance to *Phytophthora capsici* (Shi et al. 2013). The ectopic expression of *Fragaria vesca NPRL-1*, which is phylogenetically close to *NPR4* in *Arabidopsis*, confers enhanced susceptibility to *Pseudomonas syringae* pv. tomato DC3000 (Shu et al. 2018). Thus, *NPR1*, *NPR1* homologs and *NPR1*-like genes are attractive candidates for engineering plant disease resistance.

Recently, *AtNPR1* was manipulated to increase citrus plant resistance to HLB (Dutt et al. 2015). A transcriptome analysis of *AtNPR1* transgenic citrus plants against *C*Las identified 57 differentially expressed genes (DEGs). A further analysis revealed that these DEGs encoded proteins of pathogen-associated molecular patterns, leucine-rich repeat receptor kinases, transcription factors and putative ankyrin repeat-containing proteins, indicating that *AtNPR1* positively regulates innate defense mechanisms in citrus (Qiu et al. 2020). Wang et al. (2016) predicted a protein–protein interaction sub-network associated with interactions between NPR1-like proteins and TGA transcription factors by analyzing DEGs between HLB-tolerant 'Jackson' and HLB-susceptible 'Marsh' grapefruit (*C. paradisi* Macf.). The expression of one *NPR1*-like gene (*Ciclev10031749m*) was significantly up-regulated in HLB-tolerant 'Jackson' grapefruit, suggesting that this *NPR1*-like gene positively regulates HLB resistance. In this study, we genetically transformed *Ciclev10031749m* into 'Wanjincheng' orange (*C. sinensis* Osbeck) to study its potential effects on HLB resistance.

Materials and methods

Bioinformatics analysis of an *NPR1*-like gene

The full-length DNA and protein sequences of the *NPR1*-like gene (*Ciclev10031749m*) were downloaded from the citrus genome database (https://www.citrusgenomedb.org/). The exon–intron structure of the full-length DNA sequence was analyzed using GSDS2.0 (http://gsds.cbi.pku.edu.cn/).

To investigate the phylogenetic relationships between the NPR1-like protein and other *NPR1* gene family proteins, a phylogenetic tree was constructed as described by Liu et al. (2019) with minor modifications. The NPR1-like protein

sequence and other dicot NPR1-related protein sequences from the report of Liu et al. (2019) and from *C. sinensis* and *Fragaria vesca* were aligned using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw/). Subsequently, a phylogenetic tree was constructed using MEGA v7.0 (Kumar et al. 2016) with the neighbor-joining method and the following parameters: 1,000 bootstrap replicates, Poisson correction and complete deletion. Conserved domain compositions of the NPR1-like protein and other NPR1-related proteins were predicted using Pfam (http://pfam.xfam.org) and visualized using TBtools software (Chen et al. 2020). A multiple amino acid sequence alignment between NPR1-like and other known NPR3/4 proteins was performed using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/) and visualized using Jalview Version 2 (Waterhouse et al. 2009).

Construction of the transformation vector

KpnI and EcoRI sequences were added to the 5′- and 3′-ends, respectively, of the coding sequence of the *NPR1*-like gene. The modified coding sequence was ligated into the pUC57 plasmid by BGI Tech Solutions (Beijing Liu He Co., Limited, Beijing, China) to construct pUC57:CiNPR. The pUC57:CiNPR and pGN plasmids (constructed in our laboratory) were digested with KpnI and EcoRI. The coding sequence of the *NPR1*-like gene was inserted into the KpnI/EcoRI double-digested pGN to form the plant expression vector pGNCiNPR. Then, the plasmid was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. From the left to right borders of the T-DNA, the pGNCiNPR plasmid harbored the fusion of *uidA* plus neomycin phosphotransferase genes (*uidA:NPTII*) followed by the *NPR1*-like gene. Both *uidA:NPTII* and *NPR1*-like genes were driven by the Cauliflower mosaic virus 35S promoter and terminated by the nopaline synthase gene terminator.

Plant transformation

Fruit of 'Wanjincheng' orange (*C. sinensis* Osbeck) were collected from the National Citrus Germplasm Repository, Chongqing, China and sterilized with 75% ethanol. The seeds were removed from the fruit and placed on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) on a clean bench. The epicotyl transformation of 'Wanjincheng' orange was performed as described by Peng et al. (2015) with modifications. After co-cultivation, the epicotyl segments were transferred onto MS medium containing 2 mg L^{-1} 6-benzylaminopurine, 0.25 mg L^{-1} indole-3-acetic acid, 500 mg L^{-1} cefotaxime and 70 mg L^{-1} kanamycin, and then, they were placed in the dark at 28 °C for 7 d. At this time, the cultures were exposed to a 16-h photoperiod at 28 °C. Two months later, all the adventitious buds generated from the ends of explants were examined using GUS histochemical staining (Peng et al. 2019). The GUS-positive shoots were grafted onto citrange seedlings in vitro (He et al. 2011). One month later, they were grafted onto 1-year-old citrange seedlings in a greenhouse.

Molecular analysis of transgenic plants

PCR was used to detect the integration of the transgene into the citrus genome. Total genomic DNA was extracted from leaves of GUS-positive shoots and wild-type (WT) plants using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Quantitative real-time PCR (qRT-PCR) was performed to detect the expression level of the transgene in transgenic plants. Leaves of transgenic and WT plants were collected for the extraction of total RNA using an EASYspin Plant RNA Extraction Kit (Aidlab, Beijing, China). The total RNA was reverse-transcribed into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The citrus *actin* gene (*Cs1g05000*) was used as the reference gene for the qRT-PCR analysis. The relative expression level of the transgene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The primers for PCR and qRT-PCR analyses are presented in Supplemental Table 1. The expression analysis had three biological repeats.

Analysis of HLB resistance in transgenic citrus plants

Transgenic and WT plants were propagated by grafting onto a *Citrus junos* Sieb. ex Tanaka rootstock. Nine months after grafting, four plants per transgenic line, as well as the WT, having similar growth potentials and heights, were selected to be grafted with *C*Las-infected buds. Each plant was grafted with four *C*Las-infected buds, which each contained 10^6 *C*Las cells μg^{-1} citrus DNA. The inoculated plants were cultured in a 28 °C greenhouse. After grafting, the plants were observed and disease symptoms recorded monthly. Every 3 months, a qPCR analysis was performed with 50 ng DNA from leaf midrib tissue to detect the Ct values of the *C*Las 16S rDNA and citrus 18S genes. The primer pairs for 16S rDNA and 18S amplification are shown in Supplemental Table 1. The *C*Las population was calculated in accordance with the report of Zou et al. (2017) as follows: *C*Las cells μg^{-1} citrus DNA $= [10^{(-0.2718 \times Ct\ 16S\ rDNA + 10.624)} / 10^{(-0.2749 \times Ct\ 18S + 4.0531)}] \times 10^3$ ($12.7 < Ct\ 16S\ rDNA < 31.3$ and $8.4 < Ct\ 18S < 26.5$). The *C*Las population analysis was repeated three times.

Analysis of the starch content in transgenic citrus plants

The starch content in transgenic plants was determined every 6 months after inoculating with *C*Las. Briefly, 0.1 g leaf

tissue per sample was ground to a fine powder for measuring the starch content in accordance with the instructions of the Starch Content Detection Kit (G-clone, Biotechnology Co., Ltd, Beijing, China). The experiment was repeated three times.

Epoxy resin-embedded tissue sections

Leaf midribs of transgenic and WT plants, with and without *C*Las-infections, were cut into 2–5 mm length and then quickly placed into 4% paraformaldehyde universal tissue fixation solution. After being subjected to a vacuum, the tissues were rinsed with 0.1 M potassium phosphate buffer (pH 7.2) three times for 15 min per wash and then sequentially dehydrated with 30%, 50%, 70%, 85% and 95% acetone solutions for 15 min each. Afterwards, they were placed in 100% acetone solution three times for 20 min per rinse. Dehydrated midrib fragments were permeated with LR White Resin:acetone at 1:3 (v/v) for 4 h, 1:1 (v/v) overnight and 3:1 (v/v) for 4 h. Finally, they were embedded in LR White Resin overnight at 70 °C. The tissues were sectioned transversely into 0.8 µm slices using a Leica EM UC7 Ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by toluidine blue. The stained tissues were observed under an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Hormone content measurement

The plant hormones SA and jasmonic acid (JA) were measured in non- and *C*Las-infected transgenic and WT plants using an enzyme-linked immunoabsorbent assay method by Sino Best Biological Technology Co., Ltd (Shanghai, China) with three biological repeats.

RNA sequencing (RNA-seq) analysis of transgenic plants

At 18 months after *C*Las inoculation, a transgenic line, WT control plant and a non-inoculated plant of the same line were selected for an RNA-seq analysis. Total RNAs were isolated from 3.0 g leaves of each plant using an RNeasy Plant Mini Kit following the kit instructions (Qiagen, Valencia, CA, USA). The integrity levels of the total RNAs were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The qualified RNA samples with RNA integrity values > 6.5 were used for RNA-seq by Biomarker Technologies Co., Ltd (Beijing, China). Sequence reads were mapped, and the functions of DEGs were annotated using the *C. sinensis* genome, which is available online (http://citrus.hzau.edu.cn/cgi-bin/orange/search), as a reference. For the RNA-seq analysis, three biological replications were used.

A gene ontology (GO) enrichment analysis of the DEGs was implemented using the GO seq R package (Young et al, 2010). Overrepresented *p*-values in the hyper-geometric test were used to identify significantly enriched GO terms with corrected *p*-values < 0.05.

To analyze significantly enriched metabolic or signal transduction pathways of DEGs, a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed. KOBAS (Mao et al. 2005) software was used to test the statistical enrichment of DEGs. The KEGG enrichment degree was measured on the basis of the enrichment factor, *q* value and gene number enriched in the pathway. The 20 most significantly enriched pathways were selected. A pathway was determined as being significantly enriched when its corrected *p*-value was less than 0.05.

In total, 35 DEGs were selected for the validation of RNA-seq results using qRT-PCR. The selected genes and gene-specific primers are shown in Supplemental Table 2. The citrus *actin* gene (*Cs1g05000*) was used as the reference gene. The relative expression levels of tested genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Three biological replications were performed.

Data analysis

The data were analyzed using the IBM SPSS Statistics 19 software (SPSS Inc., Chicago, IL, USA) and expressed as the means ± standard deviation (SD) of three biological replicates. Significant differences were established using Duncan's test at a 0.05 level. For RNA-seq, *p*-values were adjusted using *q*-values. Thresholds of *q* value < 0.05 and |fold change| ≥ 2 were set as criteria of significant differential expression.

Results

Phylogenetic and structural analyses of NPR1-like protein

To clarify the classifications of the NPR1-like (Ciclev10031749m) proteins, phylogenetic analyses were performed. The NPR1-like protein was grouped into a clade with AtNPR3 and AtNPR4, and it was most closely related to CsNPR4, suggesting that it would be a member of the NPR4 group (Fig. 1a). Therefore, in this study, this *NPR1*-like gene was termed as *CiNPR4*. An investigation of the exon–intron distribution revealed that *CiNPR4* contains four exons and three introns.

To further understand its potential functions, the structural features of the CiNPR4 protein were analyzed. Unlike other NPR1/2 and NPR3/4 proteins in clades I and II, respectively, the CiNPR4 protein lacks an N-terminal BTB/

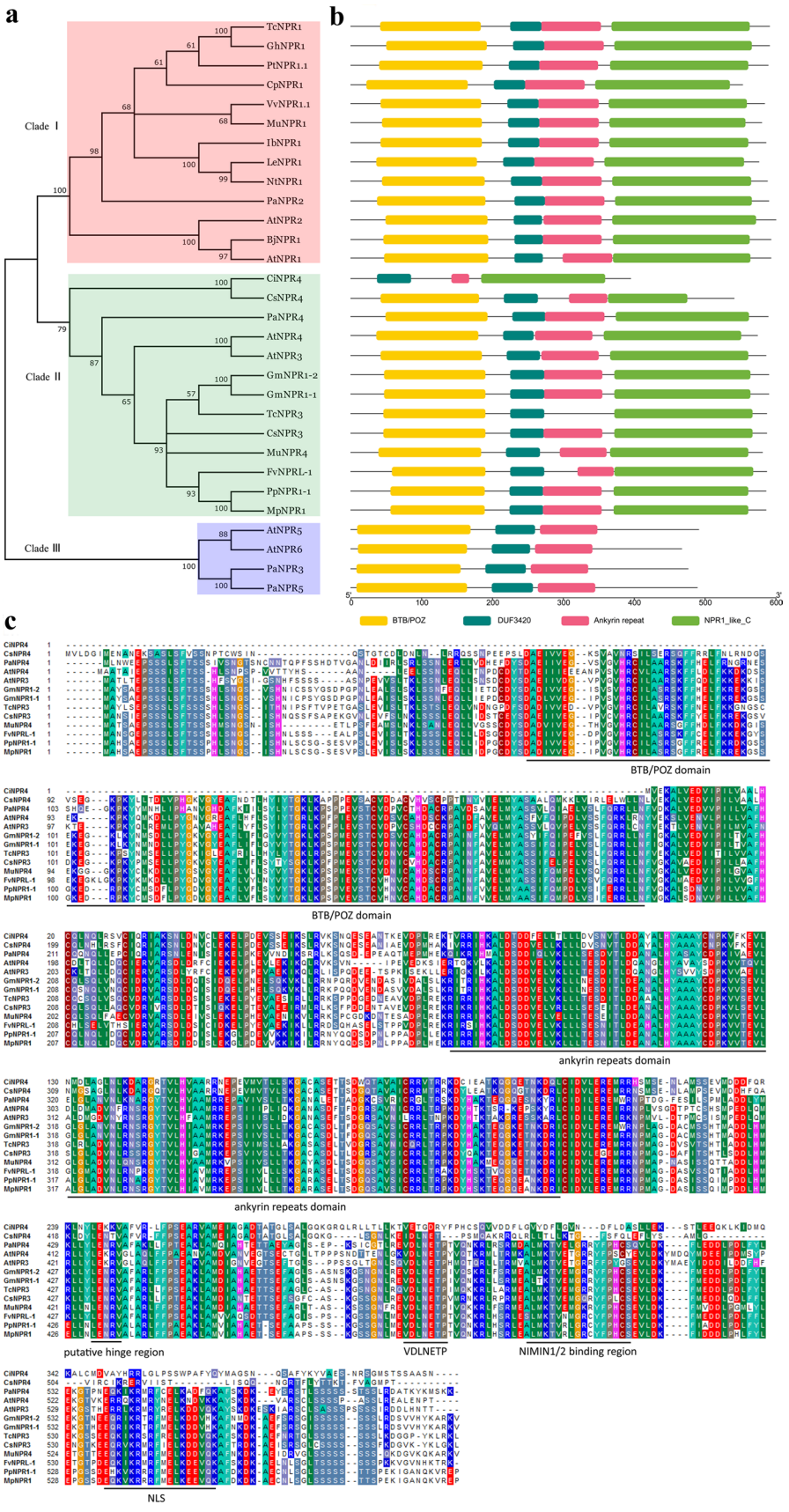

Fig. 1 Phylogenetic analysis and amino acid sequence comparison of CiNPR4 with other known NPR-like proteins. **a** Phylogenetic tree of CiNPR4 and other known NPR-like proteins. Clades I, II and III are highlighted in pink, pale blue and grey, respectively. **b** Conserved domains of BTB/POZ, DUF3402, ankyrin repeat and NPR1-like C-terminal domains presented in CiNPR4 and other known NPR-like proteins. **c** Alignment of amino acid sequences of CiNPR4 and other NPR3/4 proteins in clade II. The conserved BTB/POZ domains, ankyrin repeats, putative hinge regions, VDLNETP motifs, NIMIN1/2-binding regions and nuclear localization signals (NLSs) are marked with black lines

POZ domain and only contains ankyrin repeats in the central region and the C-terminal transactivation domain (Fig. 1b). Additionally, the EAR-like motif VDLNETP, which is required for AtNPR3/4 function as a co-repressor factor, is absent from the CiNPR4 protein (Fig. 1c). The difference in the CiNPR4 protein domain's composition and the lack of a VDLNETP motif indicated that the protein had a specific function in plant immunity.

Production and confirmation of transgenic plants

CiNPR4 was introduced into 'Wanjincheng' orange using *Agrobacterium*-mediated transformation. In total, 30 GUS-positive shoots were obtained and grafted to the rootstocks. The *uidA:NPTII* gene, in the same T-DNA with the *CiNPR4* gene, was selected to determine transgene integration. A predicted 531-bp fragment was amplified from 26 of 30 GUS-positive shoots at 3 months after grafting (Fig. S1a), indicating the successful insertion of *uidA:NPTII* into the 'Wanjincheng' orange genome. A WT plant served as the calibrator in the relative *CiNPR4* expression level analysis of 26 transgenic lines. *CiNPR4* was differentially expressed in all the transgenic lines (Fig. S1b).

Enhanced HLB resistance in transgenic plants

CiNPR4 overexpression significantly inhibited *C*Las growth. Transgenic plants also demonstrated significantly fewer HLB symptoms compared with the control. The development of *C*Las populations in all the transgenic lines after inoculation, as well as in *C*Las-inoculated and non-inoculated WT plants, was monitored by examining the Ct values of *C*Las 16S rDNA. At 3 months after inoculation (MAI), the Ct values of all the transgenic lines and the *C*Las-inoculated WT plants were not different from those of non-inoculated WT plants. However, at 6 MAI, the Ct values of all the tested transgenic lines and the *C*Las-inoculated WT plant were lower than those of non-inoculated WT plants, indicating the successful transmission of *C*Las from infected scions to transgenic and WT plants through grafting (Fig. S2). Consequently, the *C*Las-inoculated WT plants were termed *C*Las-infected WT (WTLas) plants.

The *C*Las population gradually increased from 9 to 21 MAI in WTLas plants. Statistical analyses showed that 16 of 26 *CiNPR4* transgenic lines had significantly lower *C*Las population levels compared with the WTLas plants at 9 MAI. At 15 MAI, 7 of 16 transgenic lines still exhibited significantly lower *C*Las population levels. The *C*Las population levels in these seven transgenic lines, N1, N2, N8, N12, N20, N21 and N28, remained low even at 21 MAI (Fig. 2a). Thus, the overexpression of *CiNPR4* enhanced the resistance of citrus plants to *C*Las.

To test the correlation between *C*Las population and *CiNPR4* expression level of transgenic plants, the *CiNPR4* expression levels in N1, N2, N8, N12, N20, N21 and N28 transgenic lines with and without *C*Las inoculation for 21 months were determined. The expression of *CiNPR4* was not affected by *C*Las infection. There was no difference on the level of *CiNPR4* expression in transgenic lines between before and after *C*Las inoculation for 21 months. Analyses of *CiNPR4* expression and *C*Las populations in transgenic lines at 21 MAI indicated that a higher *CiNPR4* expression level was significantly negatively correlated with a lower *C*Las population level in transgenic lines ($r = -0.553$, $P < 0.05$) (Fig. 2b). This suggested that high-level *CiNPR4* expression may significantly inhibit the proliferation of *C*Las pathogen.

At 9 MAI, HLB symptoms developed in WTLas plants and some of the transgenic lines. The newly sprouted leaves on these plants were yellow and smaller than non-infected WT (WTck) plants. Some leaves on *C*Las-infected transgenic and WT plants had prominent veins compared with old leaves on the same plant. Over time, the symptoms became more severe in WTLas plants and the majority of the transgenic lines. However, HLB symptom development in N1, N2, N8, N12, N20, N21 and N28 transgenic lines was slower, and the symptoms were less severe, than in WTLas plants. Transgenic line N1 had HLB symptoms at 18 MAI. HLB symptoms were not observed on the N2, N8, N12, N20, N21 and N28 transgenic lines at 21 MAI (Fig. 2c), suggesting that the *C*Las infection resulted in little or no damage to the phloem tissues, and, therefore, did not affect plant growth and development.

Starch content changes in transgenic plants

CiNPR4 overexpression significantly alleviated the accumulation of starch in *C*Las-infected transgenic citrus plants. The rapid accumulation of starch was observed in WTLas plants after inoculation with *C*Las (Fig. 3). The starch content in WTLas plants was always significantly greater than in the other plants throughout the entire observational period. The degree of starch-accumulation alleviation among the transgenic lines differed. At 6 MAI, none of the transgenic plants had starch contents that differed from the WTck plants, indicating that starch accumulation in transgenic plants was significantly delayed. The starch content gradually increased in the transgenic lines from 6 MAI, and most lines reached levels significantly greater than those of the WTck plants by 12 MAI. At 18 MAI, all the transgenic lines, except N2 and N21, had significantly greater starch contents than WTck plants. Lines N1 and N28 showed very sharp increases from 12 to 18 MAI.

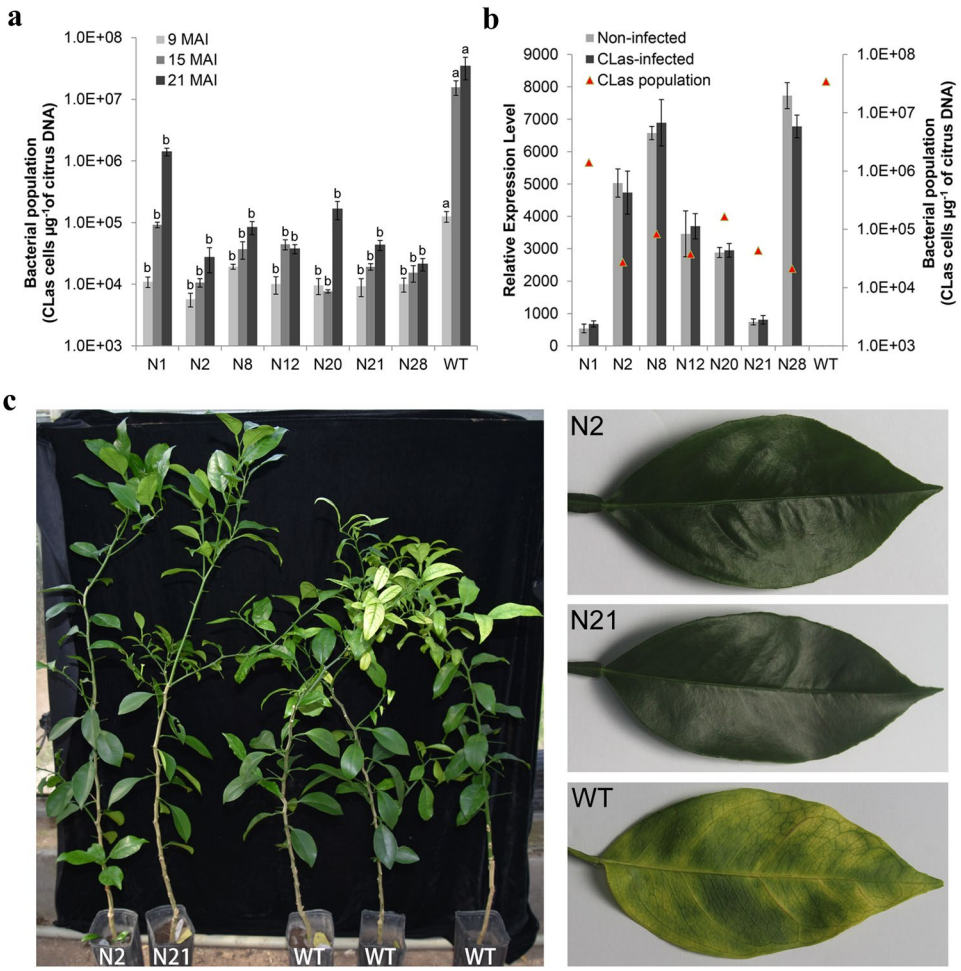

Fig. 2 Evaluation of Huanglongbing (HLB) resistance in *CiNPR4* transgenic citrus plants. **a** *Candidatus* Liberibacter asiaticus (*C*Las) populations in transgenic and wild-type (WT) plants. **b** Correlation analysis between *CiNPR4* expression levels in transgenic citrus plants and *C*Las population at 21 months after inoculation (MAI). The relative *CiNPR4* expression and *C*Las population levels are shown on the primary and secondary axes, respectively. **c** HLB symptoms observed on N2 and N21 *CiNPR4* transgenic and WT plants at 21 MAI. HLB symptom was not observed on the leaves of N2 and N21 transgenic lines. While, the newly sprouted leaves on WT plants were mottled yellow and had prominent veins. The data are shown as the means ± standard deviation of three biological replications. The different letters above the bars indicate significant differences from the WT plants at $P < 0.05$

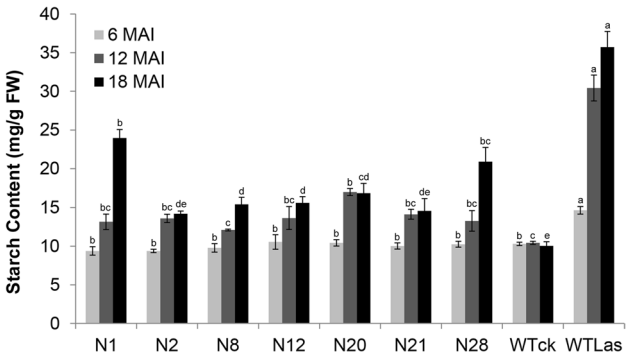

Fig. 3 The starch contents in *CiNPR4* transgenic plants. The letters above the bars indicate significant differences from the *C*Las-infected wild-type (WT) plants at the same time node on the basis of Duncan's test ($P < 0.05$). WTck and WTLas represent non-infected and *C*Las-infected WT plants, respectively. MAI, months after inoculation; FW, fresh weight

SA and JA content changes in CiNPR4 transgenic plants

CiNPR4 overexpression did not affect the SA contents, but it did significantly decrease the JA contents, of transgenic plants. Infection with *C*Las significantly increased both SA and JA contents in transgenic and WT plants (Fig. 4a, b). The SA and JA levels in transgenic and WT plants were determined at 18 MAI with *C*Las. The SA contents varied among the tested transgenic citrus lines N2, N12, N20 and N28, and WT plants, but the differences were not significant. However, significant differences in JA contents were observed among transgenic lines, and the JA contents in all the tested transgenic lines were lower than in WT plants. After *C*Las infection, both the SA and JA contents in tested transgenic lines and WT plants increased compared with non-infected control plants. However, the increased levels of SA or JA among N2, N12, N20, N28 and WT plants after *C*Las infection were not significantly different.

Changes in the anatomical phloem structures in CiNPR4 transgenic citrus plants

CiNPR4 overexpression resulted in less damage to phloem structures after *C*Las infection compared with in WTLas plants. Leaf midribs of transgenic line N2 and WT plants,

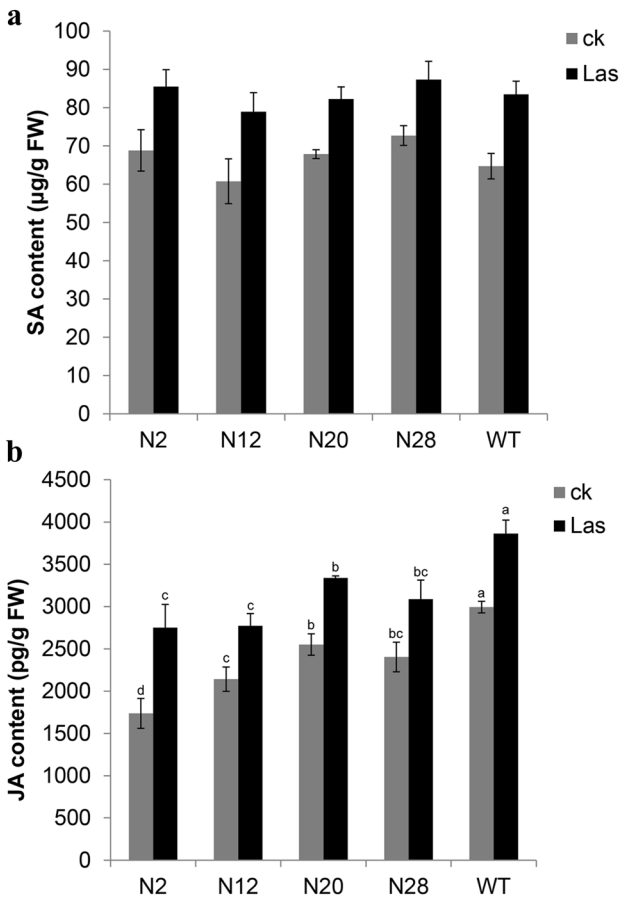

Fig. 4 Salicylic acid (SA) and jasmonic acid (JA) contents in transgenic citrus plants. **a** SA contents in transgenic citrus plants. **b** JA contents in transgenic citrus plants. The SA and JA contents were observed in N2, N12, N20, N28 and wild-type (WT) plants before and for 18 months after *C*Las inoculation. The letters above the bars indicate significant differences from the WT plants having the same treatment on the basis of Duncan's test ($P < 0.05$). ck and Las represent non-infected and *C*Las-infected plants, respectively. FW, fresh weight

with and without *C*Las-infection, were transversely sectioned to observe their phloem structures at 18 MAI. There were no differences in the phloem structures between non-infected N2 (N2ck) transgenic plants and WTck plants. Compared with non-infected plants, abnormal phloem structures, resulting from damage caused by *C*Las, were observed in both *C*Las-infected N2 (N2Las) transgenic plants and WTLas plants, which had wider phloem layers (Fig. 5). However, compared with the N2Las transgenic plants, there was greater damage to the phloem structures of WTLas plants. The phloem parenchymal cells were hypertrophic and disordered, with large spaces between parenchymal cells (Fig. 5). These observations indicated that the enhanced HLB resistance of transgenic plants contributed to the lower degree of phloem disruption.

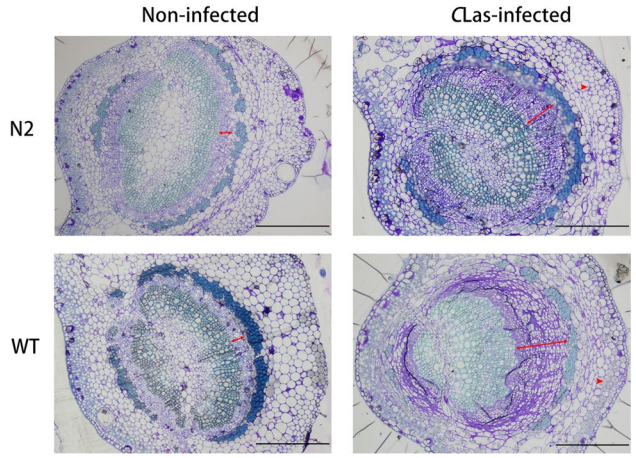

Fig. 5 Anatomical structures of leaf midribs from *CiNPR4* transgenic plants. Transverse sections of leaf midribs of transgenic line N2 and wild-type (WT) plants, with and without *C*Las-infection, were observed under a light microscope 18 months after *C*Las inoculation. The double and single arrows indicate the phloem layer and starch granules, respectively. Bar = 200 μm

Analysis of transcriptomic profiles in transgenic plants

A transcriptomic analysis was performed to dissect the functional roles of *CiNPR4* in response to *C*Las infections in citrus. N2ck and N2Las transgenic plants, as well as WTck and WTLas plants, were subjected to RNA-seq analyses with three independent biological replicates. The raw data were filtered and mapped to the *C. sinensis* reference genome. In total, as shown in Table 1, 42,777,098–57,654,044 clean

Table 1 Sample sequencing data and sequence alignment with the *Citrus sinensis* reference genome

Samples	Clean reads	Mapped reads	Uniq mapped reads
N2ck1	54,361,262	51,613,720 (94.95%)	49,126,641 (90.37%)
N2ck2	44,738,862	42,807,084 (95.68%)	40,867,726 (91.35%)
N2ck3	46,136,080	43,260,128 (93.77%)	41,082,323 (89.05%)
N2Las1	44,775,404	42,346,016 (94.57%)	40,383,098 (90.19%)
N2Las2	42,927,484	40,396,528 (94.10%)	38,295,244 (89.21%)
N2Las3	48,485,842	45,795,699 (94.45%)	43,726,791 (90.18%)
WTck1	48,634,346	46,408,227 (95.42%)	44,210,558 (90.90%)
WTck2	49,683,968	47,424,234 (95.45%)	45,241,037 (91.06%)
WTck3	42,777,098	40,291,074 (94.19%)	38,365,324 (89.69%)
WTLas1	57,654,044	54,391,754 (94.34%)	51,668,468 (89.62%)
WTLas2	55,365,698	52,778,380 (95.33%)	50,112,297 (90.51%)
WTLas3	48,137,780	45,791,694 (95.13%)	43,552,273 (90.47%)

N2ck1/2/3, N2Las1/2/3, WTck1/2/3 and WTLas1/2/3 represent three biological replications (1/2/3) of non-infected N2 transgenic line, *C*Las-infected N2 transgenic line, non-infected wild-type plants and *C*Las-infected wild-type plants, respectively

reads were obtained. Then, 93.77–95.68% of the clean reads of each sample were mapped to the reference genome, with more than 89.05% of the reads being uniquely mapped. Thus, the transcriptome data were reliable and useful for exploring the mechanism of HLB resistance in *CiNPR4* transgenic plants.

To analyze the DEGs, the RNA-seq results were divided into three groups, namely N2ck/WTck, N2Las/N2ck and WTLas/WTck. The numbers of DEGs in N2ck/WTck, N2Las/N2ck and WTLas/WTck were 2030, 2940 and 2752, respectively. The up- and down-regulated DEGs were 754 and 1236, respectively, in the N2ck/WTck group (Table S3), 2093 and 847, respectively, in the N2Las/N2ck group (Table S4), while 1567 and 1185 genes were up- and down-regulated expressed, respectively, in the WTLas/WTck group (Table S5).

A GO annotation analysis showed that in the N2ck/WTck, N2Las/N2ck and WTLas/WTck groups, 1303, 1702 and 1614 GO annotations, respectively, were obtained, accounting for 64.19%, 57.89% and 58.65%, respectively, of the total DEGs. GO cluster analyses showed that the annotated genes in each group could be divided into three categories, biological process, cell component and molecular function. In each group, genes mainly clustered in the biological process categories 'metabolic process', 'cell process' and 'single organism process', the cellular component category 'cell and cell part' and the molecular function category 'catalytic activity and binding' (Fig. S3).

To reveal differences among metabolic pathways of DEGs, a KEGG pathway enrichment analysis was performed, and the top 20 pathways having the lowest significant *q*-values were investigated. The significant enrichments in metabolic pathways (*q* value threshold < 0.05) were shown in Fig. 6. The plant hormone signal transduction pathway was significantly affected by *CiNPR4* overexpression in the N2ck/WTck group. All annotated five *JAZ* homologous genes (*Cs1g17210*, *Cs1g17220*, *Cs4g06520*, *Cs4g07130* and *Cs7g02820*) and the uniquely annotated *MYC2* gene (*orange1.1t00550*), which involved in JA signal, were significantly down-regulated by *CiNPR4* overexpression. A *TGA* gene, downstream of *NPR* gene in SA signal was also repressed. This investigation indicated that *CiNPR4* overexpression negatively regulated JA and SA signal.

CiNPR4 overexpression triggered *C*Las-induced expression of plant–pathogen interaction genes. In the N2Las/N2ck group, the DEGs were mainly enriched in the plant–pathogen interaction pathway (Fig. 6). In total, 43 genes were involved in this pathway, with 95.35% having up-regulated expression levels. These genes mainly regulate the hypersensitive response and cell wall reinforcement, and induce the expression of defense-related genes (Fig. S4). However, in the WTLas/WTck group, the DEGs were enriched in the stillbenoid, diarylheptanoid and gingerob biosynthetic pathway (Fig. S5). The significant enrichment and large number of genes involved in this pathway in transgenic line N2 after *C*Las infection indicated that the overexpression of *CiNPR4* regulated the transcription activity levels of genes involved in plant–pathogen interaction in response to *C*Las infection.

Validation of DEGs derived from RNA-seq by qRT-PCR

To verify the DEGs identified in the RNA-seq analysis, 25 common DEGs related to hormone metabolism and biotic stress in the N2ck/WTck, N2Las/N2ck and WTLas/WTck groups, as well as 10 up-regulated DEGs involved in plant–pathogen interactions in the N2Las/N2ck group, were selected for an expression analysis using qRT-PCR (Table S2). In the N2ck/WTck group, the expression pattern of *Cs5g17920*, *Cs4g07730* and *Cs5g19260* genes was not consistent with the RNA-seq results (Fig. 7a, b). In the N2Las/N2ck and WTLas/WTck groups, the expression levels of seven genes involved in SA and JA metabolism were up-regulated (Fig. 7a), consistent with the transcriptomic analysis results. All 18 biotic stress-related genes, except *Cs2g31360* and *Cs4g07730*, displayed expression patterns similar to the RNA-seq results (Fig. 7b, c). For the 10 plant–pathogen interaction-related genes in the N2Las/N2ck group, the expression level of *Cs4g08100* was not changed, while the others were up-regulated after *C*Las infection as assessed by qRT-PCR (Fig. 7d). Thus, the expression levels of 88.0%, 91.4% and 92.0% of selected genes in N2ck/WTck, N2Las/N2ck and WTLas/WTck, respectively, as determined by qRT-PCR, were in agreement with the RNA-seq results, providing further validation of the reliability of the transcriptomic data.

Discussion

The strategy to design plants resistance to microbial pathogens by enhancing their innate defense responses has been used in breeding genetically engineered citrus. *AtNPR1* was first used to enhance citrus canker resistance and, subsequently, HLB resistance (Zhang et al. 2010; Dutt et al. 2015). Its homolog in citrus, *CtNH1*, has also been used to engineer citrus canker resistance (Chen et al. 2013). The resulting transgenic plants promote the expression of defense-related genes and have enhanced resistance or even immunity to bacterial diseases. Silencing *CsNPR3* or *CsNPR4* slightly decreases the titer of the *Citrus tristeza* virus in the phloem of sour orange (Gómez-muñoz et al. 2017). Here, 7 of 26 *CiNPR4* transgenic lines exhibited significantly lower *C*Las titer levels in their phloem tissues compared with WTLas plants at 21 MAI (Fig. 2a).

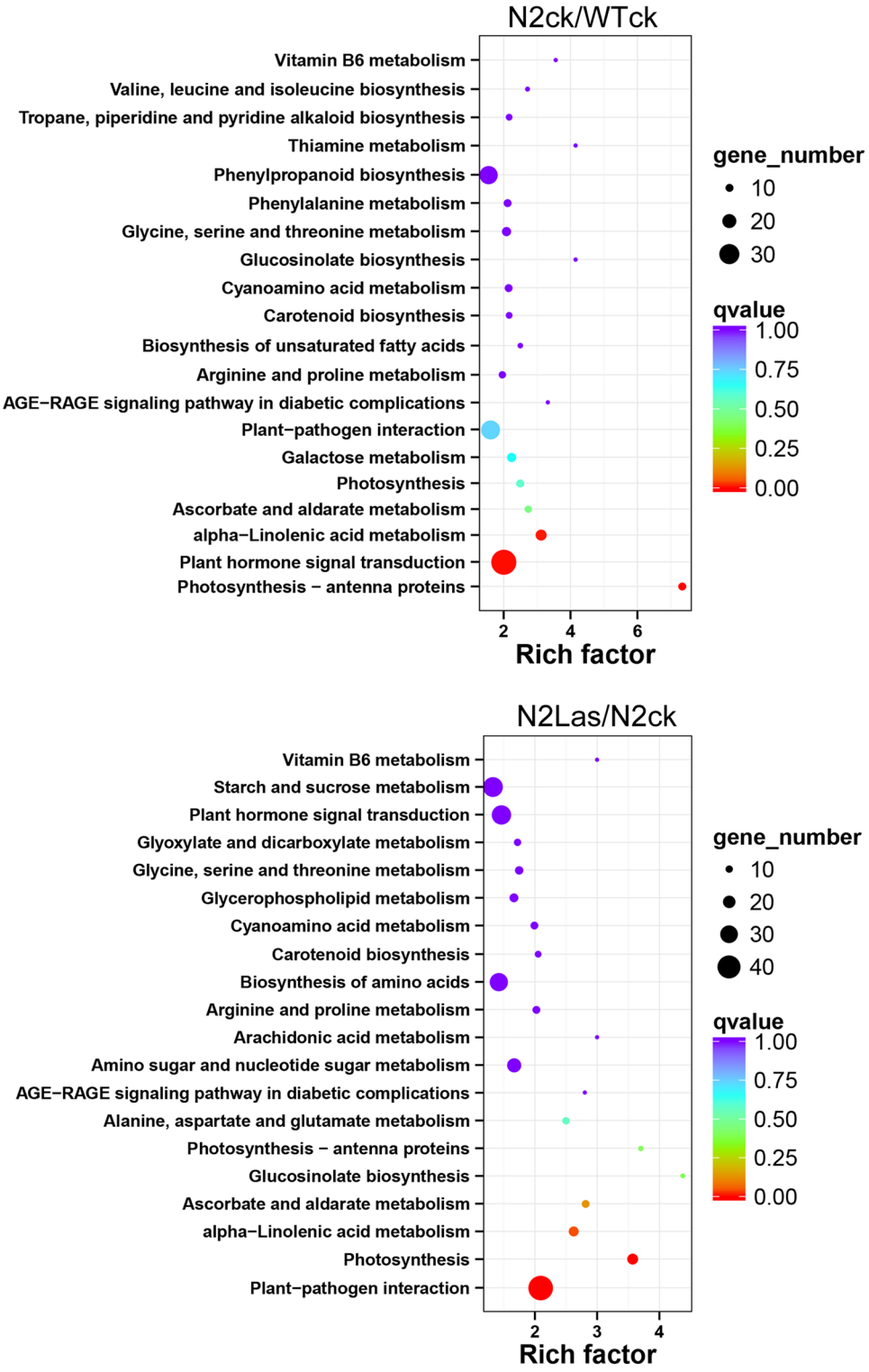

Fig. 6 Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes. The enrichment factor and the name of pathway are shown on the x-axis and y-axis, respectively. The color and dot size indicate the *q* value and the gene number, respectively. N2ck, WTck, and N2Las, non-infected transgenic line N2 and wild-type plants, and *C*Las-infected transgenic line N2, respectively

An RNA-seq analysis revealed that the enhanced disease resistance of the transgenic plants resulted from the up-regulated transcriptional activities of plant–pathogen interaction-related genes (Fig. 6). These results indicated that enhancing intrinsic immunity is an effective way for citrus plants to resist invasive pathogens.

NPR4 has been shown to function as a transcriptional repressor to negatively regulate plant immunity (Zhang et al. 2006; Ding et al. 2018). However, in the present study, a *NPR1*-like gene from 'Jackson' grapefruit, *CiNPR4*, positively regulated the defense responses in citrus. The deduced amino acid sequences of almost all

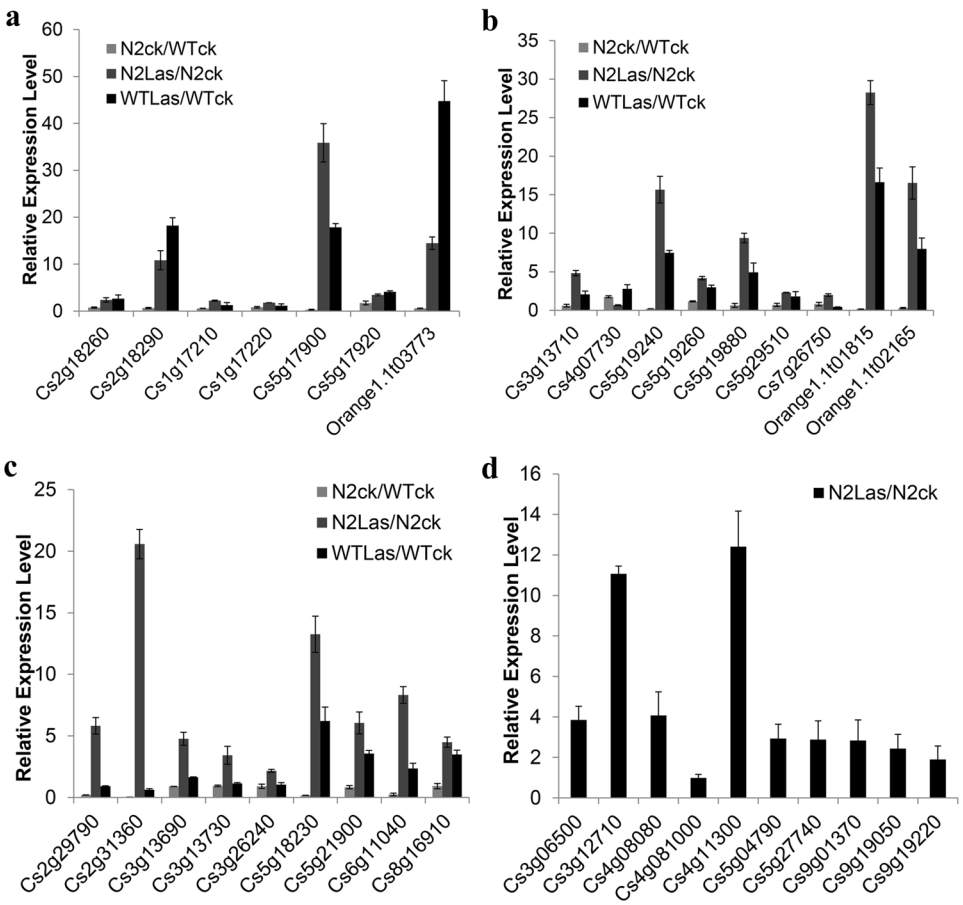

Fig. 7 Quantitative real-time PCR validation of differentially expressed genes from the RNA-seq analysis. **a–d** The relative expression levels of 7 SA and JA metabolism-related (**a**) and 18 biotic stress-related (**b**, **c**) genes in N2ck/WTck, N2Las/N2ck and WTLas/WTck groups, as well as 10 genes involved in plant–pathogen interactions (**d**) in the N2Las/N2ck group, were analyzed using the $2^{-\Delta\Delta Ct}$ method. N2ck and N2Las, non-infected and *C*Las-infected transgenic line N2; WTck and WTLas, non-infected and *C*Las-infected wild-type plants

the genes clustered with NPR3 and NPR4 contain a conserved VDLNETP motif at their C-termini (Fig. 1c). This motif shares a high similarity with the ethylene-responsive element binding factor-associated amphiphilic repression motif [L/FDLNL/F(x)P] (Ohta et al. 2001). Mutating the NPR4 amino acids "DLN" to the NPR1 "GVK" results in the loss of NPR4′s repressive function, demonstrating that the VDLNETP motif is required for NPR4′s transcriptional repressive activity and that the "DLN" amino acids play a decisive role in maintaining NPR4′s function (Ding et al. 2018). The NPR4 proteins CsNPR4 and FvNPRL-1 contain the IDLNETP and VDLNETP motifs, respectively, at their C-termini (Fig. 1c). Both proteins function as repressors in plant defense responses (Gómez-muñoz et al. 2017; Shu et al. 2018). However, compared with other NPR3 and NPR4 proteins in clade II, CiNPR4 completely lacks both of these motifs (Fig. 1c), resulting in the release of NPR4′s repressive function. The transformation of *CiNPR4* into citrus enhanced the defense responses against *C*Las, revealing the positive regulatory function of NPR4 lacking the VDLNETP motif at the C-terminus. This result was consistent with the up-regulated expression of *CiNPR4* in HLB-tolerant 'Jackson' grapefruit after *C*Las infection (Wang et al. 2016), and it confirmed that CiNPR4 functions as an activator that positively regulates disease resistance in citrus.

Salicylic acid and JA are two important plant hormones that mediate host defense responses against various pathogens in antagonistic or synergistic manners. Efforts have been made to elucidate the regulatory network mediated by SA and/or JA in response to pathogen infections in model plants. After inoculating with *P. syringae* pv. tomato DC3000, levels of both free and conjugated SA are strongly increased in WT *Arabidopsis*, but they are not changed in SA hydroxylase-expressing NahG transgenic *Arabidopsis*. However, after infection, the JA levels in WT *Arabidopsis* only slightly increase, while there is a 25-fold higher JA accumulation in NahG plants. These results indicate the presence of an antagonistic effect between SA and JA in WT *Arabidopsis* after pathogen infection (Spoel et al. 2003). In another model plant, rice (*Oryza sativa*), JA signaling plays an important role in defense systems, enhancing resistance to bacterial blight (Yamada et al. 2012). However, little is known about SA- and JA-modulated immune responses to *C*Las. Compared with *Arabidopsis* and rice (Spoel et al. 2003; Yuan et al. 2007), healthy 'Wanjincheng' orange had a very high free SA level and a very low JA level (Fig. 4a, b). Activities in the SA and JA signal transduction pathways

were triggered during *C*Las infection as revealed by the RNA-seq analysis of WTLas and WTck plants. The SA and JA contents significantly increased in WTLas plants compared with in WTck plants, but it was not a multiple-fold change (Fig. 4a, b). Thus, both SA and JA appear to be involved in regulating the basal resistance of 'Wanjincheng' orange against HLB. However, the levels of their contributions still require further study.

Significantly decreased JA levels were observed in non-infected transgenic lines compared with WTck plants (Fig. 4b). This observation differed from the report that *OsNPR1*/*NH1* overexpression did not change the JA level in rice plants (Yuan et al. 2007). The molecular mechanism responsible for *CiNPR4*'s regulation of the JA level in citrus is unknown. However, a transcriptomic analysis of the N2ck transgenic line and WTck plants demonstrated that some genes in the JA biosynthetic process had down-regulated expression levels (Table S6), suggesting that *CiNPR4* overexpression repressed JA biosynthesis. After *C*Las infection, although the increases in the JA levels of tested transgenic lines were similar to those observed in WT plants, the JA contents in all the tested transgenic lines were comparable to those of WTck plant and were significantly lower than those of WTLas plants (Fig. 4b). Additionally, both tested transgenic lines and WT plants maintained high non-significantly different SA levels at 18 MAI (Fig. 4a). These results suggested that the high SA and low JA levels are more favorable to the use of SAR to regulate the defense responses of transgenic citrus plants against HLB.

Citrus HLB is a systemic disease, and its pathogen, *C*Las, only exists in the phloem of citrus plant. Therefore, it often damages the phloem's structure, leading to phloem dysfunction, especially in photosynthate transport. Although some transgenic citrus lines demonstrated resistance after being challenged with *C*Las in this study, no transgenic plant was immune to *C*Las. To date, much research has been conducted to develop HLB-resistant citrus, but no reliable resistance to control the disease has been achieved. This study explored a new approach to enhance the HLB-resistance in citrus and investigated the introduction of HLB resistance through SA and JA-mediated defense pathways. We will investigate the differentially expressed defense-related genes identified in our RNA-seq analysis and screen candidate genes for roles in HLB resistance. Further improvements in the resistance could be developed in *CiNPR4* transgenic plants by stacking other resistant genes.

Conclusion

Resistance to HLB has been achieved by the introduction of the *CiNPR4* gene from HLB-tolerant 'Jackson' grapefruit into the *C*Las-susceptible citrus cultivar 'Wanjincheng' orange. *CiNPR4* overexpression suppressed JA signal pathway and up-regulated the expression levels of plant–pathogen interaction-related genes during *C*Las infection. The increased defense response elevated the plants' innate immunity, resulting in decreased *C*Las bacterial populations in the phloem, which reduced damage to phloem tissues. Our results demonstrated that the introduction of HLB resistance by manipulating an *NPR1* paralogous gene to regulate plant innate immunity is feasible in citrus.

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Author contributions AP constructed the plasmid, performed the resistance evaluation of transgenic plants and wrote the manuscript. XZ performed the RNA-seq analysis. YH and SC performed citrus transformations and analyzed the GUS activities of regenerants. XL performed the phylogenetic analysis and amino acid sequence alignment. JZ performed the starch content analysis. QZ and ZX observed the anatomical phloem structures. JL performed the PCR and expression analyses. XZ designed the experiments and revised the manuscript. All the authors have read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

An C, Mou Z (2011) Salicylic acid and its function in plant immunity. J Integr Plant Biol 53:412–428

Belasque J, Bassanezi RB, Yamamoto PT, Ayres AJ, Tachibana A, Violante AR, Tank A, Di Giorgi F, Tersi FEA, Menezes GM, Dragone J, Jank RH, Bové JM (2010) Lessons from Huanglongbing management in São Paulo State, Brazil. J Plant Pathol 92:285–302

Bové JM (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J Plant Pathol 88:7–37

Chen X, Barnaby JY, Sreedharan A, Huang X, Orbović V, Grosser JW, Wang N, Dong X, Song WY (2013) Over-expression of the citrus gene *CtNH1* confers resistance to bacterial canker disease. Physiol Mol Plant 84:115–122

Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R (2020) TBtools: an integrative toolkit developed for interactive analyses of big biological data. Mol Plant 13(8):1194–1202

da Graça JV, Douhan GW, Halbert SE, Keremane ML, Lee RF, Vidalakis G, Zhao H (2016) Huanglongbing: an overview of a complex pathosystem ravaging the world's citrus. J Integr Plant Biol 58(4):373–387

Ding Y, Sun T, Ao K, Peng Y, Zhang Y, Li X, Zhang Y (2018) Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant immunity. Cell 173:1454–1467

Dutt M, Barthe G, Irey M, Grosser J (2015) Transgenic citrus expressing an *Arabidopsis NPR1* gene exhibit enhanced resistance

against Huanglongbing (HLB; Citrus Greening). PLoS ONE 10(9):e0137134
Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, Dong X (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486:228–232
Gómez-muñoz N, Velázquez K, Vives MC, Ruiz-ruiz S, Pina JA, Flores R, Moreno P, Guerri J (2017) The resistance of sour orange to *Citrus tristeza virus* is mediated by both the salicylic acid and RNA silencing defence pathways. Mol Plant Pathol 18(9):1253–1266
He YR, Chen SC, Peng AH, Zou XP, Xu LZ, Lei TG, Liu XF, Yao LX (2011) Production and evaluation of transgenic sweet orange (*Citrus sinensis* Osbeck) containing bivalent antibacterial peptide genes (*Shiva* A and *Cecropin* B) via a novel *Agrobacterium*-mediated transformation of mature axillary shoots. Sci Hort 128:99–107
Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874
Le Henanff G, Heitz T, Mestre P, Mutterer J, Walter B, Chong J (2009) Characterization of *Vitis vinifera* NPR1 homologs involved in the regulation of *pathogenesis-related* gene expression. BMC Plant Biol 9:54
Liu X, Liu Z, Niu X, Xu Q, Yang L (2019) Genome-wide identification and analysis of the *NPR1*-like gene family in bread wheat and its relatives. Int J Mol Sci 20(23):5974
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408
Makandar R, Essig JS, Schapaug MA, Trick HN, Shah J (2006) Genetically engineered resistance to Fusarium head blight in wheat by expression of *Arabidopsis NPR1*. Mol Plant Microbe Interact 19:123–129
Mao X, Cai T, Olyarchuk JG, Wei L (2005) Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics 21:3787–3793
Meur G, Budatha M, Srinivasan T, Kumar KRR, Gupta AD, Kirti PB (2008) Constitutive expression of *Arabidopsis NPR1* confers enhanced resistance to the early instars of *Spodoptera litura* in transgenic tobacco. Physiol Plant 133:765–775
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell 13:1959–1968
Peng A, Xu L, He Y, Lei T, Yao L, Chen S, Zou X (2015) Efficient production of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) with enhanced resistance to citrus canker using a Cre/*loxP* site-recombination system. Plant Cell Tiss Organ Cult 123:1–13
Peng A, Zou X, Xu L, He Y, Lei T, Yao L, Li Q, Chen S (2019) Improved protocol for the transformation of adult *Citrus sinensis* Osbeck 'Tarocco' blood orange tissues. Vitro Cell Dev-Pl 55:659–667
Peraza-Echeverria S, Santamaría JM, Fuentes G, Menéndez-Cerón MDLÁ, Vallejo-Reyna MÁ, Herrera-Valencia VA (2012) The NPR1 family of transcription cofactors in papaya: insights into its structure, phylogeny and expression. Genes Genom 34:379–390
Pilotti M, Brunetti A, Gallelli A, Loreti S (2008) NPR1-like genes from cDNA of rosaceous trees: cloning strategy and genetic variation. Tree Genet Genomes 4:49–63
Qiu W, Soares J, Pang Z, Huang Y, Sun Z, Wang N, Grosser J, Dutt M (2020) Potential mechanisms of *AtNPR1* mediated resistance against Huanglongbing (HLB) in citrus. Int J Mol Sci 21(6):2009
Shao Y, Zhang H, He H, Cheng B, Xiang Y (2013) Molecular cloning and characterization of orthologues of *NPR1* gene from poplar. J Phytopathol 161:35–42
Shi Z, Zhang Y, Maximova SN, Guiltinan MJ (2013) TcNPR3 from *Theobroma cacao* functions as a repressor of the pathogen defense response. BMC Plant Biol 13:204
Shu L, Liao J, Lin N, Chung C (2018) Identification of a strawberry *NPR*-like gene involved in negative regulation of the salicylic acid-mediated defense pathway. PLoS ONE 13(10):e0205790
Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Pelt JAV, Mueller MJ, Buchala AJ, Métraux JP, Brown R, Kazan K, Loon LCV, Dong X, Pieterse CMJ (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 15:760–770
Sticher L, MauchMani B, Metraux JP (1997) Systemic acquired resistance. Annu Rev Phytopathol 35:235–270
Wally O, Jayaraj J, Punja ZK (2009) Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (*Daucus carota* L.) expressing an *Arabidopsis NPR1* gene. Planta 231:131–141
Wang Y, Zhou L, Yu X, Stover E, Luo F, Duan Y (2016) Transcriptome profiling of Huanglongbing (HLB) tolerant and susceptible citrus plants reveals the role of basal resistance in HLB tolerance. Front Plant Sci 7:1–13
Wang L, Guo Z, Zhang Y, Wang Y, Yang G, Yang L, Wang L, Wang R, Xie Z (2017) Overexpression of LhSorNPR1, a NPR1-like gene from the oriental hybrid lily 'Sorbonne', conferred enhanced resistance to Pseudomonas syringae pv. tomato DC3000 in Arabidopsis. Physiol Mol Biol Plants 23(4):793–808
Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ (2009) Jalview version 2–A multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189–1191
Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID, De Luca V, Després C (2012) The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell Rep 1:639–647
Yamada S, Kano A, Tamaoki D, Miyamoto A, Shishido H, Miyoshi S, Taniguchi S, Akimitsu K, Gomi K (2012) Involvement of OsJAZ8 in jasmonate-induced resistance to bacterial blight in rice. Plant Cell Physiol 53(12):2060–2072
Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol 11:R14
Yuan Y, Zhong S, Li Q, Zhu Z, Lou Y, Wang L, Wang J, Wang M, Li Q, Yang D, He Z (2007) Functional analysis of rice *NPR1*-like genes reveals that *OsNPR1/NH1* is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. Plant Biotechnol J 5:313–324
Zhang Y, Cheng YT, Qu N, Zhao Q, Bi D, Li X (2006) Negative regulation of defense responses in *Arabidopsis* by two NPR1 paralogs. Plant J 48:647–656
Zhang X, Francis MI, Dawson WO, Graham JH, Orbović V, Triplett EW, Mou Z (2010) Over-expression of the *Arabidopsis NPR1* gene in citrus increases resistance to citrus canker. Eur J Plant Pathol 128:91–100
Zou X, Jiang X, Xu L, Lei T, Peng A, He Y, Yao L, Chen S (2017) Transgenic citrus expressing synthesized *cecropin* B genes in the phloem exhibits decreased susceptibility to Huanglongbing. Plant Mol Biol 93:341–353
Zou X, Bai X, Wen Q, Xie Z, Wu L, Peng A, He Y, Xu L, Chen S (2019) Comparative analysis of tolerant and susceptible citrus reveals the role of methyl salicylate signaling in the response to Huanglongbing. J Plant Growth Regul 38:1516–1528

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检 索 报 告

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认证单位：教育部科技查新工作站 N08

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作者: Peng, Aihong; Zou, Xiuping; He, Yongrui; 等.
PLANT CELL REPORTS　卷: 40　期: 3　页: 529-541　出版年: MAR 2021</td></tr>
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附件 1：SCI 收录情况

序号	作者排名	题名	检索号	影响因子	中科院 JCR 类别	JCR 分区	出版时间	语种	出版商地址
1	1	Overexpressing a NPR1-like gene from Citrus paradisi enhanced Huanglongbing resistance in C. sinensis	000604082900001	IF_{2020}=4.57	小类 植物科学 2 区 大类 生物 2 区		2021 年	英语	国外

附件 2：SCI 检索结果记录

第 1 条，共 1 条

标题: Overexpressing a NPR1-like gene from Citrus paradisi enhanced Huanglongbing resistance in C. sinensis

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