

RESEARCH ARTICLE

Overexpressing *GH3.1* and *GH3.1L* reduces susceptibility to *Xanthomonas citri* subsp. *citri* by repressing auxin signaling in citrus (*Citrus sinensis* Osbeck)

Xiuping Zou^{1,2*}, Junhong Long^{1,2}, Ke Zhao^{1,2}, Aihong Peng^{1,2}, Min Chen^{1,2}, Qin Long^{1,2}, Yongrui He^{1,2}, Shanchun Chen^{1,2*}

1 Citrus Research Institute, Chinese Academy of Agricultural Sciences and National Center for Citrus Variety Improvement, Chongqing, P. R. China, **2** Citrus Research Institute, Southwest University, Chongqing, P. R. China

* zouxiping@cric.cn (XZ); chenshanchun@cric.cn (SC)



OPEN ACCESS

Citation: Zou X, Long J, Zhao K, Peng A, Chen M, Long Q, et al. (2019) Overexpressing *GH3.1* and *GH3.1L* reduces susceptibility to *Xanthomonas citri* subsp. *citri* by repressing auxin signaling in citrus (*Citrus sinensis* Osbeck). PLoS ONE 14(12): e0220017. <https://doi.org/10.1371/journal.pone.0220017>

Editor: Ji-Hong Liu, Key Laboratory of Horticultural Plant Biology (MOE), CHINA

Received: June 27, 2019

Accepted: November 16, 2019

Published: December 12, 2019

Copyright: © 2019 Zou et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data available in the Supporting Information files and from the SRA database (accession number PRJNA576978, <https://www.ncbi.nlm.nih.gov/sra/PRJNA576978>).

Funding: This work was supported by the National Key Research and Development Program of China (2018YFD1000300), the Natural Science Foundation Project of CQ (cstc2017jcyjBX0020), the Earmarked Fund for China Agriculture Research

Abstract

The auxin early response gene Gretchen Hagen3 (*GH3*) plays dual roles in plant development and responses to biotic or abiotic stress. It functions in regulating hormone homeostasis through the conjugation of free auxin to amino acids. In citrus, *GH3.1* and *GH3.1L* play important roles in responding to *Xanthomonas citri* subsp. *citri* (Xcc). Here, in Wanjingcheng orange (*Citrus sinensis* Osbeck), the overexpression of *CsGH3.1* and *CsGH3.1L* caused increased branching and drooping dwarfism, as well as smaller, thinner and upward curling leaves compared with wild-type. Hormone determinations showed that overexpressing *CsGH3.1* and *CsGH3.1L* decreased the free auxin contents and accelerated the Xcc-induced decline of free auxin levels in transgenic plants. A resistance analysis showed that transgenic plants had reduced susceptibility to citrus canker, and a transcriptomic analysis revealed that hormone signal transduction-related pathways were significantly affected by the overexpression of *CsGH3.1* and *CsGH3.1L*. A MapMan analysis further showed that overexpressing either of these two genes significantly downregulated the expression levels of the annotated auxin/indole-3-acetic acid family genes and significantly upregulated biotic stress-related functions and pathways. Salicylic acid, jasmonic acid, abscisic acid, ethylene and zeatin levels in transgenic plants displayed obvious changes compared with wild-type. In particular, the salicylic acid and ethylene levels involved in plant resistance responses markedly increased in transgenic plants. Thus, the overexpression of *CsGH3.1* and *CsGH3.1L* reduces plant susceptibility to citrus canker by repressing auxin signaling and enhancing defense responses. Our study demonstrates auxin homeostasis' potential in engineering disease resistance in citrus.

System (CARS-27 to S. Chen) and the Science and Technology Major Project of Guangxi (Gui Ke AA18118046).

Competing interests: The authors have declared that no competing interests exist.

Introduction

Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (Xcc), is an important disease of citrus. Xcc affects various citrus species and most of the economically important cultivars, including orange, grapefruit, lime, lemon, pomelo and citrus rootstock [1]. The canker's development includes the initial appearance of oily looking spots, usually on the abaxial leaf surface, outbursts of white or yellow spongy pustules and finally the formation of brown corky cankers [2]. Pustule formation (excessive cell division) in the infected tissues plays a vital role in citrus canker development and pathogen spread [1, 3–5]. The inhibition or disruption of pustule development can efficiently repressed pathogen spread and even confer plant resistance to citrus canker [6, 7], indicating that the manipulation of pustule development is a potential strategy for the efficient management of citrus canker. Thus, understanding the molecular mechanisms involved in responding to pathogen-induced pustule formation in citrus could stimulate renewed efforts to develop more effective and economical control methods of citrus canker management.

Auxin, a critical plant hormone that controls a range of plant growth and developmental processes, including cell division and expansion, has long been recognized as a regulator of plant defenses [8, 9]. The effector AvrRpt2 from *Pseudomonas syringae* elicits auxin biosynthesis in plants and promotes disease in *Arabidopsis thaliana* [10]. The flagellin Flg22 from *P. syringae* induces the microRNA mi393 to degrade the RNAs of the auxin receptor gene *TIR1*, resulting in immune responses in *Arabidopsis* [11]. Auxin represses the expression of pathogenesis-related (*PR*) genes to impair defense responses, and the inhibition of auxin signaling is a part of the salicylic acid (SA)-mediated resistance mechanism [12–14]. Indole-3-acetic acid (IAA) is the major form of auxin in most plants, including citrus. Many plant pathogens can either secrete IAA into host tissues or enhance the host's IAA synthesis, and the elevated IAA levels increase cell wall loosening and remodeling to favor pathogen invasion and spread [9]. Conversely, plant disease resistance is enhanced by repressing auxin signaling or decreasing the IAA content [5, 15].

Xcc increases cell division and expansion in host-infected sites through the regulation of auxin to increase bacterial growth [3, 5]. In the initial stages of canker development, naphthalene acetic acid treatments significantly enhanced the water soaking phenomenon on citrus leaves [5]. Additionally, Costacurta et al. [16] reported that the Xcc pathogen produces IAA through the indole-3-pyruvic acid pathway, and that this IAA biosynthesis is enhanced by citrus leaf extracts. However, the molecular mechanisms of the host governing auxin responses to citrus canker remain to be elucidated.

The normal physiologic function of auxin depends on the spatiotemporal fine-tuning of hormone levels. The maintenance of IAA homeostasis is regulated by several groups of auxin-responsive genes, including those of the auxin/IAA (*Aux/IAA*), Gretchen Hagen3 (*GH3*) and small auxin-up RNA (*SAUR*) families [17]. *GH3* is an amido synthetase that conjugates IAA to amino acids (such as Asp, Ala, and Phe), thus inactivating free IAA [18]. *GH3* proteins are classified into three groups in *Arabidopsis*. Group I has jasmonic acid (JA)-amino synthetase activity, whereas group II is able to catalyze IAA conjugation to amino acids [18]. No adenylation activity on the substrates tested has been found for group III members. Some *GH3* proteins conjugate SA to amino acids [18]. At present, many *GH3* genes have been identified in bean, apple, maize, tomato, rice and *Medicago sativa* [19–21]. In addition to their functions in plant growth and development, *GH3* genes participate in disease resistance. *AtGH3.12* regulates SA-dependent defense responses by controlling pathogen-inducible SA levels [22]. *AtGH3.5* has a dual regulatory role in *Arabidopsis* SA and auxin signaling during pathogen infection [13, 23]. *Oryza sativa* *GH3-8* and *GH3-1* promote fungal resistance through the

regulation of auxin levels [24, 25], while *GH3-2* mediates a broad-spectrum resistance to bacterial and fungal diseases [15].

In the early stage of this experiment, the transcriptomes of Newhall navel orange (*Citrus sinensis* Osbeck) and Calamondin (*Citrus madurensis*), with susceptibility and resistance to Xcc, respectively, were constructed (Unpublished). The transcriptomic analysis showed that GH3 group II *CsGH3.1* genes were induced significantly by Xcc and had high expression levels in the Newhall navel orange [26], indicating that this group's members play important roles in responding to citrus canker. Here, to understand the roles of *CsGH3.1* in regulating host responses to citrus canker, we constructed transgenic Wanjingcheng orange (*C. sinensis* Osbeck) plants independently overexpressing *CsGH3.1* and *CsGH3.1L*. Their overexpression reduced endogenous auxin levels, altered plant architecture and enhanced host defense responses to citrus canker. We then explored the effects of *CsGH3.1* and *CsGH3.1L* overexpression in transgenic plants using high-throughput transcriptome sequencing.

Materials and methods

Plant materials and growth conditions

Wanjincheng orange (*C. sinensis* Osbeck) used in this study were planted in a greenhouse at the National Citrus Germplasm Repository, Chongqing, China.

Vector construction

The coding sequences of the *CsGH3.1* (Cs1g22140) and *CsGH3.1L* (Cs8g04610) genes were obtained from the *C. sinensis* genome database (<http://citrus.hzau.edu.cn/orange/>). The pGEM plasmids independently containing the *CsGH3.1* and *CsGH3.1L* genes [26] and the plant expression vector pGN [27], from our laboratory, were used to construct plant overexpression vectors for this study. *CsGH3.1* and *CsGH3.1L* were digested from the pGEM vectors with *Sall/Bam*HI, and then independently inserted into *Sall/Bam*HI-digested pGN. Finally, two plant overexpression vectors containing *CsGH3.1* and *CsGH3.1L*, respectively, were obtained (S1 Fig). They were transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Citrus transformation

The epicotyls of Wanjincheng orange were used as explants for citrus transformation. The transformation protocol was performed according to Peng et al. [6]. Putative transgenic shoots were screened using GUS histochemical staining [27]. The recovery of GUS-positive plants was performed by grafting onto Troyer citrange [*Poncirus trifoliata* (L.) Raf. × *C. sinensis* Osbeck] seedlings *in vitro*. The integration of *CsGH3.1* and *CsGH3.1L* into plants was further confirmed by a PCR analysis. The primers for the PCR confirmation of transgenic plants are presented in S1 Table. All transgenic and wild-type (WT) plants were grown in a netted greenhouse at 28°C.

Real-time quantitative PCR (qPCR) analysis

Citrus total RNA was isolated using the EASYspin Plant RNA Extraction Kit following the manufacturer's instructions (Aidlab, Beijing, China). RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The detection of gene expression was performed by qPCR using the iQ™ SYBR Green Supermix (Bio-Rad). The PCR reactions were carried out as follows: a pretreatment (94°C for 5 min), followed by 40 amplification cycles (94°C for 20 s and 60°C for 60 s). Experiments were repeated three times. Using

the citrus Actin gene for normalization, the relative expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method [28].

Measurement of hormone contents

Hormones IAA, SA, zeatin (ZT), abscisic acid (ABA), ethylene (ET) and JA were extracted from the leaves of citrus plants was determined at Chongqing Bono Heng Biotechnology Co., Ltd (Chongqing, China). Contents of IAA, SA, ZT, ABA, and JA was simultaneously measured as described by [29, 30]. In brief, Tissue samples (1 g fresh weight) were frozen in liquid nitrogen, ground to a fine powder, extracted with 80% methanol overnight and then centrifuged at 13,000 \times g for 10 min. The supernatant was evaporated and then redissolved in 1% acetic acid. Hormones were purified on Oasis cartridges (Waters, Milford, MA, USA) in accordance with the manufacturer's instructions. The extracted hormones were redissolved in 10% methanol, and then IAA, SA, ZT, ABA and JA levels were determined using high-performance liquid chromatography. To measure ET contents, 1 g leaf tissue were placed in a gas-tight container equipped with septa, and 1 mL of headspace gas was sampled using a gas syringe [31]. The ethylene production was measured using gas chromatograph. The test was repeated three times.

Evaluation of transgenic plants resistance to citrus canker

The disease resistance assay for transgenic plants against citrus canker was performed according to Peng et al. [6]. A Xcc strain, XccYN1 [6], was used to investigate plant disease resistance. Three mature healthy leaves per plant were tested. In total, 24 punctures were made per leaf with a needle containing the bacterial suspension (0.5×10^5 CFU ml⁻¹). The inoculated leaves were maintained at 28°C under a 16-h light/8-h dark photoperiod with 45 μ mol m⁻² s⁻¹ illumination and 60% relative humidity. Photographs were taken at 10 dpi. The area of all diseased spots was assessed with ImageJ 2.0 software (National Institutes of Health, Bethesda, MD, USA). The disease intensity of an individual line was based on the mean values of the diseased areas surrounding the 24 punctures on three leaves using the rating index described by Peng et al. [6]. The test was repeated three times.

The Xcc population levels in transgenic plants were determined as described by Peng et al. [32]. At 1, 3, 5, 7 and 9 dpi, bacterial colonies were counted and used to estimate the number of bacterial cells per unit leaf area (cm²).

Construction of RNA-Seq libraries and high-throughput sequencing

In this experiment, three biological replicates per selected transgenic and WT plants were performed. Total RNA from fully mature leaves was extracted using a TRIzol kit (Invitrogen, Thermo Fisher Scientific, Shanghai, China) in accordance with the user manual. RNA quality was determined using a NanoDrop 2000 spectrophotometer (Thermo) and an Agilent 2100 Bioanalyzer (Agilent Technologies Canada Inc, Mississauga, ON, Canada). Sequencing libraries were constructed from 1 μ g of total RNA using NEBNext Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) following manufacturer's recommendations. The libraries were sequenced using the Illumina HiSeq 2500 platform (BioMarker Technologies Illumina, Inc, Shanghai, China).

Analysis of RNA-Seq data

Approximately 6.4 Gb of high-quality clean reads were generated from each library after removing adaptor sequences and filtering low-quality sequences. All the clean reads were

mapped to the reference genome of sweet orange (*C. sinensis*, <http://citrus.hzau.edu.cn/orange/index.php>) by TopHat2 with default parameters [33]. Gene function was annotated based on Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KO and gene ontology (GO) databases. Gene expression levels in all the biological replicates were estimated using the FPKM method [34]. A differential expression analysis between transgenic lines and WT was performed using the DESeq R package 1.10.1 [35]. Genes with adjusted P-values < 0.05 found by DESeq were assigned as differentially expressed genes (DEGs). A GO enrichment analysis of the DEGs was performed using the Goseq R package [36]. A KEGG pathway enrichment analysis of DEGs was performed using KOBAS software [37].

MapMan software [38] was also used to analyze citrus gene expression data. At the end, the citrus genes from the reference genome of sweet orange (*C. sinensis*, <http://citrus.hzau.edu.cn/orange/index.php>) were assigned to BINs using the Mercator automated annotation pipeline (<http://mapman.gabipd.org/web/guest/mercator>), and then, the pathways, which were affected by DEGs, were analyzed using MapMan. Differentially represented MapMan pathways were defined using a two-tailed Wilcoxon rank sum test corrected using the Benjamin–Hochberg method (false discovery rate ≤ 0.05).

Results

Overexpression of *CsGH3.1* and *CsGH3.1L* in citrus causes abnormal phenotypes

To understand the functions of *CsGH3.1* and *CsGH3.1L* in citrus responses to citrus canker, the two genes, under the control of CaMV 35S promoter (S1 Fig), were introduced separately into Wanjincheng orange by *Agrobacterium*-mediated epicotyl transformation. Transgenic plants were identified using β -glucuronidase (GUS) histochemical staining and PCR analysis (S1 Fig). Expression levels of *CsGH3.1* and *CsGH3.1L* in transgenic plants were evaluated by qPCR analyses (S2 Fig). Based on the data, the pLGN-GH3.1 lines 1–3, -4, -5, -6, -8 and -9, and the pLGN-GH3.1L lines L-2, -5 and -6 showed high expression levels.

After the transgenic lines were planted in a greenhouse, their phenotypes were investigated. In the early stage (~6 months after planting), most transgenic plants showed leaf drooping and upward curling (Fig 1a). Gradual increased branching and leaf curling were detected as transgenic plants grew (S3 Fig). Lines 1–3, 1–4, 1–5, 1–9, L-2, L-5 and L-6, having high gene expression levels, had severe malformations, while lines 1–1, 1–2 and 1–10, having low gene expression levels, showed no obvious differences compared with WT (S3 Fig). Line 1–8 also showed no obvious difference, while line L-1 displayed mild changes in its phenotype compared with WT (S3 Fig). After one year, lines 1–3 and 1–4 died. After two years, transgenic plants displayed a bushy dwarf phenotype with smaller, drooping and upward curling leaves and branch softening and drooping (Fig 1b and S4 Fig). Moreover, transgenic leaves were significantly thinner, and both their longitudinal and transverse diameters were significantly shorter compared with WT (Fig 1c–1e). Such abnormal phenotypes indicated that overexpressing *CsGH3.1* and *CsGH3.1L* affected the basic plant development.

The decreased free IAA level in transgenic plants

To investigate the auxin content, the free IAA level in each transgenic line was measured. The free IAA levels in the 1–5, 1–8, 1–9, L-2, L-5 and L-6 transgenic lines were significantly lower than in WT plants before exposure to Xcc (Fig 2). Other lines showed no differences in free IAA levels compared with WT. After Xcc inoculation, free IAA levels in these transgenic lines

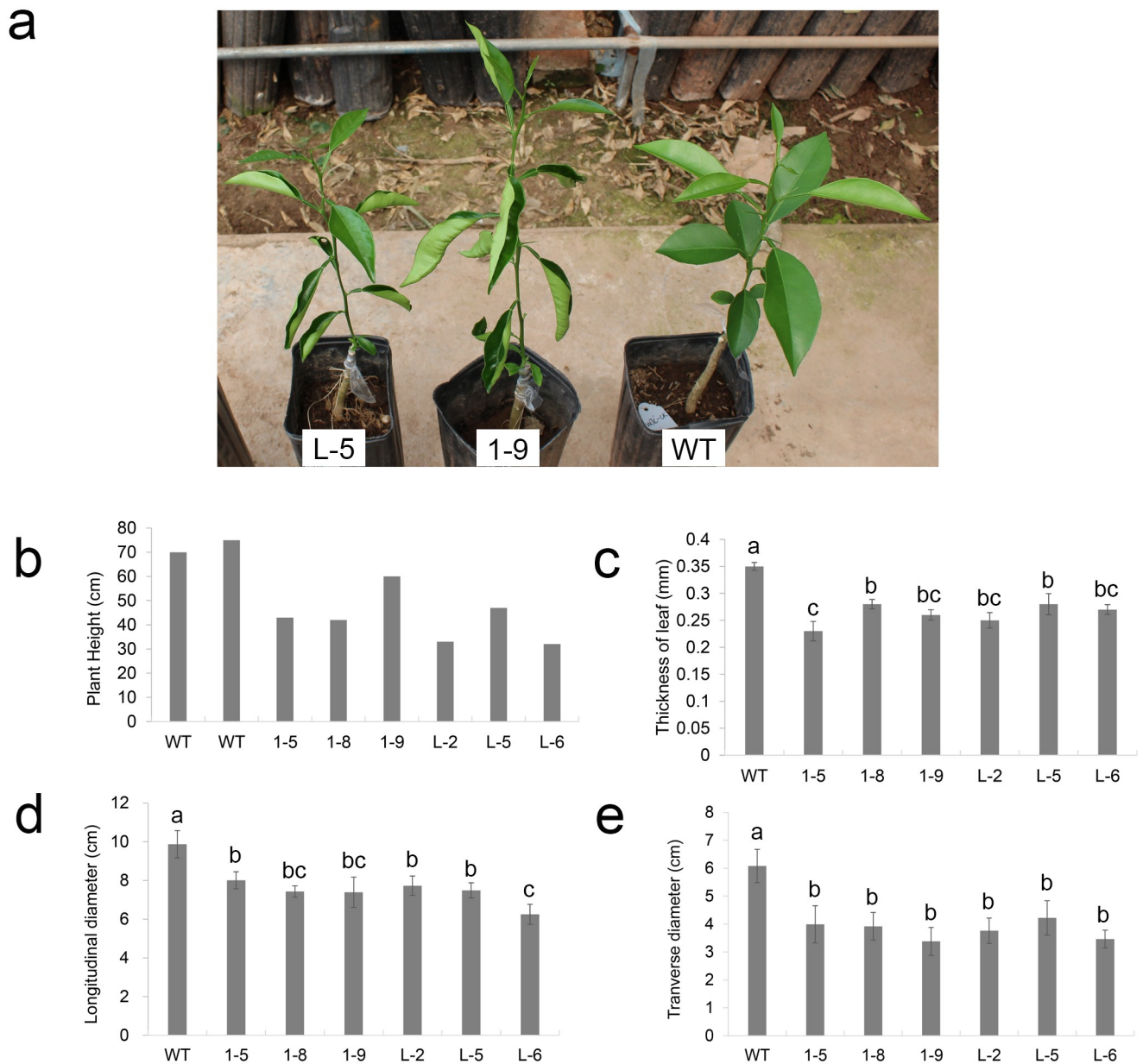


Fig 1. Phenotypic analysis of transgenic citrus independently overexpressing *CsGH3.1* (1-#) and *CsGH3.1L* (L-#). (a) Phenotypes of transgenic and wild-type (WT) plants after growing for six months in a greenhouse. (b) The heights of transgenic plants grown in a greenhouse for two years after grafting. The thickness (c), and the longitudinal (d) and transverse (e) diameters of leaves from transgenic plants grown in a greenhouse for 2 years after grafting were evaluated using 20 leaves per line. Error bars represent the mean standard errors. Different letters on top of the bars represent significant differences from WT controls based on a Tukey's test ($P < 0.05$).

<https://doi.org/10.1371/journal.pone.0220017.g001>

were still significantly lower than in WT, although the levels were markedly decreased in both transgenic lines and WT after *Xcc* inoculations (Fig 2).

Overexpression of *CsGH3* in citrus reduced susceptibility to *Xcc*

To evaluate the citrus canker resistance levels of transgenic plants, the 1-5, 1-8, 1-9, L-2, L-5 and L-6 transgenic lines were inoculated with *Xcc* by *in vitro* pinpricks. The diseased areas were determined 10 d after *Xcc* infection. Lesions around the pinprick sites in transgenic lines

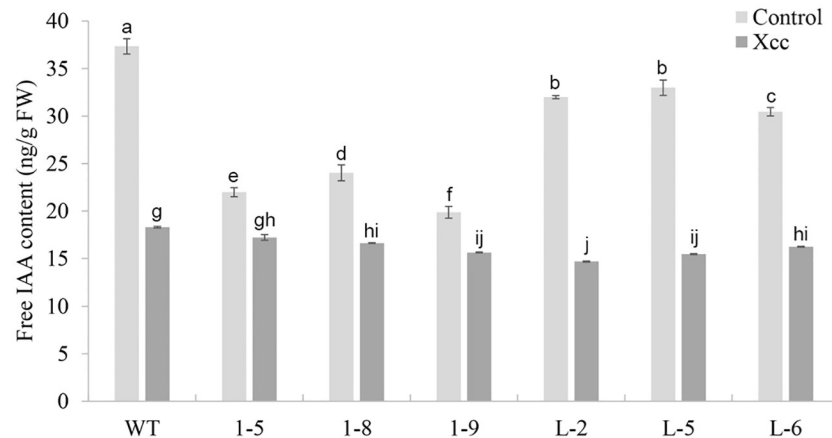


Fig 2. Determination of the IAA contents in transgenic citrus independently overexpressing *CsGH3.1* (1-#) and *CsGH3.1L* (L-#). IAA was isolated from six fully expanded intact leaves per line. The presented IAA values are the averages of three independent measurements per line before *Xanthomonas citri* subsp. *citri* (Xcc) infection (control) and 3 d after Xcc infection. The overexpression of *CsGH3.1* or *CsGH3.1L* decreased the IAA contents in transgenic plant before and after pathogen exposure. Error bars represent the mean standard errors. WT: wild-type. Different letters on top of bars represent significant differences from WT controls based on Tukey's test ($P < 0.05$).

<https://doi.org/10.1371/journal.pone.0220017.g002>

were significantly smaller compared with WT (Fig 3a and 3b). The disease indices of these transgenic lines decreased significantly compared with WT (Fig 3c), indicating that these transgenic lines had enhanced citrus canker resistance. The lines 1–9 and L-5 showed the strongest resistance levels to citrus canker (Fig 3c). Moreover, bacterial growth assays showed that Xcc populations in the 1–9 and L-5 lines were smaller than those in the WT 5 d after inoculation (Fig 3d), indicating that bacterial growth in transgenic plants was delayed. Thus, overexpressing *CsGH3.1* and *CsGH3.1L* enhanced host defense responses to citrus canker.

An overview of the transcriptional responses in transgenic citrus lines

To reveal the molecular mechanisms underlying canker resistance in *CsGH3*-overexpressing plants, global transcriptional profiling of lines 1–9 and L-5 showing high levels of resistance to citrus canker and a WT plant were compared using RNA-Seq (Fig 4a and S2 Table). In total, 1,560 and 1,037 genes were identified as DEGs in the 1–9 and L-5 transgenic lines, respectively, when compared with a WT plant (S3 Table). There were more upregulated DEGs than downregulated DEGs in both the 1–9 and L-5 lines (Fig 4b and S3 Table). In the GO annotation, most of the DEGs were classified into metabolic process, cellular process, single-organism process, response to stimulus, and biological regulation (S4 Table and S5 Fig). The KEGG pathway enrichment analysis further revealed that the DEGs in the 1–9 and L-5 transgenic lines were assigned to 88 and 83 KEGG pathways (Fig 4c), respectively. Notably, 34 and 31 DEGs were significantly ($q\text{-value} < 0.05$) assigned to “plant hormone signaling transduction (KO04075)” (Fig 4c).

To further survey the pathways or functions that were affected by the DEGs in the 1–9 and L-5 transgenic lines, the RNA-Seq data were visualized using the MapMan tool (Fig 5). A complete list of MapMan pathways differentially represented in the transgenic lines is provided in S5 Table. Based on these data, cell wall, secondary metabolism, hormone metabolism, stress, RNA and signaling were the major pathways or functions that were significantly regulated by the overexpression of *CsGH3.1* and *CsGH3.1L*. Differences in differentially represented pathways or functions were also detected between the transgenic lines. Importantly, flavonol metabolism, cytokinin metabolism and synthesis-degradation, biotic stress, touch or

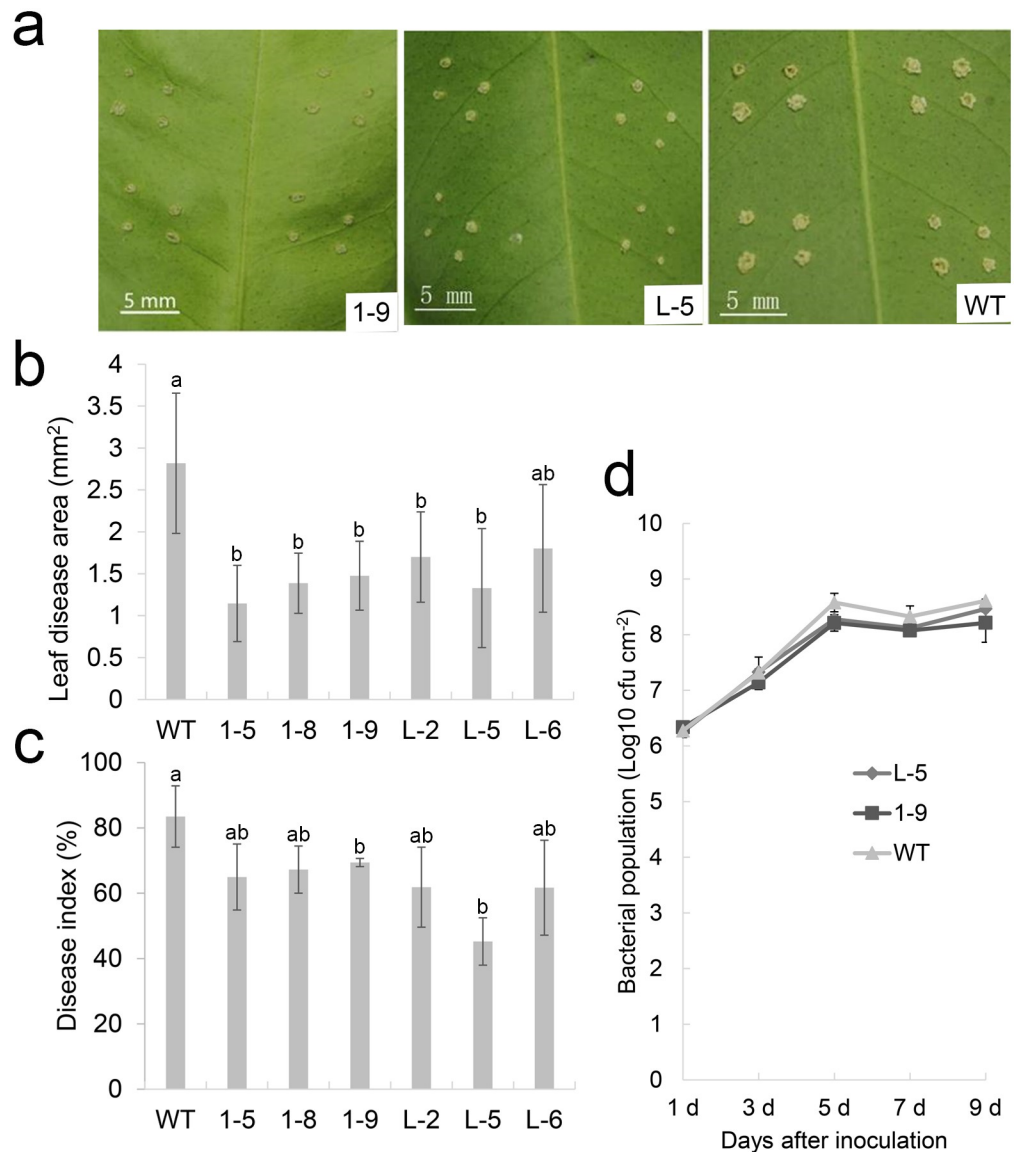


Fig 3. Evaluation of citrus canker resistance in transgenic citrus independently overexpressing *CsGH3.1* (1-#) and *CsGH3.1L* (L-#). (a) Citrus canker leaf symptoms in transgenic and non-transgenic lines 10 d after pin-puncture inoculation with *Xanthomonas citri* subsp. *citri* (Xcc). (b) Lesion areas and (c) and disease indices in transgenic plants. Diseased areas in leaves were counted 10 d after Xcc inoculation in WT and transgenic lines. (d) Growth of Xcc in leaves of the transgenic 1-9 and L-5 lines. Each column represents the mean of nine leaves from three independent experiments. Error bars represent the mean standard errors. Different letters on top of bars represent significant differences from wild-type (WT) controls based on Tukey's test ($P < 0.05$).

<https://doi.org/10.1371/journal.pone.0220017.g003>

wounding, Aux/IAA family, signaling, and unknown categories displayed significant changes in both the 1-9 and L-5 transgenic lines compared with WT. Biotic stress-related pathways were positively affected, while Aux/IAA family genes were negatively affected by both *CsGH3.1* and *CsGH3.1L* (Fig 5). Aux/IAA family genes have vital roles in auxin signaling [9]. The survey clearly showed that the overexpression of *CsGH3.1* and *CsGH3.1L* repressed auxin signaling and enhanced biotic stress responses in citrus. Thus, the DEGs involved in auxin metabolism and signaling, as well as biotic stress responses, were studied in more detail.

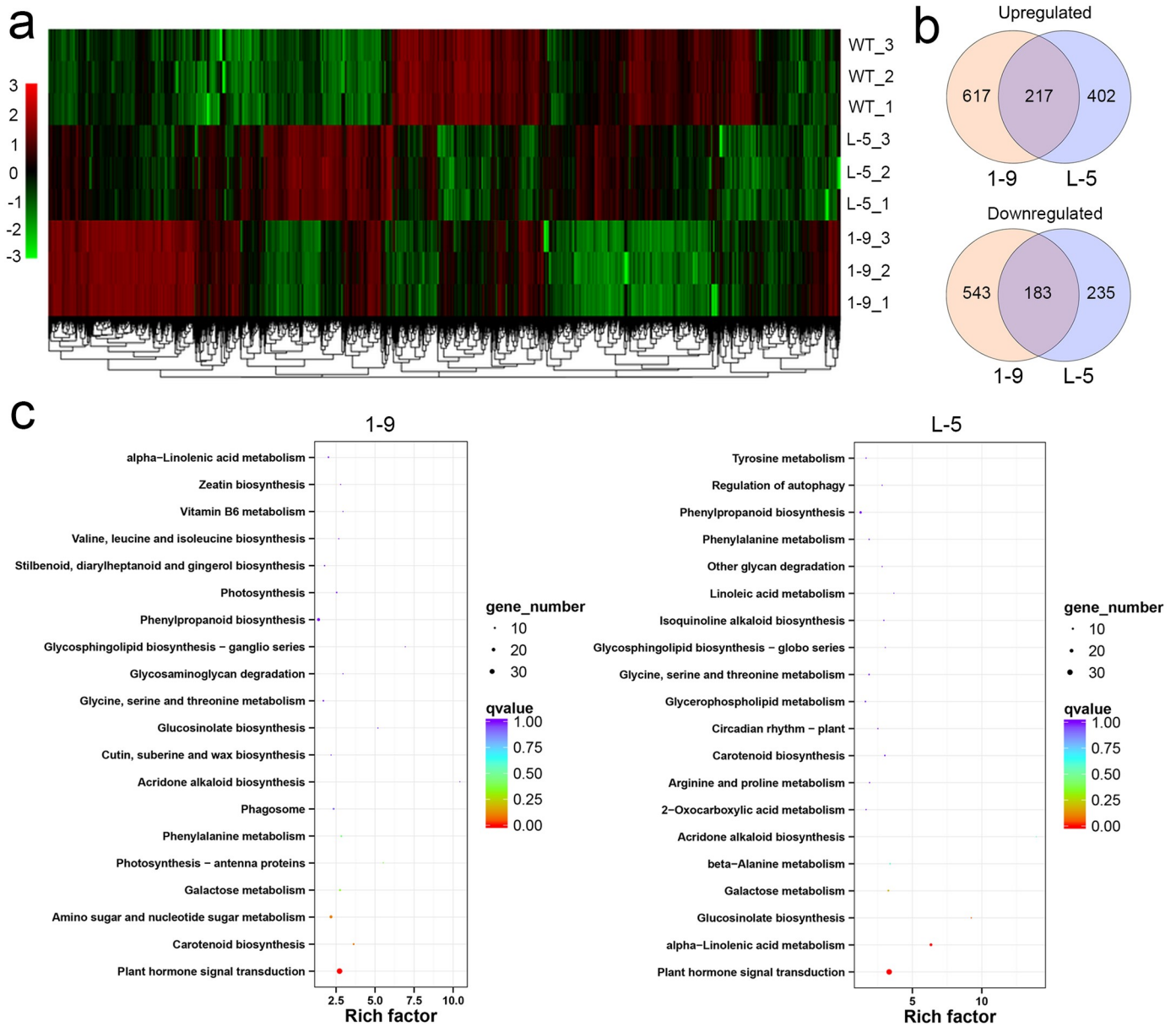


Fig 4. Global gene expression profiles of transgenic citrus independently overexpressing *CsGH3.1* (1-9) and *CsGH3.1L* (L-5). (a) Heat map analysis of the differentially expressed genes (DEGs) among 1-9 and L-5 transgenic and wild-type lines. The 1-9 and L-5 transgenic lines showed a similar hierarchical cluster pattern. (b) Venn diagrams showing the overlaps of differentially expressed genes between transgenic lines. In total, 400 of 2,745 DEGs showed similar expression profiles among these lines. (c) KEGG pathway enrichment of the DEGs between transgenic lines. The overexpression of *CsGH3.1* or *CsGH3.1L* significantly affected hormone signal transduction.

<https://doi.org/10.1371/journal.pone.0220017.g004>

Auxin-related genes

We investigated the DEGs related to auxin homeostasis, perception and signaling in transgenic lines using a MapMan analysis. Most of the 28 auxin-related DEGs showed significantly downregulated expression levels in both the 1-9 and L-5 transgenic lines, and most of these genes were assigned to auxin signaling transduction (Table 1). All 12 Aux/IAA family members, a group of auxin-induced genes, showed significantly downregulated expression levels in the transgenic plants. In addition, two of four SAUR-like auxin-responsive protein family

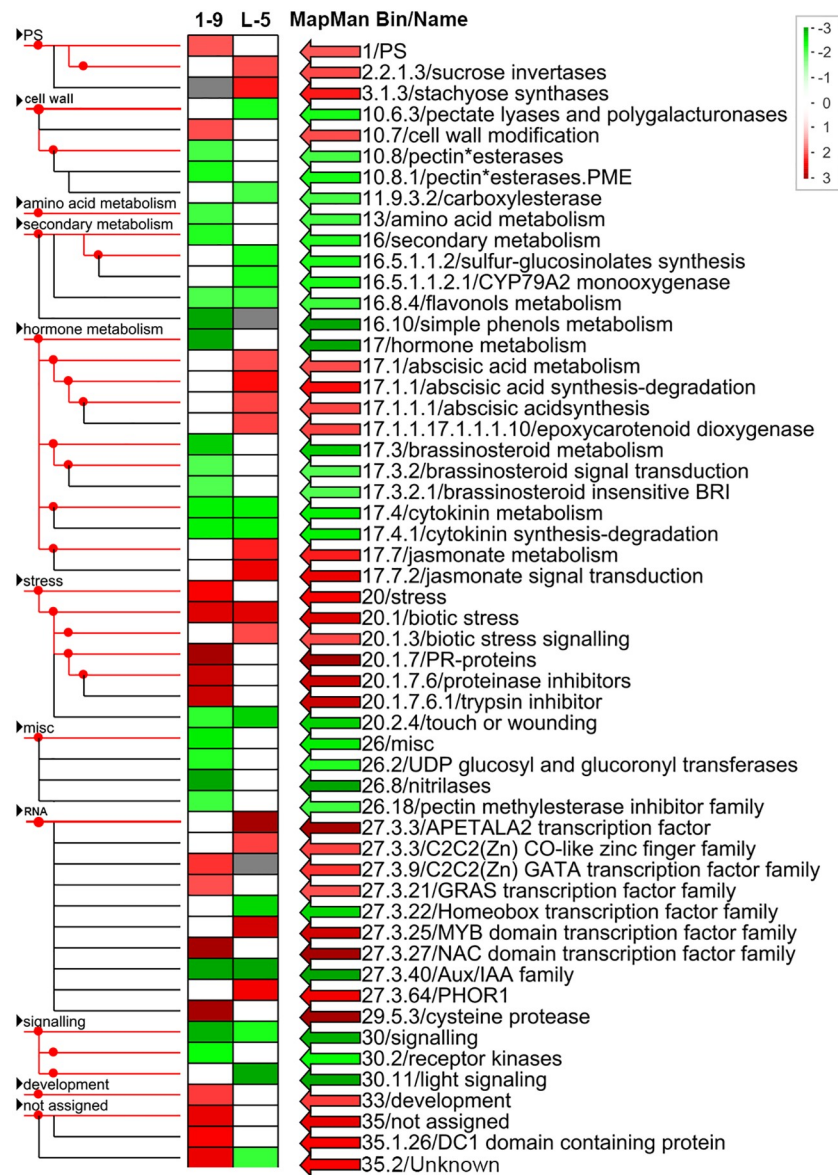


Fig 5. MapMan visualization of differentially represented pathways and functional categories between the 1-9 and L-5 transgenic citrus lines overexpressing *CsGH3.1* and *CsGH3.1L*, respectively. Each colored rectangular block denotes a MapMan pathway or functional category. Upregulated and downregulated categories are shown in red and green, respectively. The scale bar represents fold change values. The categories differentially represented in the transgenic plants are indicated on the right.

<https://doi.org/10.1371/journal.pone.0220017.g005>

members (ARG7), and one auxin response factor (ARF10) also showed significantly downregulated expression levels in the transgenic plants. The expression levels of three genes (PIN1, PIN3 and AUX1-like protein 3) involved in auxin transport were significantly downregulated in transgenic plants (Table 1). However, four genes (Cs7g08110, Cs5g20420, Cs3g19760 and Cs7g08080) involved in auxin synthesis-degradation were significantly induced by the independent overexpression of *CsGH3.1* and *CsGH3.1L* (Table 1). Overall, the overexpression of *CsGH3.1* and *CsGH3.1L* significantly repressed the expression levels of auxin transport and

Table 1. Differentially expressed genes related to auxin biosynthesis and signaling in transgenic citrus independently overexpressing *CsGH3.1* and *CsGH3.1L*.

Gene ID	Putative function	Log ₂ (Fold change)* (Transgenic line vs WT)	
		1-9 line	L-5 line
Auxin synthesis-degradation			
Cs7g08110	UDP-glycosyltransferase (UGT) 74B1	2.332	1.632
cs5g20420	UDP-glycosyltransferase (UGT) 75B1	1.048	ND.
cs3g19760	IAA amino acid conjugate hydrolase	ND.	1.878
cs7g08080	IAA-amino acid conjugate hydrolase	ND.	1.899
Auxin transport			
Orange1.1t00089	Auxin efflux carrier component 1, PIN1	-1.224	-1.119
Cs2g16620	Auxin efflux carrier component 3, PIN3	-1.289	-2.156
cs2g06880	TIR1/AFB auxin receptor protein	1.239	ND.
cs3g19250	Auxin transporter-like protein 3 (AUX1-like 3)	-1.563	ND.
Auxin signaling			
Cs5g29060	AUX/IAA family Auxin induced gene, IAA11	-1.630	-1.396
Cs9g09120	AUX/IAA family Auxin induced gene, IAA13	-1.209	-1.144
Cs5g30380	AUX/IAA family Auxin-induced protein, IAA16	-7.380	-5.406
Cs5g30390	AUX/IAA family Auxin-induced protein; IAA4	-1.994	-2.445
Cs7g05540	AUX/IAA family Auxin-induced protein; IAA29	-3.151	-1.735
Cs9g08100	AUX/IAA family Auxin-induced protein; IAA4	-4.057	-3.351
Cs9g08110	AUX/IAA family IAA14-like	-3.146	-4.451
Cs1g13970	AUX/IAA family Auxin-induced protein; IAA19	-4.305	-5.132
Cs4g18240	AUX/IAA family Auxin-induced protein; IAA29	-3.919	-2.829
cs1g13960	AUX/IAA family Auxin-inducible AUX/IAA gene	-1.972	ND.
cs3g10920	AUX/IAA family AUXIN INDUCIBLE 2-11 (ATAUX2-11)	-1.619	ND.
cs3g10930	AUX/IAA family early auxin-induced (IAA16)	ND.	-1.399
Orange1.1t04221	SAUR-like auxin-responsive protein family (ARG7)	-3.446	-2.909
cs1g16790	SAUR-like auxin-responsive protein family	-2.084	ND.
orange1.1t02550	SAUR-like auxin-responsive protein family	1.056	1.035
cs4g12720	SAUR-like auxin-responsive protein family	ND.	2.624
Cs8g16440	Auxin response factor 10 (ARF10)	-2.241	-2.089
orange1.1t00464	indole-3-acetic acid-amido synthetase GH3.5	ND.	1.301
cs1g22140	CsGH3.1	12.353	7.818
cs8g04610	CsGH3.1L	-3.452	7.296

*Differentially expressed genes having |Log₂ (Fold change)| ≥ 1 are presented. ND, not detected.

<https://doi.org/10.1371/journal.pone.0220017.t001>

signaling-related genes. Interestingly, *CsGH3.1*'s overexpression downregulated *CsGH3.1L*'s expression, while *CsGH3.1L*'s overexpression upregulated *CsGH3.1*'s expression (Table 1).

Disease response-related genes

Fig 6 presents an overview of the MapMan functional categories for genes involved in disease responses. Their functional products could be sorted into the following major classes: hormone signaling, cell wall, proteolysis, signaling, PR proteins, redox state, transcription factors, heat shock proteins and secondary metabolites. Among the disease response-related genes, the number (90) of upregulated genes was obvious more than that (57) of downregulated genes. In total, 45 and 23 genes, including resistance, stress recognition, signal receptor and transduction, and PR protein, were directly assigned into the “stress. biotic” MapMan category in the

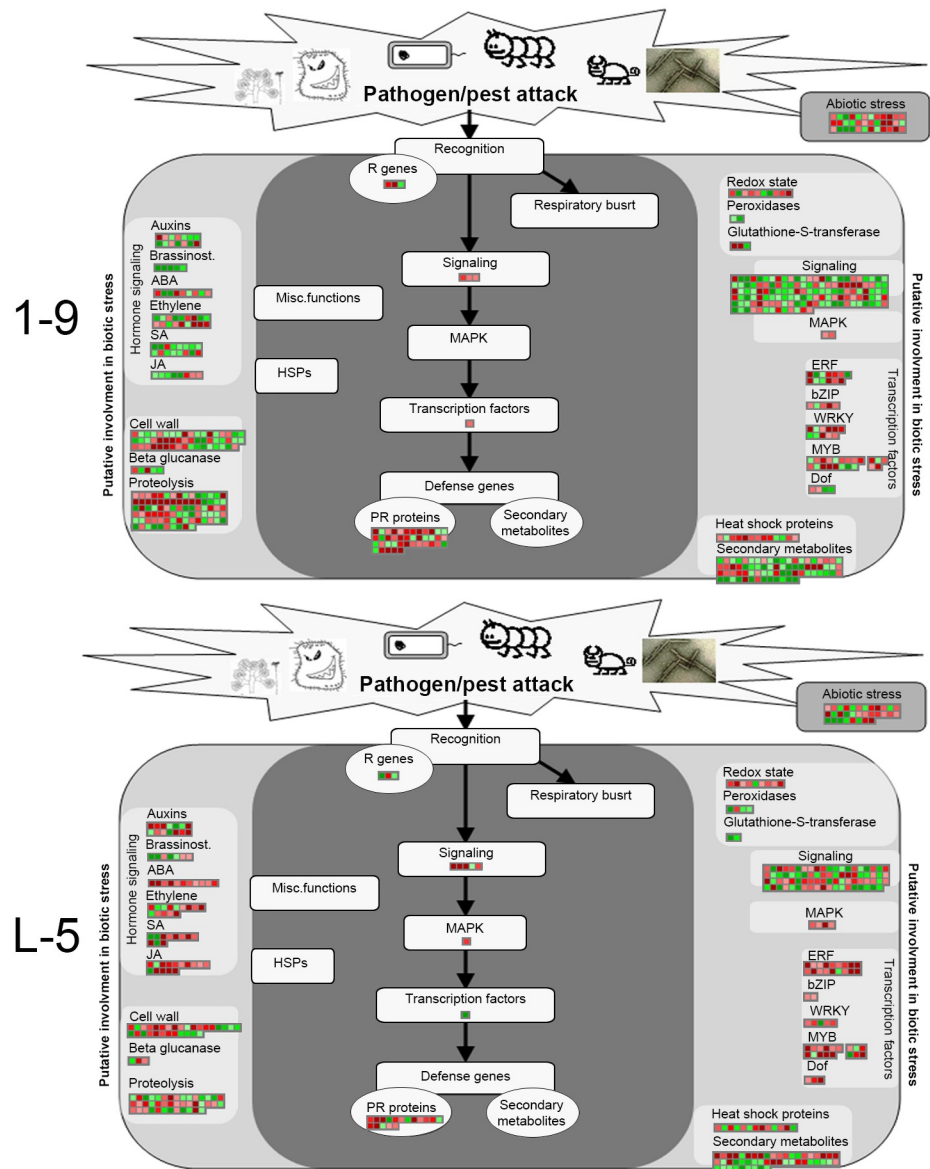


Fig 6. MapMan visualization of the functional categories of genes differentially expressed in response to biotic stress in the 1-9 and L-5 transgenic citrus lines overexpressing *CsGH3.1* and *CsGH3.1L*, respectively. Significantly upregulated and downregulated genes are displayed in red and green, respectively.

<https://doi.org/10.1371/journal.pone.0220017.g006>

1-9 and L-5 transgenic lines, respectively (S6 Table). Among these genes, 33 and 18 genes showed significantly upregulated expression levels in the 1-9 and L-5 lines, respectively (S6 Table).

Table 2 displays the important DEGs correlated with biotic stress in transgenic plants based on the MapMan annotation. The induced genes included a Toll-Interleukin-Resistance (TIR) domain family member (Cs5g18230) involved in innate immune responses, three defense PR genes (Cs6g01070, NB-ARC domain-containing disease resistance protein; Cs9g18740, disease resistance family protein/LRR family protein; and Cs5g19240, TIR-NBS-LRR disease resistance protein). Moreover, all 10 transcription factor genes had upregulated expression levels, and these genes mainly included the AP2/EREPB, MYB and WRKY families. Thus, gene

Table 2. Differentially expressed genes related to biotic stress in transgenic citrus independently overexpressing *CsGH3.1* and *CsGH3.1L*.

Gene ID	Putative function	Log ₂ Fold change* (Transgenic line vs WT)	
		1-9	L-5
Stress recognition			
Orange1.1t02076	Disease resistance-responsive family protein	-1.005	-2.029
Orange1.1t03601	LRR receptor-like kinase FLS2	2.755	1.412
Cs2g16870	MLP-like protein 31 (MLP31)	2.198	3.223
Cs2g17820	ARM repeat superfamily protein	1.177	3.669
Cs2g29780	HXXXD-type acyl-transferase family protein	1.436	3.488
Cs2g31450	Acetyl coa:(Z)-3-hexen-1-ol acetyltransferase (CHAT)	-1.365	1.685
Signal receptor			
Cs5g18230	Toll-Interleukin-Resistance (TIR) domain family protein	2.044	1.717
Cs5g27950	Leucine-rich repeat I receptor kinase	-2.207	-2.005
Cs5g15420	Leucine-rich repeat VIII (VIII-2) receptor kinase	1.262	1.429
Cs9g14980	Leucine-rich repeat XI receptor kinase	-1.142	-2.069
Orange1.1t01442	Leucine-rich repeat XI receptor kinase	-2.353	-1.839
Orange1.1t03106	Leucine-rich repeat XI receptor kinase	1.394	1.342
Cs4g07400	Serine/threonine receptor kinase	3.036	1.785
Orange1.1t01406	DUF 26receptor kinase	-1.357	-1.596
Signal transduction			
Cs1g17210	JAZ1 involved in jasmonate signaling	1.173	2.883
Cs1g17220	JAZ1 involved in jasmonate signaling	1.143	2.371
Cs6g17590	Calmodulin-like protein	1.321	1.482
Cs7g27120	Calmodulin binding protein-like	1.230	1.591
Cs5g07160	Calmodulin like 37 (CML37)	1.288	1.996
Cs2g15970	Rac-like GTP-binding protein RAC2	-2.151	-1.370
Cs2g21150	Mitogen-activated protein kinase kinase kinase 18 (MAPKKK18)	1.286	3.530
Orange1.1t01873	Tobacco Rapid Alkalinization Factor (RALF)	-1.357	-1.438
Cs9g05280	AHP1 Arabidopsis thaliana histidine phosphotransfer protein	-1.134	-1.235
Cs7g09390	Phototropic-responsive NPH3 family protein	-1.633	-1.696
Cs9g07670	Chlorophyll A-B binding family protein	-1.136	-2.031
Defense response gene			
Cs6g01070	NB-ARC domain-containing disease resistance protein	2.089	1.506
Cs9g18740	Disease resistance family protein / LRR family protein defense	2.640	2.459
Cs8g14950	Glucan endo-1,3-beta-glucosidase	-1.567	1.177
Cs3g06300	Receptor like protein 6 (RLP6)	-1.089	1.200
Cs5g19240	Disease resistance protein (TIR-NBS-LRR class)	2.180	1.563
Redox metabolism			
Cs2g17910	Cytochrome b561/ferric reductase transmembrane protein family	-1.646	-1.768
Cs5g32580	Thioredoxin superfamily protein	1.316	1.028
Cs2g16150	GRX480, the glutaredoxin family that regulates protein redox state	1.436	1.806
Orange1.1t03455	Probable glutathione S-transferase, gsts, Auxin-induced protein	-1.777	-1.624
Cs2g15310	Peroxidase superfamily protein	-1.918	-1.385
Responsive transcription factor			
Cs6g15360	The DREB subfamily A-1 of ERF/AP2 transcription factor (CBF4)	2.032	4.256
Cs9g16810	The DREB subfamily A-1 of ERF/AP2 transcription factor (CBF4)	1.725	2.039
Orange1.1t01154	The DREB subfamily A-1 of ERF/AP2 transcription factor (CBF2)	1.543	2.840
Cs1g07950	The ERF subfamily B-1 of ERF/AP2 transcription factor (ATERF-4)	1.913	2.078

(Continued)

Table 2. (Continued)

Gene ID	Putative function	Log ₂ Fold change* (Transgenic line vs WT)	
		1-9	L-5
Cs4g07040	The DREB subfamily A-5 of ERF/AP2 transcription factor family	5.413	2.803
Cs5g29830	Member of the R2R3 factor gene family (MYB14)	1.249	2.333
Cs2g27410	Member of the R2R3 factor gene family (MYB58)	3.896	4.470
Cs3g23070	Member of the R2R3 factor MYB gene family (MYBR1)	2.909	2.012
Cs3g23950	Member of the R2R3 factor gene family (MYB73)	2.235	3.114
Cs1g03870	Group II-c WRKY Transcription Factor (WRKY51)	3.168	1.313
Protein degradation			
Cs2g18910	Encode a protein similar to subtilisin-like serine protease	-1.763	-2.831
Cs2g27790	Cysteine proteinases superfamily protein	3.370	1.277
Cs9g06150	Eukaryotic aspartyl protease family protein	1.971	3.298
Cs2g29430	Skp2-like F-box family protein	-2.364	-2.155
Cs3g14660	F-box family protein; RNI-like superfamily protein	1.338	1.078
Cs6g13640	E3 ubiquitin ligase protein involved in PAMP-triggered immunity	1.070	2.153

*Differentially expressed genes having |Log₂ (Fold change)| ≥ 1 are presented.

<https://doi.org/10.1371/journal.pone.0220017.t002>

expression associated with responses to biotic stress was clearly activated by the overexpression of *CsGH3.1* and *CsGH3.1L*.

Data presented in Table 3 show that 11 cell wall-related genes were differentially expressed, with 8 being upregulated by the overexpression of *CsGH3*. These upregulated genes were mostly involved in cell wall biosynthesis and modification, such as xyloglucan endotransglycosylase, cellulose synthase, and wax and cutin synthesis. Among them, two genes encoding FAS-CICLIN-like arabinogalactan-protein 12 (Cs8g16830) and a xylem-specific cellulose synthase (Cs4g02000), showed highly induced expression levels (fold change > 5). Three genes

Table 3. Differentially expressed genes related to cell wall in transgenic citrus independently overexpressing *CsGH3.1* and *CsGH3.1L*.

Gene ID	Putative function	Log ₂ (Fold change)* (Transgenic line vs WT)	
		1-9	L-5
Cell wall biosynthesis			
Cs8g16830	FASCICLIN-like arabinogalactan-protein 12 (FLA12)	5.439	5.357
Cs4g03060	Xyloglucan endotransglycosylase-related protein (XTR6)	2.591	1.90
Cs4g03130	Xyloglucan endotransglycosylase-related protein (XTR6)	2.031	1.393
Cs4g03140	Xyloglucan endotransglycosylase-related protein (XTR6)	2.227	1.673
Cs4g02000	Xylem-specific cellulose synthase	7.304	6.993
Cs9g02720	Putative membrane-anchored cell wall protein, COBRA-like protein-7	1.113	1.570
Cs5g28330	Long chain acyl-coa synthetase involved in cutin synthesis	1.576	1.109
Orange1.t00556	Condensing enzyme KCS1 involved in wax biosynthesis	1.408	2.712
Cell wall degradation			
Cs1g05510	Plant invertase/pectin methylesterase inhibitor superfamily	-1.940	-1.797
Cs5g07854	EXLB1 (expansin-like B1), a member of the expansin family	-1.393	-1.692
Cs7g08620	Polygalacturonase 2 (PG2)	-2.432	-1.802

*Differentially expressed genes having |Log₂ (Fold change)| ≥ 1 are presented.

<https://doi.org/10.1371/journal.pone.0220017.t003>

(pectinesterase inhibitor Cs1g05510, expansin Cs5g07854 and polygalacturonase Cs7g08620), assigned to cell wall degradation, were downregulated when CsGH3 was overexpressed.

Effects of overexpression of *CsGH3* on hormone contents of transgenic lines

Because the transgenic plants displayed severe dwarfism (S4 Fig) and obvious changes in transcriptional profiles involved in hormone metabolism (Fig 6 and S7 Table), we investigated SA, JA, ABA, ZT and ET levels in the 1–9 and L-5 transgenic lines (Fig 7). The SA and ET contents in both 1–9 and L-5 transgenic lines were significantly greater than in WT. The JA, ABA and ZT contents in the 1–9 transgenic line were significantly lower than in WT. No significant difference in JA, ABA or ZT level was detected in the L-5 line compared with WT, although the ABA and ZT levels were decreased in this transgenic line.

Discussion

The *GH3* gene family maintains hormonal homeostasis by conjugating free hormones to amino acids during exposure to biotic and abiotic stresses. Our work first showed that the overexpression of *CsGH3.1* and *CsGH3.1L* decreased free IAA levels. Correspondingly, transgenic plants displayed a bushy dwarf phenotype that is associated with an auxin shortage [25]. Additionally, naphthalene acetic acid treatments significantly induced the expression of *CsGH3.1* in citrus leaves [26]. Thus, *CsGH3.1* and *CsGH3.1L* appear to be functional IAA-amido synthetase genes involved in the regulation of free IAA levels in citrus. *CsGH3.1* and *CsGH3.1L* belong to the group II proteins of the GH3 family. *Arabidopsis GH3.5* and rice *GH3.1* and *sGH3.8* of this group positively regulate pathogen-induced defense responses through the depletion of free IAA [13, 24, 25]. Similarly, the overexpression of *CsGH3.1* and *CsGH3.1L* enhances plant resistance to citrus canker. Moreover, we showed that the overexpression of *CsGH3.1* and *CsGH3.1L* accelerated the Xcc-induced decline of free IAA levels in transgenic plants. Based on these data, our results indicated that the overexpression of *CsGH3.1* and *CsGH3.1L* reduces the susceptibility to Xcc by decreasing free IAA levels both before and after pathogen infection in citrus.

The transcriptomic data showed that the decrease in the free IAA level significantly repressed the expression levels of auxin signaling-related genes in transgenic plants. For example, AUX/IAA family members, SAUR-like auxin-responsive protein family members, and ARF10, PIN1, PIN3 and AUX1-like protein genes, which are involved in auxin signal transduction, were significantly downregulated in transgenic plants. In particular, the expression levels of the AUX/IAA family members annotated by our transcriptomic data were significantly repressed by the overexpression of CsGH3. The AUX/IAA family encodes a group of primary auxin-responsive proteins, which regulate ARF expression through ubiquitin-mediated degradation [17]. When cellular auxin concentrations are below a certain threshold, AUX/IAA proteins inhibit ARF transcription factors to activate auxin responsive genes (such as *AUX/IAA*, *GH3* and *SAUR*) and subsequently suppress auxin responses [9]. This suppression further represses AUX/IAA transcript levels. The decreased expression of PIN1 and PIN3 indicated that the auxin efflux was inhibited in transgenic plants, suggesting that the overexpression of *CsGH3.1* and *CsGH3.1L* affected the auxin distribution in plants. The transcriptomic data also showed that no auxin biosynthesis-related genes (such as those related to tryptophan biosynthesis or metabolism) were affected by the decrease in the free IAA content, while the two genes encoding UDP-glycosyltransferase (*UGT74B1* and *UGT75B1*), which conjugate auxin to glucoside [39], displayed significantly increased expression levels in transgenic plants. Thus, the unchanged expression levels of auxin biosynthesis-related genes and

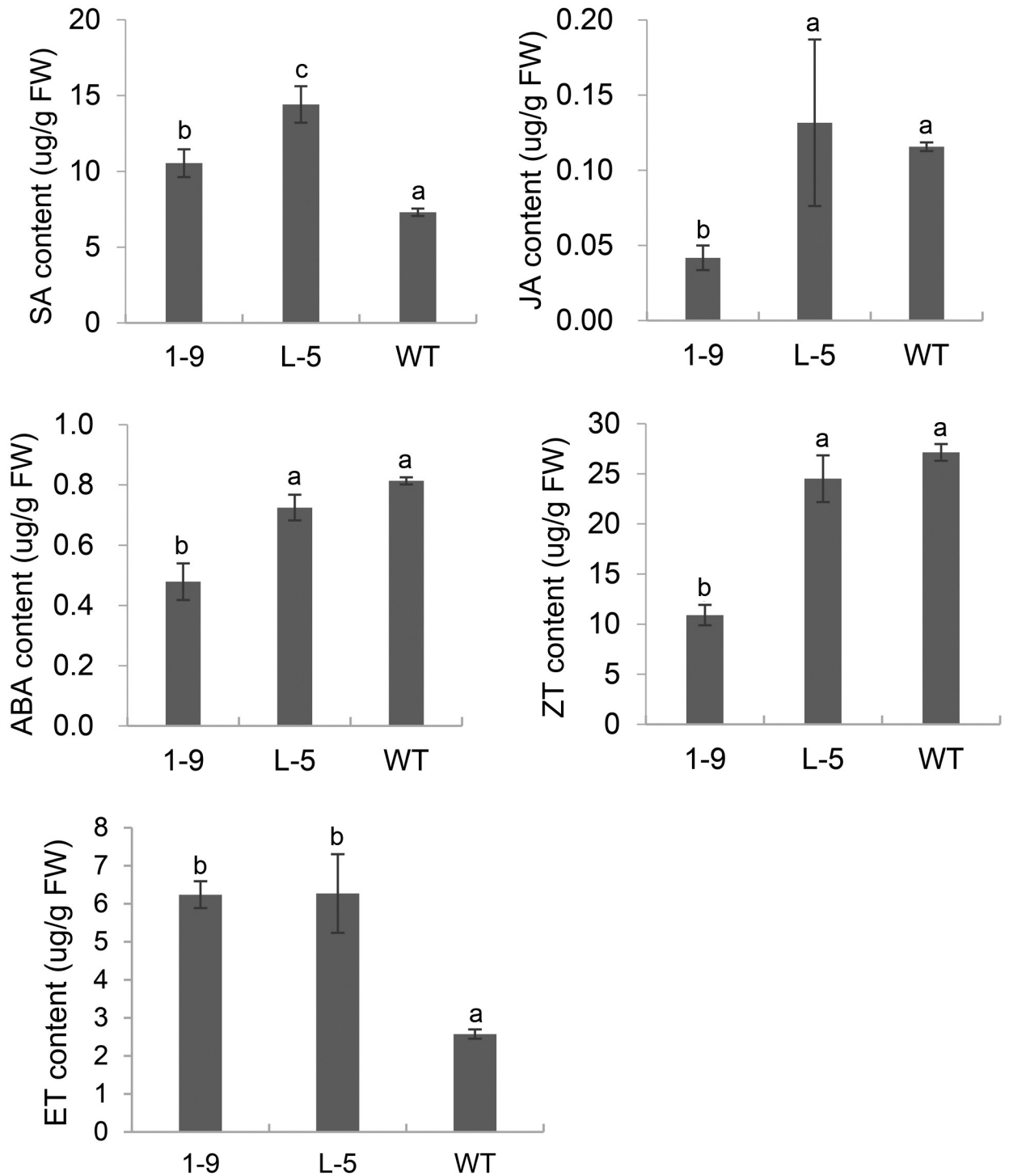


Fig 7. Determination of SA, JA, ABA, ZT and ET contents in the 1-9 and L-5 transgenic citrus lines overexpressing *CsGH3.1* and *CsGH3.1L*, respectively. Hormones were isolated from six fully expanded intact leaves per line. Error bars represent the mean standard errors of three independent measurements. Different letters on top of bars represent significant differences from wild-type (WT) controls based on Tukey's test ($P < 0.05$).

<https://doi.org/10.1371/journal.pone.0220017.g007>

activation of auxin glucosylation-related genes further favored decreases in the free IAA contents of transgenic plants. Additionally, the IAA–amino acid conjugated hydrolase genes (Cs3g19760 and Cs7g08080), which can rapidly regenerate IAA from IAA–amino acid conjugates to help maintain auxin homeostasis [40], displayed increased expression levels in the L-5 line, which could be an antagonistic response to depleted free auxin levels.

The constitutive expression of *CsGH3.1* and *CsGH3.1L* affected the establishment of citrus architecture. Abnormal phenotypes also occurred in transgenic rice overexpressing *OsGH3.1* [25] and *OsGH3.8* [24]. The GO annotation revealed that many of the DEGs in transgenic lines were classified into metabolic process, cellular process and development. Cell walls play critical roles in establishing plant architecture. In our study, most genes that were involved in cell wall biosynthesis, were profoundly upregulated by the overexpression of *CsGH3.1* and *CsGH3.1L*. Additionally, three genes (pectinesterase inhibitor, expansin and polygalacturonase), related to cell wall loosening, had decreased expression levels. However, Domingo et al. [25] showed that most of the genes involved in both cell wall biosynthesis and loosening were downregulated by *OsGH3.1*'s overexpression in rice. This transcriptional differences of these genes between the two studies indicated that GH3.1 has various functions in the regulation of cell wall-related genes in different species. However, it is clear that overexpressing GH3.1 can result in similar dwarf phenotypes in both rice and citrus.

Auxin is believed to act as a pathogenic factor in pustule formation during citrus canker development [5, 16, 26]. The transcriptional activator-like effectors, PthAs, secreted by Xcc manipulate multiple disease susceptibility genes or their products to regulate pustule formation in infected sites [41]. For example, the PthA-induced expression of the susceptibility genes *CsLOB1*, *CsLOB2* and *CsDiox* regulate citrus cell division and growth to increase pustule formation [3, 4, 41]. PthA effectors also interact with CsCYP and CsMAF1 proteins, which are repressors of citrus RNA polymerase (Pol) II and III, respectively, to activate the transcription of host genes involved in cell division and growth [42]. Auxin inhibits the translocation of CsMAF1 from the nucleoplasm to nucleolus [43], which decreased the accumulation of CsMAF1 in the nucleolus. This decreased CsMAF1 content in the nucleolus is beneficial for Pol III's activation of host genes' transcription as well as for the PthA effectors' enhancement of *CsLOB1*, *CsLOB2* and *CsDiox* expression levels, which both increase symptom development. Thus, conversely, this depletion of free IAA in transgenic plants is favorable for CsMAF1's entry into the nucleolus to antagonize Pol III-mediated gene expression and finally represses disease development. Moreover, *CsGH3*-mediated auxin homeostasis probably affects *CsLOB1* functions in citrus canker development. The cell wall is the first line of plant defense against pathogen invasion and pathogen-induced host cell wall loosening plays an important role in symptom development [44, 45]. *CsLOB1* upregulates the expression of pectate lyase, extension, α -expansin and cellulose genes [3], which are involved in the cell wall loosening induced by pathogen infection [1]. Auxin triggers cell wall loosening by rapidly acidifying cell walls [46]. Thus, pathogen-induced increases in auxin may have synergistic roles in the cell wall loosening induced by *CsLOB1*. Our transcriptomic data also showed that a decrease in free auxin significantly repressed the expression levels of cell wall loosening-related genes. Similarly, Cernadas and Benedetti [5] showed that the auxin transport inhibitor naphthyl-phthalamic acid repressed pustule formation and the expression of cell wall loosening-related genes induced by Xcc infection in the sweet orange (*C. sinensis*) "Pêra" cultivar. Thus, the data showed that interfering with auxin homeostasis, as seen with *CsGH3*, can weaken pathogen effector-induced host pustule formation and finally enhance plant resistance.

The MapMan analysis showed that biotic stress-related pathways were significantly upregulated by the overexpression of *CsGH3.1* and *CsGH3.1L*. In addition, a considerable number of induced genes were disease-resistance response genes, such as TIR, PR, LRR and

TIR-NBS-LRR family members, suggesting that the depletion of the IAA content enhanced the defense response. In the complex network of regulatory interactions during plant resistance responses, an antagonistic relationship between SA and JA signaling pathways is evident, and generally, plant responses to bacterial infections involve activating SA signaling, which represses JA signaling [47, 48]. In citrus, SA treatments enhance resistance to citrus canker [49]. Moreover, SA also inhibits pathogen spread in plants by repressing auxin signaling [14], and *Arabidopsis GH3.5* enhances the SA-mediated defense response [50]. Thus, we evaluated the effects of overexpressing *CsGH3.1* and *CsGH3.1L* on the hormone contents of transgenic citrus. The transgenic lines had significantly increased SA levels. Additionally, the transcriptomic analysis showed that three of four genes encoding S-adenosyl-L-methionine-dependent methyltransferase superfamily proteins, which convert active SA to non-active methyl SA, were repressed in transgenic lines, which was consistent with increased levels of SA. This indicated that the depletion of the IAA content enhanced the SA accumulation by regulating the conversion of SA and methyl SA. In JA signaling, the 12-oxophytodienoic acid reductase and cystathionine Beta-synthase genes participating in JA biosynthesis were downregulated in the 1–9 line, which was consistent with the decreased JA level in this line. However, the JA content was not significantly different compared with WT plants, although the two genes were induced in the L-5 line. Thus, our data showed that the overexpression of *CsGH3.1* and *CsGH3.1L* enhanced SA signaling and partially repressed JA signaling, which may activate the expression of disease resistance genes in transgenic plants. The data also showed that ET levels were significantly induced by the overexpression of *CsGH3.1* in transgenic lines. Plants produce ET in response to most biotic and abiotic stresses. In some cases, the role of ET in plant defense contributes to pathogen resistance. In *A. thaliana*, SA and ET function together to coordinately induce several defense-related genes, and ET treatments potentiate the SA-mediated induction of PR-1 [48, 51]. However, the ET signaling pathway may also negatively affect SA-dependent resistance [47, 52, 53]. In our study, *CsGH3.1* and *CsGH3.1L* positively regulated SA and ET accumulations in citrus, which should improve resistance to citrus disease.

In this study, transgenic plants overexpressing *CsGH3.1* and *CsGH3.1L* displayed a similar altered morphology, decreased free IAA levels and enhanced citrus canker resistance. However, a transcriptomic analysis showed that there were obvious differences in the affected MapMan pathways between lines 1–9 and L-5, indicating that *CsGH3.1* and *CsGH3.1L* have different roles in the regulation of auxin signaling. Based on the findings, we hypothesized that *CsGH3.1* and *CsGH3.1L* can increase resistance against citrus canker in citrus plants by inhibiting the accumulation of active auxin, revealing a potential role for the *GH3* gene in citrus breeding to improve citrus canker resistance.

Supporting information

S1 Fig. Production of transgenic citrus plants.

(DOCX)

S2 Fig. Quantitative PCR analysis of the expression of *GH3.1* and *GH3.1L* in transgenic plants.

(DOCX)

S3 Fig. Phenotype analysis of transgenic citrus plants growing for one year in the greenhouse.

(DOCX)

S4 Fig. Phenotype analysis of transgenic citrus plants growing for two years in the greenhouse.

(DOCX)

S5 Fig. GO classification map of the differentially expressed genes in transgenic plants.

(DOCX)

S1 Table. The primers used in the study.

(DOCX)

S2 Table. Function analysis of unigenes in the 1–9 and L-5 transgenic line.

(XLS)

S3 Table. Statistic analysis of different expression genes (DEG) in transgenic plants.

(DOCX)

S4 Table. Differentially expressed genes showing similar expression profile between the 1–9 and L-5 transgenic line.

(XLS)

S5 Table. MapMan BINs enriched in the 1–9 and L-5 transgenic line using MapMan tool.

(XLS)

S6 Table. Differentially expressed genes directly involved in biostress in the 1–9 and L-5 transgenic line using MapMan tool.

(XLS)

S7 Table. Differentially expressed genes involved in hormone metabolism in the 1–9 and L-5 transgenic line using MapMan. All RNA sequencing files are available from the SRA database (accession number PRJNA576978, <https://www.ncbi.nlm.nih.gov/sra/PRJNA576978>).

(XLS)

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2018YFD1000300), the Natural Science Foundation Project of CQ (cstc2017jcyjBX0020), the Earmarked Fund for China Agriculture Research System (CARS-27 to S. Chen) and the Science and Technology Major Project of Guangxi (Gui Ke AA18118046). We thank Lesley Benyon, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Author Contributions

Formal analysis: Junhong Long, Ke Zhao, Aihong Peng, Min Chen, Qin Long, Yongrui He, Shanchun Chen.

Writing – original draft: Xiuping Zou.

References

1. Brunings AM, Gabriel DW. *Xanthomonas citri*: breaking the surface. *Molecular Plant Pathology*. 2003; 4(3):141–57. <https://doi.org/10.1046/j.1364-3703.2003.00163.x> PMID: 20569374.
2. Mendonça LBD, Zambolim L, Badel JL. Bacterial citrus diseases: Major threats and recent progress. *Journal of Bacteriology & Mycology*. 2017; 5:340–50. <https://doi.org/10.15406/jbmoa.2017.05.00143>
3. Hu Y, Zhang JL, Jia HG, Sosso D, Li T, Frommer WB, et al. *Lateral organ boundaries 1* is a disease susceptibility gene for citrus bacterial canker disease. *Proceedings of the National Academy of Sciences of*

- the United States of America. 2014; 111(4):E521–E9. <https://doi.org/10.1073/pnas.1313271111> PMID: 24474801
4. Li Z, Zou L, Ye G, Xiong L, Ji Z, Zakria M, et al. A potential disease susceptibility gene *CsLOB* of citrus is targeted by a major virulence effector PthA of *Xanthomonas citri* subsp. *citri*. *Molecular plant*. 2014; 7(5):912–5. <https://doi.org/10.1093/mp/sst176> PMID: 24398629
 5. Cernadas RA, Benedetti CE. Role of auxin and gibberellin in citrus canker development and in the transcriptional control of cell-wall remodeling genes modulated by *Xanthomonas axonopodis* pv. *citri*. *Plant Science*. 2009; 177(3):190–5. <https://doi.org/10.1016/j.plantsci.2009.05.006>
 6. Peng AH, Chen SC, Lei TG, Xu LZ, He YR, Wu L, et al. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. *Plant biotechnology journal*. 2017; 15:1509–19. <https://doi.org/10.1111/pbi.12733> PMID: 28371200
 7. Jia H, Zhang Y, Orbovic V, Xu J, White F, Jones J, et al. Genome editing of the disease susceptibility gene *CsLOB1* in citrus confers resistance to citrus canker. *Plant biotechnology journal*. 2017; 15:817–23. <https://doi.org/10.1111/pbi.12677> PMID: 27936512.
 8. Glickmann E, Gardan L, Jacquet S, Hussain S, Elasmri M, Petit A, et al. Auxin production is a common feature of most pathogens of *Pseudomonas syringae*. *Molecular Plant-Microbe Interactions*. 1998; 11(2):156–62. <https://doi.org/10.1094/MPMI.1998.11.2.156> PMID: 9450337
 9. Kazan K, Manners JM. Linking development to defense: auxin in plant–pathogen interactions. *Trends in Plant Science*. 2009; 14(7):373–82. <https://doi.org/10.1016/j.tplants.2009.04.005> PMID: 19559643
 10. Chen ZY, Agnew JL, Cohen JD, He P, Shan LB, Sheen J, et al. *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104(50):20131–6. <https://doi.org/10.1073/pnas.0704901104> PMID: 18056646
 11. Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, et al. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science*. 2006; 312(5772):436–9. <https://doi.org/10.1126/science.aae0382> PMID: 16627744.
 12. Truman WM, Bennett MH, Turnbull CGN, Grant MR. *Arabidopsis* auxin mutants are compromised in systemic acquired resistance and exhibit aberrant accumulation of various indolic compounds. *Plant physiology*. 2010; 152(3):1562–73. <https://doi.org/10.1104/pp.109.152173> PMID: 20081042
 13. Zhang Z, Li Q, Li Z, Staswick PE, Wang M, Zhu Y, et al. Dual regulation role of GH3.5 in salicylic acid and auxin signaling during *Arabidopsis-Pseudomonas syringae* interaction. *Plant physiology*. 2007; 145(2):450–64. <https://doi.org/10.1104/pp.107.106021> PMID: 17704230
 14. Wang D, Pajeroska-Mukhtar K, Culler AH, Dong XN. Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. *Current Biology*. 2007; 17(20):1784–90. <https://doi.org/10.1016/j.cub.2007.09.025> PMID: 17919906
 15. Fu J, Liu HB, Li Y, Yu HH, Li XH, Xiao JH, et al. Manipulating broad-spectrum disease resistance by suppressing pathogen-induced auxin accumulation in rice. *Plant physiology*. 2011; 155(1):589–602. <https://doi.org/10.1104/pp.110.163774> PMID: 21071600
 16. Costacurta A, Mazzafera P, Rosato YB. Indole-3-acetic acid biosynthesis by *Xanthomonas axonopodis* pv. *citri* is increased in the presence of plant leaf extracts. *FEMS Microbiology Letters*. 2010; 159(2):215–20. <https://doi.org/10.1111/j.1574-6968.1998.tb12863.x>
 17. Hagen G, Guilfoyle T. Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant molecular biology*. 2002; 49(3):373–85. <https://doi.org/10.1023/a:1015207114117>
 18. Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT, Maldonado MC, et al. Characterization of an *Arabidopsis* enzyme family that conjugates amino acids to indole-3-acetic acid. *The Plant Cell*. 2005; 17(2):616–27. <https://doi.org/10.1105/tpc.104.026690> PMID: 15659623
 19. Yuan H, Zhao K, Lei H, Shen X, Liu Y, Liao X, et al. Genome-wide analysis of the GH3 family in apple (*Malus × domestica*). *BMC Genomics*. 2013; 14(1):297. <https://doi.org/10.1186/1471-2164-14-297> PMID: 23638690
 20. Feng S, Yue R, Tao S, Yang Y, Zhang L, Xu M, et al. Genome-wide identification, expression analysis of auxin-responsive GH3 family genes in maize (*Zea mays* L.) under abiotic stresses. *Journal of Integrative Plant Biology*. 2015; 57(9):783–95. <https://doi.org/10.1111/jipb.12327> PMID: 25557253
 21. Yang Y, Yue R, Sun T, Zhang L, Chen W, Zeng H, et al. Genome-wide identification, expression analysis of GH3 family genes in *Medicago truncatula* under stress-related hormones and *Sinorhizobium meliloti* infection. *Applied Microbiology and Biotechnology*. 2015; 99(2):841–54. <https://doi.org/10.1007/s00253-014-6311-5> PMID: 25529315
 22. Nobuta K, Okrent RA, Stoutemyer M, Rodibaugh N, Kempema L, Wildermuth MC, et al. The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent defense responses in *Arabidopsis*. *Plant physiology*. 2007; 144(2):1144–56. <https://doi.org/10.1104/pp.107.097691> PMID: 17468220.

23. Westfall CS, Sherp AM, Zubieta C, Alvarez S, Schraft E, Marcellin R, et al. *Arabidopsis thaliana* GH3.5 acyl acid amido synthetase mediates metabolic crosstalk in auxin and salicylic acid homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*. 2016; 113(48):13917–22. Epub 11/14. <https://doi.org/10.1073/pnas.1612635113> PMID: 27849615.
24. Ding X, Cao Y, Huang L, Zhao J, Xu C, Li X, et al. Activation of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. *The Plant Cell*. 2008; 20(1):228–40. <https://doi.org/10.1105/tpc.107.055657> PMID: 18192436.
25. Domingo C, Andres F, Tharreau D, Iglesias DJ, Talon M. Constitutive expression of *OsGH3.1* reduces auxin content and enhances defense response and resistance to a fungal pathogen in rice. *Molecular Plant-Microbe Interactions*. 2009; 22(2):201–10. <https://doi.org/10.1094/MPMI-22-2-0201> PMID: 19132872.
26. Chen M, Yongrui HE, Lanzhen XU, Peng A, Lei T, Yao L, et al. Cloning and Expression Analysis of Citrus Genes *CsGH3.1* and *CsGH3.6* Responding to *Xanthomonas axonopodis* pv. *citri* Infection. *Horticultural Plant Journal*. 2016; 2(04):18–27. <https://doi.org/10.1016/j.hpj.2016.10.001>
27. Zou X, Jiang X, Xu L, Lei T, Peng A, He Y, et al. Transgenic citrus expressing synthesized cecropin B genes in the phloem exhibits decreased susceptibility to Huanglongbing. *Plant molecular biology*. 2017; 93:341–53. <https://doi.org/10.1007/s11103-016-0565-5> PMID: 27866312.
28. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods*. 2001; 25(4):402–8. <https://doi.org/10.1006/meth.2001.1262> PMID: 11846609
29. Wu Y, Hu B. Simultaneous determination of several phytohormones in natural coconut juice by hollow fiber-based liquid-liquid-liquid microextraction-high performance liquid chromatography. *Journal of Chromatography A*. 2009; 1216(45):7657–63. <https://doi.org/10.1016/j.chroma.2009.09.008> PMID: 19783255
30. Górká B, Wiczorek PP. Simultaneous determination of nine phytohormones in seaweed and algae extracts by HPLC-PDA. *Journal of Chromatography B*. 2017; 1057:32–9. <https://doi.org/10.1016/j.jchromb.2017.04.048> PMID: 28499204
31. Meigh DF, Norris KH, Craft CC, Lieberman M. Ethylene Production by Tomato and Apple Fruits. *Nature*. 1960; 186(4728):902–3. <https://doi.org/10.1038/186902a0>
32. Peng A, Xu L, He Y, Lei T, Yao L, Chen S, et al. 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, Tissue and Organ Culture (PCTOC)*. 2015; 123(1):1–13. <https://doi.org/10.1007/s11240-015-0799-y>
33. Kim D, Perteá G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*. 2013; 14(4):R36. <https://doi.org/10.1186/gb-2013-14-4-r36> PMID: 23618408
34. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods*. 2008; 5:621–8. <https://doi.org/10.1038/nmeth.1226> PMID: 18516045
35. Wang L, Wang X, Wang X, Zhang X, Feng Z. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics*. 2009; 26(1):136–8. <https://doi.org/10.1093/bioinformatics/btp612> PMID: 19855105
36. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology*. 2010; 11(2):R14. <https://doi.org/10.1186/gb-2010-11-2-r14> PMID: 20132535
37. Mao X, Tao CJGO, Wei L. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics*. 2005; 21(19):3787–93. <https://doi.org/10.1093/bioinformatics/bti430> PMID: 15817693
38. Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, et al. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal*. 2010; 37(6):914–39. <https://doi.org/10.1111/j.1365-3113.2004.02016.x> PMID: 14996223
39. Ludwigmüller J. Auxin conjugates: their role for plant development and in the evolution of land plants. *Journal of Experimental Botany*. 2011; 62(6):1757–73. <https://doi.org/10.1093/jxb/erq412> PMID: 21307383
40. Sherry L, Rosie T, RebekahA R, T MS, Bonnie B. Characterization of a family of IAA-amino acid conjugate hydrolases from *Arabidopsis*. *Journal of Biological Chemistry*. 2002; 277(23):20446–52. <https://doi.org/10.1074/jbc.M111955200> PMID: 11923288
41. Pereira ALA, Carazzolle MF, Abe VY, de Oliveira MLP, Domingues MN, Silva JC, et al. Identification of putative TAL effector targets of the citrus canker pathogens shows functional convergence underlying

- disease development and defense response. *BMC Genomics*. 2014; 15:157. <https://doi.org/10.1186/1471-2164-15-157> PMID: 24564253
42. Soprano AS, Abe VY, Smetana JH, Benedetti CE. Citrus MAF1, a repressor of RNA polymerase III, binds the *Xanthomonas citri* canker elicitor PthA4 and suppresses citrus canker development. *Plant physiology*. 2013; 163(1):232–42. <https://doi.org/10.1104/pp.113.224642> PMID: 23898043.
 43. Soprano AS, Giuseppe PO, Shimo HM, Lima TB, Batista FAH, Righetto GL, et al. Crystal Structure and Regulation of the Citrus Pol III Repressor MAF1 by Auxin and Phosphorylation. *Structure*. 2017; 25(9):1360–70. <https://doi.org/10.1016/j.str.2017.07.004> PMID: 28781084.
 44. Rashid A. Defense responses of plant cell wall non-catalytic proteins against pathogens. *Physiological and Molecular Plant Pathology*. 2016; 94:38–46. <https://doi.org/10.1016/j.pmp.2016.03.009>
 45. Bellincampi D, Cervone F, Lionetti V. Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. *Frontiers in plant science*. 2014; 5:228. <https://doi.org/10.3389/fpls.2014.00228> PMID: 24904623.
 46. Fendrych M, Leung J, Friml J. TIR1/AFB-Aux/IAA auxin perception mediates rapid cell wall acidification and growth of *Arabidopsis* hypocotyls. *eLife*. 2016; 5:e19048. <https://doi.org/10.7554/eLife.19048> PMID: 27627746
 47. Kunkel BN, Brooks DM. Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology*. 2002; 5(4):325–31. [https://doi.org/10.1016/s1369-5266\(02\)00275-3](https://doi.org/10.1016/s1369-5266(02)00275-3) PMID: 12179966
 48. Di X, Gomila J, Takken FLW. Involvement of salicylic acid, ethylene and jasmonic acid signalling pathways in the susceptibility of tomato to *Fusarium oxysporum*. *Molecular Plant Pathology*. 2017; 18(7):1024–35. <https://doi.org/10.1111/mpp.12559> PMID: 28390170
 49. Francis MI, Redondo A, Burns JK, Graham JH. Soil application of imidacloprid and related SAR-inducing compounds produces effective and persistent control of citrus canker. *European Journal of Plant Pathology*. 2009; 124(2):283–92. <https://doi.org/10.1007/s10658-008-9415-x>
 50. Zhongqin Z, Muyang W, Zhimiao L, Qun L, Zuhua H. *Arabidopsis* GH3.5 regulates salicylic acid-dependent and both NPR1-dependent and independent defense responses. *Plant Signaling & Behavior*. 2008; 3(8):537–42. <https://doi.org/10.4161/psb.3.8.5748> PMID: 19513247
 51. Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, et al. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97(21):11655–60. <https://doi.org/10.1073/pnas.97.21.11655> PMID: 11027363.
 52. O'Donnell PJ, Jones JB, Antoine FR, Ciardi J, Klee HJ. Ethylene-dependent salicylic acid regulates an expanded cell death response to a plant pathogen. *The Plant Journal*. 2001; 25(3):315–23. <https://doi.org/10.1046/j.1365-313x.2001.00968.x> PMID: 11208023
 53. Lawton KA, Potter SL, Uknes S, Ryals J. Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. *The Plant Cell*. 1994; 6(5):581–8. <https://doi.org/10.1105/tpc.6.5.581> PMID: 12244251