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

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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.
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 secret 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
CsGH3 regulates susceptibility to citrus canker in citrus

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 SalI/BamHI, and then independently inserted into SalI/BamHI-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 iScriptTM 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 $2^{-\Delta\Delta Ct}$ 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 × 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 ($0.5 \times 10^5$ CFU ml$^{-1}$). The inoculated leaves were maintained at 28°C under a 16-h light/8-h dark photoperiod with 45 μmol 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 (cm$^2$).

**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 μg 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 KOBASE 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
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
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-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
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.

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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 transgenic1-9 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 ($P < 0.05$).
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 down-regulated 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
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

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.

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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

### 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                              | Log2 (Fold change) | 1–9 line | L-5 line |
|-------------|-----------------------------------------------|---------------------|----------|----------|
| Cs7g08110   | UDP-glycosyltransferase (UGT) 74B1            | 2.332               | 1.632    |          |
| cs5g20420   | UDP-glycosyltransferase (UGT) 75B1            | 1.048               | ND.      |          |
| cs4g19760   | IAA amino acid conjugate hydrolase            | ND.                 | 1.878    |          |
| cs7g08080   | IAA-amino acid conjugate hydrolase            | ND.                 | 1.899    |          |

**Auxin transport**

Orange1.1/0089 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

Cs1g3970 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

Orange1.1/04221 SAUR-like auxin-responsive protein family (ARG7) -3.446 -2.909

cs1g16790 SAUR-like auxin-responsive protein family -2.084 ND.

orange1.1/02550 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

orange1.1/00464 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.

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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/E 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 Fold change*(Transgenic line vs WT)* |
|------------------|-------------------------------------------------------------|------------------------------------------|
|                  |                                                              | 1–9                                      |
|                  |                                                              | L-5                                      |
| Stress recognition | Orange1.1h02076                                             | Disease resistance-responsive family protein | -1.005              |
|                  | Orange1.1h03601                                             | LRR receptor-like kinase FLS2            | 2.755               |
|                  | Cs2g16870                                                   | MLP-like protein 31 (MLP31)              | 2.198               |
|                  | Cs2g17820                                                   | ARM repeat superfamily protein           | 1.177               |
|                  | Cs2g29780                                                   | HXXXD-type acetyl-transferase family protein | 1.436               |
|                  | Cs2g31450                                                   | Acetyl coa(Z)-3-hexen-1-ol acetyltransferase (CHAT) | -1.365              |
| Signal receptor  | Cs5g18230                                                   | Toll-Interleukin-Resistance (TIR) domain family protein | 2.044               |
|                  | Cs5g27950                                                   | Leucine-rich repeat I receptor kinase     | -2.207              |
|                  | Cs5g15420                                                   | Leucine-rich repeat VIII (VIII-2) receptor kinase | 1.262               |
|                  | Cs9g14980                                                   | Leucine-rich repeat XI receptor kinase    | -1.142              |
|                  | Orange1.1h01442                                             | Leucine-rich repeat XI receptor kinase    | -2.353              |
|                  | Orange1.1h03106                                             | Leucine-rich repeat XI receptor kinase    | 1.394               |
|                  | Cs4g0740                                                    | Serine/threonine receptor kinase         | 3.036               |
|                  | Orange1.1h01406                                             | DUF 26 receptor kinase                   | -1.357              |
| Signal transduction | Cs1g17210                                                   | JAZ1 involved in jasmonate signaling     | 1.173               |
|                  | Cs1g17220                                                   | JAZ1 involved in jasmonate signaling     | 1.143               |
|                  | Cs6g17590                                                   | Calmodulin-like protein                  | 1.321               |
|                  | Cs7g27120                                                   | Calmodulin binding protein-like          | 1.230               |
|                  | Cs5g07160                                                   | Calmodulin like 37 (CML37)              | 1.288               |
|                  | Cs2g15970                                                   | Rac-like GTP-binding protein RAC2        | -2.151              |
| Defense response gene | Cs2g231150                                                   | Mitogen-activated protein kinase kinase 18 (MAPKKK18) | 1.286               |
|                  | Orange1.1h01873                                             | Tobacco Rapid Alkalization Factor (RALF)  | -1.357              |
|                  | Cs9g05280                                                   | Arabidopsis thaliana histidine phosphotransfer protein | -1.134              |
|                  | Cs7g09390                                                   | Phototropic-responsive NPH3 family protein | -1.633              |
|                  | Cs9g07670                                                   | Chlorophyll A-B binding family protein   | -1.136              |
| Redox metabolism | Cs6g01070                                                   | NB-ARC domain-containing disease resistance protein | 2.089               |
|                  | Cs9g18740                                                   | Disease resistance family / LRR family protein defense | 2.640               |
|                  | Cs8g14950                                                   | Glucan endo-1,3-beta-glucosidase         | -1.567              |
|                  | Cs3g06300                                                   | Receptor like protein 6 (RLP6)           | -1.089              |
|                  | Cs5g19240                                                   | Disease resistance protein (TIR-NBS-LRR class) | 2.180               |
| Responsive transcription factor | Cs2g17910                                                   | Cytochrome b561/ferric reductase transmembrane protein family | -1.646              |
|                  | Cs5g32580                                                   | Thioredoxin superfamily protein          | 1.316               |
|                  | Cs2g16150                                                   | GRX480, the glutaredoxin family that regulates protein redox state | 1.436               |
|                  | Orange1.1h03455                                             | Probable glutathione S-transferase, gsts, Auxin-induced protein | -1.777              |
|                  | Cs2g15310                                                   | Peroxidase superfamily protein           | -1.918              |
|                  |                                                              |                                          | -1.385              |
| (Continued)
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 FASCICLIN-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                                      | Log₂ Fold change* (Transgenic line vs WT) |
|------------|--------------------------------------------------------|------------------------------------------|
|            |                                                        | 1–9                                      | L–5                                      |
| 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                                    |
| Orange1.1100556 | Condensing enzyme KCS1 involved in wax biosynthesis        | 1.408                                    | 2.712                                    |

*Differently expressed genes having |Log₂ (Fold change)| ≥ 1 are presented.
(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 ($P < 0.05$).

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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 repress 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) “Péra” 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)

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