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## Research article

Overexpression of a cotton cyclophilin gene (*GhCyp1*) in transgenic tobacco plants confers dual tolerance to salt stress and *Pseudomonas syringae* pv. *tabaci* infectionChuanfeng Zhu<sup>1</sup>, Yixue Wang<sup>1</sup>, Yuanbao Li, Khizar Hayat Bhatti, Yingchuan Tian, Jiahe Wu\*

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## ABSTRACT

The full-length cDNA of a cyclophilin-like gene was cloned from *Gossypium hirsutum* using rapid amplification of cDNA ends and was designated as *GhCyp1*, a member of the immunophilin protein family. *GhCyp1* expression level was higher in roots and stems than in other tissues of cotton, as determined by real-time reverse transcription polymerase chain reaction (RT-PCR). To characterize the *GhCyp1* gene, tobacco (*Nicotiana tabacum*) was transformed via *Agrobacterium tumefaciens* with a vector to express the gene under the control of a strong constitutive promoter, CaMV35S (Cauliflower Mosaic Virus). Based on analyses of tolerance to salinity stress and *Pseudomonas syringae* pv. *tabaci* (*Pst*) infection, the overexpression of *GhCyp1* in transgenic plants conferred higher tolerance to salt stress and *Pst* infection compared with control plants. Therefore, we suggest that *GhCyp1* may be a suitable candidate gene to produce transgenic plants with tolerance to abiotic and biotic stresses.

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## 1. Introduction

Cyclophilins (Cyps) constitute a family of ubiquitous proteins found in Archaea, Bacteria, and Eukarya domains [1]. They possess peptidyl-prolyl isomerase activity (PPIase, EC: 5.2.1.8), which catalyzes the *cis/trans* isomerization of peptide bonds at proline residues and protein folding [2]. Human Cyp-A (hCypA) was initially identified as a receptor for the immunosuppressive drug, cyclosporine A (CsA) [3], and also for its peptidyl-prolyl *cis-trans* isomerization activity [4]. Cyps are broadly classified into single-domain (SD) and multiple-domain (MD) protein families based on amino acid sequences. SD Cyps contain only the Cyp-catalytic domain, while MD Cyps have numerous additional domains, such as the tetratricopeptide, WD40 repeat, RNA recognition motif, and nuclear localization signal, in addition to the catalytic domain [5]. Additionally, conserved amino acid residues important for PPIase and CsA binding activity have been reported [6].

Cyps are frequently found in cellular compartments of diverse tissues and perform numerous functions. For example, in animals, Cyps were identified as cellular targets for immunosuppressant drugs to block T-cell activation and inhibit the activity of calcineurin [5]. In fungi, Cyps act as virulence determinants [7,8], and in

plants, Cyps have been reported to regulate various processes, including signaling [2,9,10], transcription regulation [11], pre-mRNA splicing [12], and cell division [13].

The first plant Cyp was identified in a tomato [14]. Thereafter, numerous Cyps have been identified and characterized in other plants, such as *Arabidopsis*, bean (*Glycine max*), maize (*Zea mays*), rice (*Oryza sativa*), and many others [5,15,16]. To date, as many as 29 Cyp-like proteins have been reported for *Arabidopsis*, and are predicted to localize in the cytosol, nucleus, secretory pathway, endoplasmic reticulum, chloroplast, and mitochondria [5]. Differential expression patterns of *cyp* genes are dependent on tissue type and developmental phases, therefore, their expression is thought to be developmentally regulated [17].

Expression levels of plant *cyp* genes have also been demonstrated to be induced in response to various stresses, including heat shock, low temperature, salt stress, light, wounding, chemical elicitors, and pathogens [15]. The mechanism of the Cyp response to abiotic and biotic stresses has not yet been fully elucidated, although some reports have suggested some molecular mechanisms. Molecular chaperones and catalytic isomerases, in particular, protein disulphide isomerases and PPIases, are present in diverse organisms and act by assisting the folding and assembly of newly synthesized proteins [16,18–20]. Cyps have been implicated in diverse cellular mechanisms such as signaling [21], transfer of reducing power [22], and preservation of protein structure [16]. Thus, these studies suggest that Cyps play important roles in adaptation to various stressful environments in plants.

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Cotton is one of the most economically important crops and has been cultivated worldwide for centuries. Environmental factors that occasionally affect the quantity and the quality of cotton fiber include salinity, ultraviolet radiation, drought, temperature extremes, mineral deficiency, herbicides, air pollution, heavy metals, and attack by pathogens. Recent studies reported that Cyp proteins in rice, tobacco, and yeast play a very important role in increasing tolerance to salt stress [15,17] and infection by pathogens [10,23]. Although expression studies using *cyp* genes have been carried out in saline habitats for some plants, expression profiles of the *cyp* genes and their physiological impact on cotton under salt stress have not been reported. To determine salt stress-regulated genes, we employed a suppression subtractive hybridization (SSH) technique, which isolated 500 expressed sequence tags (ESTs). Among these, one (designated as *GhCyp1*) was found to share high homology with hCypA. The full-length cDNA of *GhCyp1* was cloned by employing 5'- and 3'-RACE (rapid amplification of cDNA ends) and was introduced into tobacco cells. The physiological role of *GhCyp1* in transgenic tobacco plants under high salinity and fire-blight pathogenic (*Pseudomonas syringae* pv. *tabaci*, *Pst*) stress was investigated in this study.

## 2. Results

### 2.1. Isolation and characterization of *GhCyp1* in cotton

To identify and isolate salt stress-regulated genes, we employed an SSH-PCR (polymerase chain reaction) technique in cotton. As many as 500 clones were obtained and sequenced, and one clone was found to be highly homologous to hCypA, and referred to as *GhCyp1*. Thus, the full-length *GhCyp1* was cloned using previously identified clone sequences from SSH and reverse transcription (RT)-PCR. Consequently, the cDNA fragment was obtained and extended using both the 5'- and 3'-RACE techniques. The cDNA sequence of the cloned gene was confirmed by sequence analysis. The *GhCyp1* sequence was submitted to the GenBank database (GenBank accession number: GQ292530.1). The resultant full-length cDNA clone contained a 788 bp gene fragment with an open reading frame (ORF) of 522 bp that putatively encodes a protein of 173 amino acids with a predicted molecular weight of 18.15 kDa and isoelectric point of 8.76.

The current study employed the BLASTp search engine to determine similarities between the query sequence and those found in the NCBI nonredundant protein sequences. There are many Cyps in the plant kingdom; therefore, we focused on homologies with BLAST scores of >260. Further homology analyses indicated that some members of the SD Cyps from *Arabidopsis thaliana*, *O. sativa*, and *Homo sapiens*, specifically, AtROC3 (NP\_179251.1), *A. thaliana*; AtCyp2 (NP\_179709.1), *A. thaliana*; AtROC1 (NP\_195585.1), *A. thaliana*; AtROC5 (NP\_195213.1), *A. thaliana*; OsCyp2 (NP\_001063993.1), *O. sativa*; AtROC2 (NP\_191166.1), *A. thaliana*; hCypA (NP\_066953.1), *H. sapiens*, share high homology with *GhCyp1*. Sequence analysis of the *GhCyp1* protein revealed that it contained a stretch of 11 amino acids typical of SD Cyps found in plants, indicating a close relationship to the SD Cyp cluster [1] (Fig. 1A). Sequence alignment analysis was conducted to investigate the relationship between *GhCyp1* and other members of the SD Cyps (Fig. 1A). This indicated that a Csa binding-site (W) and three amino acids (R, F, and H) required for PPlase catalysis were conserved in different Cyps, including *GhCyp1* [24]. The *GhCyp1* protein shares 75–86% amino acid identity with five *Arabidopsis* Cyps, AtCyp2 (NP\_179709.1), AtROC5 (NP\_195213.1), AtROC3 (NP\_179251.1), AtROC1 (NP\_195585.1), and AtROC2 (NP\_191166.1); 74% with rice-Cyps, OsCyp2 (NP\_001063993.1); and 70% with hCypA (NP\_066953.1) (Fig. 1B). These findings suggest that *GhCyp1* is more

closely related to Cyps of the dicotyledonous plant *Arabidopsis* compared with the monocotyledonous plant rice.

Tissue-specific expression levels of *GhCyp1* in different tissues of cotton plants were determined by real-time RT-PCR. Expression of *GhCyp1* was found in all tissues of cotton plants, namely roots, stems, leaves, cotyledons, petals, anthers, and developing fibers (Fig. 2). A relatively higher level of *GhCyp1* expression was found in the roots, stems, and fibers at 12 days post-anthesis (DPA) than in other tissues, which suggests that these tissues are the typical sites of *GhCyp1* action.

### 2.2. Molecular characterization of transgenic tobacco plants

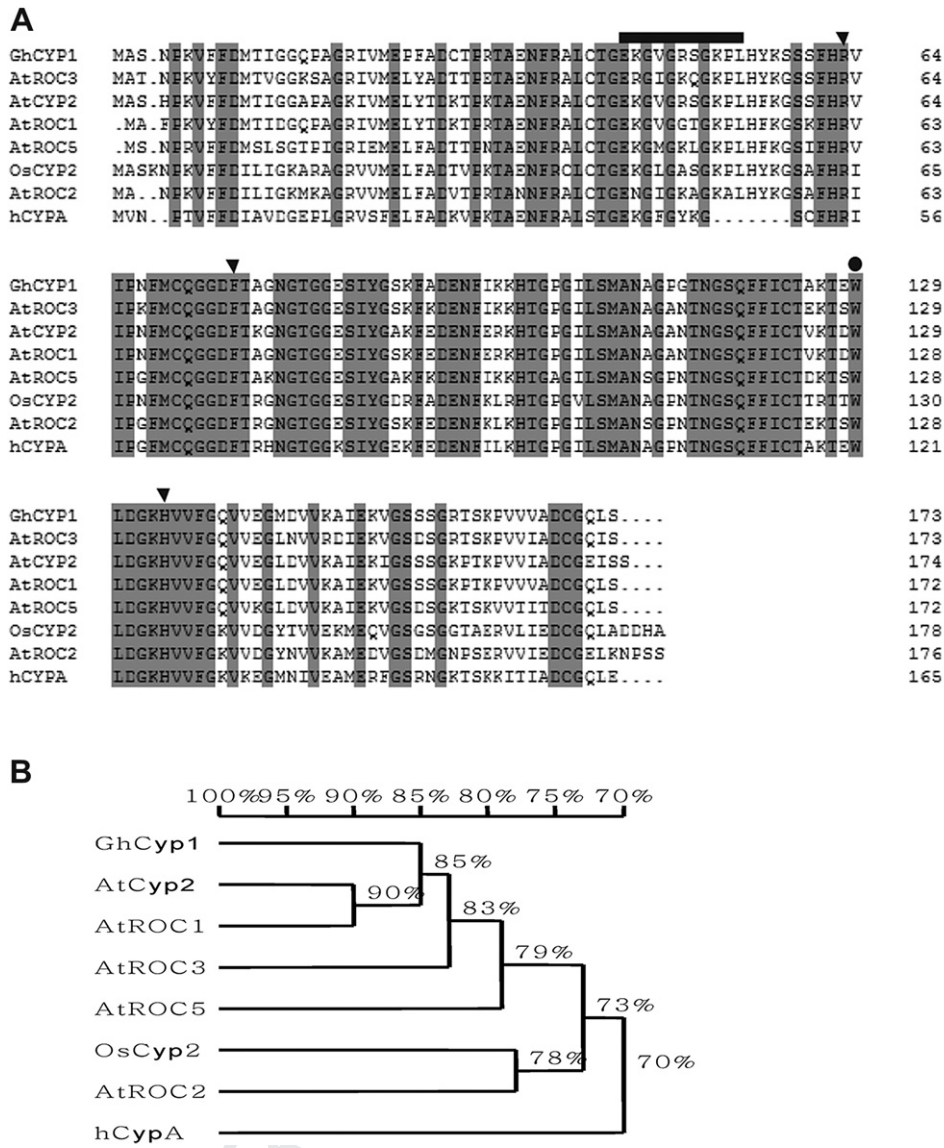
As many as 54 independent kanamycin resistant tobacco transformants were screened by PCR for the presence of the *GhCyp1* sequence. A 538-bp PCR amplicon corresponding to the *GhCyp1* PCR product size from transgenic tobacco DNA was obtained, but no such product was amplified from nontransformed (NT) control plants (Fig. 3A). Expression of *GhCyp1* mRNA was also detected in 38 of 54 transgenic tobacco events, but no such signal was detected for NT control plants (Fig. 3B). These results indicate that *GhCyp1* was integrated into transgenic tobacco events and the transcript was expressed at least at the transcriptional level.

### 2.3. Overexpression of *GhCyp1* in transgenic tobacco increased tolerance to salt stress

The expression of *cyp* genes in many plants has been shown to be upregulated when subjected to various stress stimuli, such as salinity, temperature extremes, light, wounding, some phytohormones, and fungal infection [4]. In this study, to evaluate salt tolerance, the three transgenic tobacco events (referred to as OE2, OE6, and OE7), with abundant transcripts of *GhCyp1* and no obvious phenotypic changes, were selected as representatives and subjected to salt stress assays. The phenotype of the transgenic and the NT plants was similar on normal 1/2 MS medium (Fig. 4A); however, the growth of NT plants was strongly inhibited on the medium containing 150 mM NaCl (Fig. 4A). Specifically, NT control plants exhibited dwarfism and chlorosis under salt stress, while the transgenic *GhCyp1* plants showed no such signs (Fig. 4A). Based on the measure of total chlorophyll content of leaves, the chlorophyll content of the three transgenic plants was significantly higher ( $P < 0.01$ ) than that of NT controls (at 1.61, 1.56, and 1.49 mg/g fresh leaf weight [FW] in OE2, OE6, and OE7, respectively, while at 0.40 mg/g FW in the NT control), although chlorophyll content decreased for all plants upon salt stress compared with those not under salt stress (Fig. 4B). These results imply that overexpression of *GhCyp1* confers higher tolerance to salt stress in transgenic plants.

Membrane integrity is generally disrupted in plants under salinity stress. Thus, to evaluate the membrane integrity of transgenic tobacco plants under salt stress, the leakage of cytoplasmic solutes from leaf discs was measured. A significant difference ( $P < 0.05$ ) in the relative electrolyte leakage was observed between transgenic plants and NT control plants treated with salt stress as well as untreated controls, although the relative electrolyte leakage increased in both transgenic and NT plants under salinity stress (Fig. 4C). The lack of difference between treatment and control plants was hypothesized to be due to wounding stress by cutting. To avoid the disruption of wounding stress, whole leaves of transgenic and NT plants treated with salt as well as controls were used to elucidate membrane integrity. Transgenic plants overexpressing *GhCyp1* were shown to maintain membrane stability at <150 mM NaCl treatment, and electrolyte leakages were similar to untreated controls. However, the electrolyte leakage of NT plants treated with

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**Fig. 1.** Alignment of the predicted Cyp protein sequences and phylogenetic tree. (A) Alignment of the deduced amino acid sequences of the GhCyp1 protein and other selected cytosolic Cyps. Identical and similar amino acid residues are shaded black, and dashes indicate the gaps to optimize alignment. Black triangles, necessary amino acids required for PPIase catalysis; black circles, amino acids necessary for CsA binding. A stretch of 11 amino acids, typical of single-domain Cyps in plant, is shown with the black bar. (B) Phylogenetic tree of GhCyp1 and other selected cytosolic Cyps.

salt was significantly higher than plants not treated with salt. These findings suggest that the higher membrane stability in transgenic plants was responsible for the higher tolerance to salinity stress compared with the NT control.

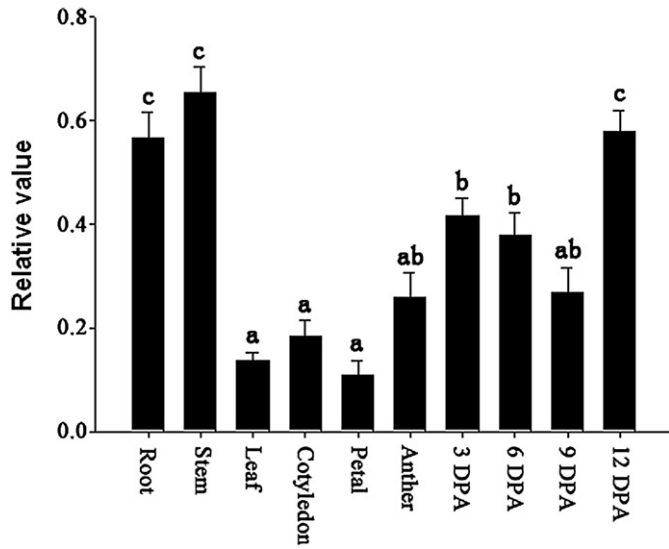
#### 2.4. Overexpression of GhCyp1 in transgenic tobacco increased tolerance to *Pst*

To elucidate the function of GhCyp1 protein in tolerance to infection by pathogens, transgenic tobacco plants and the NT control were challenged with *Pst*, the cause of fire-blight disease. The leaves of NT plants and three transgenic tobacco events, OE2, OE6, and OE7, as the representatives were inoculated with *Pst* at  $10^6$  cfu/mL of inocula. Typical chlorosis was observed at the site of leaf inoculation after 36 h of *Pst* infection at  $10^6$  cfu/mL inocula and eventually developed into severe necrotic lesion(s) at 7 days post-inoculation (DPI) in NT plants (Fig. 5A). The development of chlorosis was remarkably delayed, and necrotic lesions were still slight

at 7 DPI in the three transgenic events compared with the NT control (Fig. 5A). However, chlorosis also developed into severe necrotic lesions up to 10 DPI in the three transgenic events, as well as those in NT control (data not shown). These data suggest that GhCyp1 can delay necrotic lesion development in transgenic plants.

To further elucidate the tolerance of transgenic plants to *Pst*, low levels of inocula were used. Transgenic plants OE2, OE6, and OE7 were shown to have only trace or no necrotic lesions at 7 DPI at  $10^3$  cfu/mL inocula, although obvious necrotic lesions developed in the NT plants (Fig. 5B). At 10 DPI, no necrotic lesions were observed in transgenic plants.

Based on the data of infection of plants with *Pst* at  $10^6$  and  $10^3$  cfu/mL inocula, the development of necrotic lesions was retarded or prevented, likely owing to reduced growth of *Pst* in the infected tissues in the three transgenic events. Our data show that there was no difference in bacterial growth between transgenic event OE6 and NT plants at 0 DPI at  $10^6$  cfu/mL inocula. However, the growth rate of *Pst* was relatively low at 2 DPI in transgenic

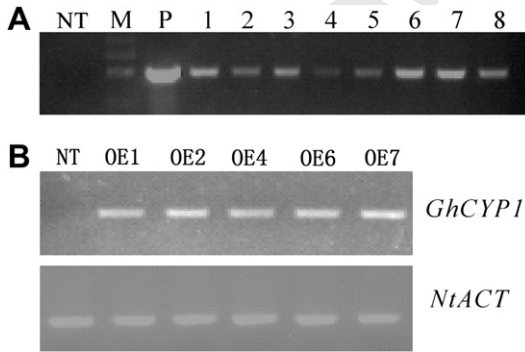


**Fig. 2.** Real-time RT-PCR analysis of expression profiles of the *GhCyp1* gene in cotton tissues. Relative value of *GhCyp1* gene expression in cotton tissues was shown as rate of *GhUBI* expression activity. Error bars represent standard deviation for three replicates. *GhUBI1* was used as an internal control for amplification. The data on transcript level of *GhCyp1* in the different tissues had been analyzed by ANOVA. Means with standard deviation were separated using Duncan's multiple comparison test at  $P < 0.05$ , and means labeled with different letters were of significant difference at  $P < 0.05$ .

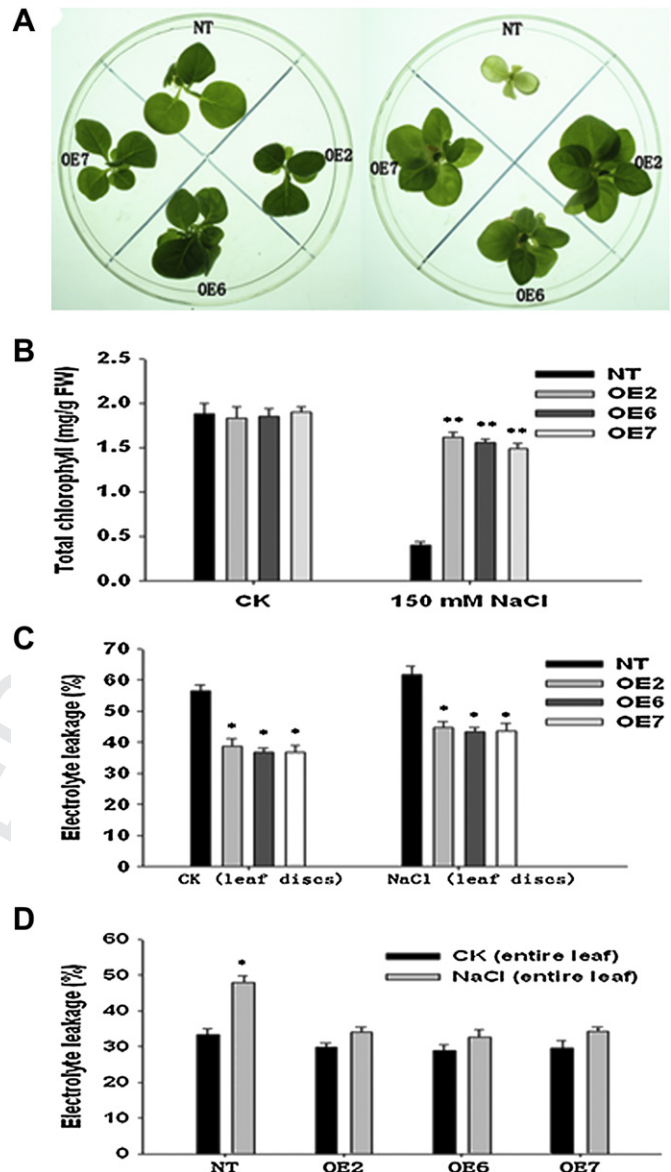
events than in the control. Furthermore, at 4 DPI, growth declined 15-fold in the transgenic events compared with the control (Fig. 5B). Similar results were obtained from the highly over-expressing *GhCyp1* transgenic events, OE2 and OE7. The growth of *Pst* in the transgenic plant infected at  $10^3$  cfu/mL inoculum had stopped at 2 DPI, and was not detected at 4 DPI; however, the number of bacteria continuously increased in NT plants (data not shown).

### 3. Discussion

Our results are consistent with studies on other plant species. For example, ectopic expression of *ThCyp1* was able to confer salt



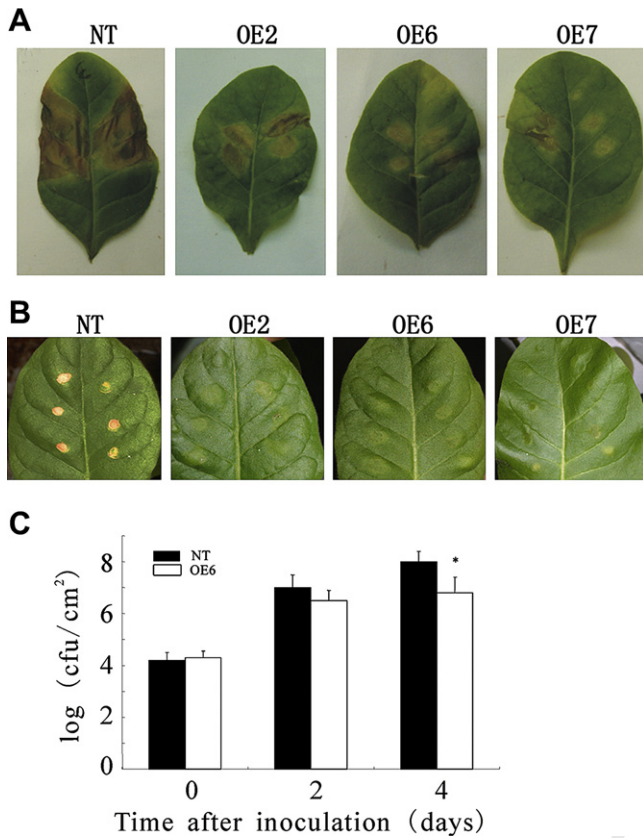
**Fig. 3.** Molecular characterization of transgenic tobacco events. (A) PCR analysis of *GhCyp1* in transgenic tobacco events. Genomic DNA was extracted from leaves of tobacco transformants and NT plants. Suitable primer pairs were designed from sequence of *GhCyp1* to amplify the desired PCR product. Lane NT, nontransformed tobacco plants; lane M, DNA Marker DL2000; lane P, pBI121-*GhCyp1* plasmid control; lanes 1–8, representative transgenic tobacco events. (B) Analysis of overexpression *GhCyp1* in five representative transgenic tobacco events using semi-quantitative RT-PCR. *NtACT* was used as a control for normalization (22 cycles for RT-PCR). Lane NT, nontransformed tobacco plants; lanes OE1, OE2, OE4, OE6, and OE7, randomly selected transgenic tobacco events (28 cycles for RT-PCR).



**Fig. 4.** Improvement of salt tolerance in the transgenic tobacco plants with over-expression of *GhCyp1*. (A) Effect of salt stress on growth of *GhCyp1* transgenic and control plants. Growth of transgenic tobacco events and NT plants on 1/2 MS medium without salt stress (left) and with 150 mM NaCl (right), respectively (B, C and D). Effect of salt stress on total chlorophyll content (B), relative electrolyte leakage of leaf discs (C) and relative electrolyte leakage of whole leaves (D). Data represent the mean  $\pm$  SD of three independent experiments ( $n = 12$ ). Student's *t*-test was used for the comparison of two means, transgenic event and NT control, in (B) and (C), and treated and untreated with 150 mM NaCl in (D). \* $P < 0.05$  level, \*\* $P < 0.01$  level.

tolerance in fission yeast (*Schizosaccharomyces pombe*) and tobacco BY-2 cells [17]. Increased tolerance to salinity was also reported in yeast (*Saccharomyces cerevisiae*) and *Escherichia coli*, which were transformed with the *OsCyp2* gene [15]. Moreover, abundant transcript expression was observed in most tissues of *Pokkali* (salt-tolerant cultivar) than that in *IR64* (salt-sensitive cultivar) rice [15]. These data support the finding that overexpression of *cyp* genes may impart enhanced tolerance to salt stress in transgenic plants. The *GhCyp1* gene could also be considered a suitable candidate gene for introducing tolerance to salinity stress in transgenic plants similar to studies of *OsCyp2* [15].

The underlying molecular mechanism for salt tolerance by Cyps, however, remains largely unknown. Some Cyps have been found to



**Fig. 5.** Improvement of disease tolerance in the transgenic tobacco plants with overexpression of *GhCyp1*. Representative disease symptoms in NT and selected transgenic tobacco plants inoculated with *Pst* at  $10^6$  cfu/mL inoculum (A) and at  $10^3$  cfu/mL inoculum (B). Fully expanded tobacco leaves were inoculated with *Pst* at two different levels of inocula. The experiment was repeated three times. Photos were taken on 7 DPI (days post-inoculation). (C) Quantification of *Pst* from infected tobacco leaves. Leaf discs were collected at different time-points after inoculation. Values are the mean  $\pm$  SD of three replicates ( $n = 8$ ), and  $^*P < 0.05$  for Student's *t*-test for comparison of the means of the transgenic event (OE6) and NT control.

possess PPIase and a chaperone-like activity, which are capable of refolding proteins and protecting them from degradation and aggregation [25]. A number of predicted cytosol-localized Cyps were shown to shuttle and target the nucleus by escalating interactions with nucleic acids [26]. Meanwhile, some Cyps were shown to be localized in the nucleus and to interact with transcription factors, as well as RNA polymerases, to control gene expression, a property that may help plants grow successfully under saline conditions [11,27,28]. Although a preliminary understanding of the mechanism for induction of stress tolerance in plants through Cyps was developed, further studies are necessary to establish their physiological role in various stresses.

In this study, the membrane integrity of transgenic tobacco plants was significantly improved compared with NT plants based on the results of relative electrolyte leakage of whole leaves, which indicated that the *GhCyp1* protein can play an important role in maintaining membrane protein stability, especially under stress. However, based on the measure of the relative electrolyte leakage of leaf discs, we found that the membrane integrity of transgenic plants was higher under wounding stress compared with NT plants, which suggested that the transgenic plants with overexpression of *GhCyp1* can also enhance tolerance to wounding stress.

The transcription of *cyp* genes was induced by infection with pathogens, which indicated that the Cyp protein can play an important role in tolerance to pathogens in plants [16,28,29]. In this

study, on the basis of slower bacterial growth and lessened necrotic lesion symptoms on the leaves during *Pst* infection in the transgenic tobacco compared with those in the NT control, we speculate that *GhCyp1* may have a protective role in retarding bacterial growth and reducing pathogen-induced damage. These data suggest that overexpression of *GhCyp1* may increase tolerance against *Pst* infection in transgenic tobacco plants, as previously demonstrated in other plant species, such as maize [30,31], beans [16], potatoes [29], *Arabidopsis* [32], and peppers [28].

The mechanism of the protective response to biotic stress has not been determined, although some reports have discovered various functions of Cyps in plant defense, where some Cyps have been implicated in mediating apoptotic DNA degradation [33]. Additionally, in *Arabidopsis*, the Cyp ROC1 was shown to activate the bacterial effector, AvrRpt2, through its PPIase activity. ROC1 induced the self-cleavage of AvrRpt2, triggering limited cleavage of *A. thaliana* RIN4 in plant cells to initiate RPS2-mediated resistance [23,34]. However, *GhCyp1* is not likely to increase resistance to pathogens by activating bacterial effectors, since overexpression of *GhCyp1* can reduce bacterial growth and reduce necrotic lesions in transgenic plants inoculated with either high or low levels of inocula. This resulted in increased tolerance to *Pst*, but was not likely to be the result of effector-triggered immunity. Instead, the increased tolerance of transgenic plants overexpressing *GhCyp1* to *Pst* is similar to plants that display characteristic systematic acquired resistance. Interestingly, infection by pathogens and/or treatment with SA,  $H_2O_2$ , and wounding have all been reported to induce expression of *cyp* genes [16,28,29], therefore suggesting a role for Cyps in the response of plants to pathogen attack. It has been shown that Cyp expression either leads to hastened folding and assembly of nascent proteins under stressful conditions or can act as chaperone-like molecules to protect other proteins from degradation and/or aggregation during pathogenic infection in plants [16,20].

Based on data presented for the *GhCyp1* protein conferring tolerance to salt stress and fire-blight disease in the transgenic tobacco, *GhCyp1* may be a suitable candidate gene for the development of transgenic plants to confer increased tolerance to abiotic and biotic stresses.

## 4. Materials and methods

### 4.1. Plant materials and growth conditions

Seeds of cotton (*Gossypium hirsutum* cv. Zhongmian 35) were surface-sterilized with 70% ethanol for 30 s and 10% NaOCl for 10 min and washed three times with sterile double-distilled water (ddH<sub>2</sub>O). Seeds were sown in plastic pots (10 cm diameter) containing sterilized wet soil, and seedlings were grown at 28 °C under a 12 h photoperiod to maturation in a greenhouse. Samples from developing fibers (3, 6, 9, and 12 DPA), anthers (–1 DPA), petals (0 DPA), leaves (25 d post-germination), cotyledons (5 d post-germination), stems (25 d post-germination), and roots (25 d post-germination) were harvested and immediately frozen in liquid nitrogen and stored at –80 °C for RNA isolation and RT-PCR.

### 4.2. Cloning of full-length *GhCyp1* cDNA

A subtractive cDNA library of cotton plants treated and untreated with salt stress was constructed by subtractive hybridization, according to methods proposed by Mathews et al. [35]. Partial ESTs of *GhCyp1* were obtained through SSH and the primer pair GR1: 5'-cggatttgatggagccttc-3' and GR2: 5'-gaactccgctccctctgta-3' was used to PCR to confirm the presence of *GhCyp1* sequence under salt induction. However, for rapid amplification of cDNA 5'- and 3'-ends,

5'-Full RACE (Takara Biotechnology, Dalian, China), and 3'-Full RACE Core Set (Ver. 2.0; Takara Biotechnology, Dalian, China) reactions were employed to extend *GhCyp1* cDNA sequences, according to the manufacturer's instructions. The amplicons were cloned into pGEM-T Easy vector (Promega, Madison, WI) and the nucleotide sequence was confirmed. Primers used were synthesized by Beijing Sunbio (Beijing, China). Sequences of the selected nucleotides and deduced amino acids were analyzed using DNAMAN (ver. 5.2; Lynnon Bio-Soft, Quebec, Canada).

#### 4.3. Phylogenetic analysis

BLASTp (Basic Local Alignment Search Tool) (<http://blast.ncbi.nlm.nih.gov/>) was used to search for homologies in the non-redundant protein sequence in NCBI databank to the *GhCyp1* putative sequence. The corresponding protein sequences were aligned and a phylogenetic tree was constructed using DNAMAN 5.2. CDART (Conserved Domain Architectural Retrieval Tool; <http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) was used to identify and retrieve conserved domain sequences. The following sequences from GenBank were used in multiple-sequence alignment analysis: *GhCyp1* (GQ292530.1), *G. hirsutum*; AtROC3 (NP\_179251.1), *A. thaliana*; AtCyp2 (NP\_179709.1), *A. thaliana*; AtROC1 (NP\_195585.1), *A. thaliana*; AtROC5 (NP\_195213.1), *A. thaliana*; OsCyp2 (NP\_001063993.1), *O. sativa*; AtROC2 (NP\_191166.1), *A. thaliana*; hCypA (NP\_066953.1), *H. sapiens*.

#### 4.4. RNA preparation and transcriptional analysis in cotton

Total RNA of high quality was isolated from cotton plant organs and tissues using Trizol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Promega, Madison, WI). Using the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA), cDNA was synthesized from 2 µg of the total RNA. Expression of the *GhCyp1* gene in cotton tissues was analyzed by RT-PCR using the fluorescent intercalating dye SYBR-Green in a LightCycler detection system (Roche, Indianapolis, IN).

The cDNA (1 µL) was amplified and its expression was monitored using a primer pair located in the 3'-UTR, Cyp-utrF: 5'-gtgttaagctgggatgtgtc-3' and Cyp-utrR: 5'-gaaaaccaccaacc-cagtg-3', and the size of amplicon was 104 bp. The fragments of cotton *GhUBI1* (GenBank accession number: EU604080) were amplified from the cDNA as an internal control to normalize the expression and quantity of cDNA, using a primer pair UBI-F: 5'-gagacgtagttagaaggaag-3' and UBI-R: 5'-agtacgttccattccggaac-3'. ddH<sub>2</sub>O was used as the control.

Real-time PCR was performed using Light Cycler–FastStart DNA Master SYBR-Green I kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. The Ct (cycle threshold), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, was used as a measure of the starting copy number of the target genes. Relative quantities of the target *GhCyp1* expression level were performed using the comparative Ct method (Roche LightCycler System). PCR products were confirmed by agarose gel electrophoresis.

Relative values of *GhCyp1* gene expression in cotton tissues were shown as relative to *GhUBI* expression activity. Error bars represent standard deviations for three biological replicates. The data for the transcript levels of *GhCyp1* in different tissues were analyzed by one-tailed ANOVA (analysis of variance). Means with standard deviations were separated using Duncan's multiple comparison test at  $P < 0.05$ , and means labeled with different letters were significantly different at  $P < 0.05$ .

#### 4.5. Vector construction and *Agrobacterium*-mediated tobacco transformation

The cDNA (1 µL) obtained from the SuperScript™ First-Strand Synthesis System was employed and the ORF of *GhCyp1* was amplified by PCR using the primers pair Cyp-2F: 5'-ggatccgccac-catggcttcaaatccaagg-3' and Cyp-2R: 5'-gagctcctaagagagctgtccgcag-3', and *Bam*HI and *Sac*I restriction sites (underlined) were introduced. After denaturing the DNA at 94 °C for 5 min, PCR was performed for 30 cycles followed by 94 °C, 30 s; 56 °C, 30 s; and 72 °C, 5 min. *GhCYP1* was introduced into the *Bam*HI/*Sac*I restriction site of pBI121 (Clontech Laboratories, CA) to generate a *CaMV35S::GhCyp1* plant transformation construct. Tobacco (*Nicotiana tabacum* cv. SR1) was transformed using *Agrobacterium tumefaciens* (LBA4404) harboring the *GhCyp1* transgene, according to a previously described method [36]. *GhCyp1*-transgenic T<sub>0</sub> seedlings were transferred to greenhouse conditions in soil for seed setting. T<sub>1</sub> progeny seedlings from T<sub>0</sub> transformant seeds were selected on 1/2 MS medium containing kanamycin (200 mg/L), and green plantlets were transferred to soil. The T<sub>2</sub> transgenic and control plants grown under greenhouse conditions were used for the disease-tolerance assay.

#### 4.6. PCR detection and transcription level of *GhCyp1* of transgenic tobacco plants

High-quality genomic DNA was isolated from leaves of putatively transformed T<sub>0</sub> and T<sub>1</sub> independent tobacco plants [37]. PCR was carried out using 20 ng of genomic DNA as template with primers Cyp-1F: 5'-tggcttcaaatccaagg-3' and Cyp-1R: 5'-ccggtgtgcttcttgatga-3' to amplify the transgene fragment. Analysis of the amplicons was performed by agarose gel electrophoresis and visualized using ethidium bromide.

The RNA isolation and cDNA synthesis from the leaves of transgenic tobacco and NT plants were performed as described for cotton (Section 4.4). The cDNA (1 µL) was amplified and *GhCyp1* expression was monitored using the primer pair Cyp-1F: 5'-tggcttcaaatccaagg-3' and Cyp-1R: 5'-ccggtgtgcttcttgatga-3'. The tobacco *NtACT actin* (GenBank accession number: GQ281246) gene was amplified from the cDNA as a control to normalize the expression and quantity of cDNA using the *NtACT* primer pair ACT-F: 5'-caatgaactctgtgtgctcc-3' and ACT-R: 5'-cggaatctctcag-caccaatg-3'.

#### 4.7. Analysis of tolerance to salt stress in transgenic tobacco events

Seeds of NT plants and three transgenic events (OE2, OE6, and OE7) with abundant expression of *GhCyp1* and no obvious phenotypic changes were surface-sterilized with 70% ethanol for 30 s, 10% NaOCl for 5 min, and washed three times with sterile ddH<sub>2</sub>O. The sterilized seeds were grown on 1/2 MS medium containing 3% sucrose and 2.5 g/L Phytogel with selectable antibiotic kanamycin (200 mg/L). On appearance of leaves, the seedlings were transferred to 1/2 solid medium with or without 150 mM NaCl and maintained at 28–30 °C under 16/8 h light/dark cycle greenhouse conditions. Thereafter, photos of the treated and control plants were taken at 10 days post-salt stress. The experiments were essentially repeated three times with similar findings.

#### 4.8. Leaf total chlorophyll content assay

The transgenic and NT seedlings transferred on medium with 0 and 150 mM NaCl were used in this experiment. The total leaf chlorophyll content was measured after 10 days of subjecting the plants to salt stress. The chlorophyll content was determined

according to a method proposed by Tang et al. [38]. Three replicates were sampled for this test.

#### 4.9. Relative electrolyte leakage (REL) assay in transgenic tobacco and NT control plants

The measure of REL was performed according to a method proposed by Yang et al. [39]. Twelve leaf discs (1 cm diameter) obtained from the transgenic tobacco and NT control plants, cultured on media with 0 and 150 mM NaCl for 10 days by stiletto, were immersed in a falcon tube with 10 mL ddH<sub>2</sub>O, placed in a vacuum for 30 min, and surged for 3 h to measure initial electronic conductance (E<sub>i</sub>) using a conductivity meter (Model DDS-307, USA). The tubes were then transferred into a 95 °C water bath and incubated for 30 min to determine the final electronic conductance (E<sub>f</sub>). Three replicates were performed for this test. REL was calculated according to the following formula:

$$REL(\%) = E_i/E_f \times 100$$

The electrolyte leakage of the whole leaf (three leaves, nearly 40 cm<sup>2</sup> per sample) was vacuumed for 1 h and surged for 24 h to measure initial electronic conductance, and then E<sub>f</sub> was tested and REL was calculated according to methods described above.

#### 4.10. Analysis of tolerance to *Pst* infection in transgenic tobacco events

Transgenic T<sub>2</sub> and NT control seedlings were transferred into plastic pots containing loamy soil and farm yard manure (3:1 ratio) and kept at 28 °C and grown under a 12 h photoperiod for 1 month in a greenhouse, for disease-tolerance assays. *Pst* bacteria were cultured on solid KMB (King's Medium B) supplemented with 20 mg/L rifamycin and kept overnight. Moreover, these were made to grow on KMB for 24 h, harvested and washed twice with sterile distilled water, and then resuspended in 10 mM MgCl<sub>2</sub> solution. The inoculum concentration was determined by spectrophotometer and adjusted to 0.1–0.2 at OD<sub>600</sub>. Two solutions of different bacterial concentrations, at 10<sup>6</sup> and 10<sup>3</sup> cfu/mL, were infiltrated into fully expanded tobacco leaves using a 5 mL needle-less plastic syringe [40]. The bacterial population was determined in the tobacco leaf tissues infected at 10<sup>6</sup> cfu/mL inoculum, according to a method proposed by Bhatt et al. [41]. These experiments were repeated three times.

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