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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* infection

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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.

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 PPIase 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