Electronic Journal of Biotechnology 18 (2015) 368–375

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Electronic Journal of Biotechnology

Overexpression of a cysteine proteinase inhibitor gene from *Jatropha curcas* confers enhanced tolerance to salinity stress

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ARTICLE INFO

Article history:
Received 31 March 2015
Accepted 12 June 2015
Available online 2 September 2015

Keywords:
Cysteine proteinase inhibitor
Jatropha curcas
Salinity stress

ABSTRACT

Background: Cysteine proteinase inhibitor (cystatin, CPI) is one of the most important molecules involved in plant development and defense, especially in the regulation of stress responses. However, it is still unclear whether the *Jatropha curcas* CPI (*JcCPI*) gene functions in salinity response and tolerance. In this study, the sequence of the *JcCPI* gene, its expression pattern, and the effects of overexpression in *Escherichia coli* and *Nicotiana benthamiana* were examined. The purpose of this study was to evaluate the regulatory role of *JcCPI* in salinity stress tolerance.
Results: The CPI gene, designated *JcCPI*, was cloned from *J. curcas*; its sequence shared conserved domains with other plant cystatins. Based on a transcription pattern analysis, *JcCPI* was expressed in all tissues examined, but its expression was highest in the petiole. Additionally, the expression of *JcCPI* was induced by salinity stress. A potential role of *JcCPI* was detected in transgenic *E. coli*, which exhibited strong CPI activity and high salinity tolerance. *JcCPI* was also transferred to tobacco plants. In comparison to wild-type plants, transgenic plants expressing *JcCPI* exhibited increased salinity resistance, better growth performance, lower malondialdehyde (MDA) contents, higher anti-oxidase activity, and higher cell viability under salinity stress.
Conclusions: Based on the results of this study, overexpression of *JcCPI* in *E. coli* and *N. benthamiana* conferred salinity stress tolerance by blocking cysteine proteinase activity. The *JcCPI* gene cloned in this study will be very useful for the development of stress-tolerant crops.

1. Introduction

Plants can be seriously affected by abiotic stresses, such as high salinity, drought, and low temperature, at the morphological, physiological, biochemical, and molecular levels. Various genes and biochemical–molecular control mechanisms are activated to eliminate or reduce the effects of these stresses [1]. Plant proteinase inhibitors (PIs), which function in the regulation of proteolysis and inhibition of uncontrolled proteolysis, have been suggested to play a significant role in plant responses to abiotic stress [2]. PIs are classified according to their reaction mechanism (competitive, non-competitive, uncompetitive, and suicide PIs) or the kind of protease that is inhibited (cysteine, serine, aspartic, and metallo-PIs) [3]. Among these, plant cysteine PIs, also known as phytocystatins (PhyCys), have been studied extensively, particularly with respect to their regulatory and protective functions in plant tissues [4]. PhyCys inactivate proteinases by trapping them in an irreversible, tight equimolar complex [5]. They were originally identified from rice seed [6] and have since been detected in many higher plant species including Chinese cabbage [7], barley [8], *Arabidopsis thaliana* [9], cowpea [10], and sugar beet [11]. Most PhyCys are small proteins with molecular masses between 12 kDa and 16 kDa, but some are up to 23 kDa due to a carboxy-terminal extension [12,13].

PhyCys are considered particularly important in regulating endogenous proteolytic activity in protein turnover during seed maturation and germination [9,14,15,16] and in programmed cell death (PCD) [9,17]. Recently, PhyCys have been found to be involved in responses to abiotic stresses as evidenced by their high expression in harsh conditions such as cold, heat, drought, salinity, alkali, and oxidant stress [18,19,20,21,22]. They are induced by intercellular signaling molecules such as abscisic acid [23]. Furthermore, PhyCys can significantly improve the ability of plants to tolerate abiotic stresses by acting against the proteolytic cysteine protease, which is activated by various abiotic stresses and leads to the acceleration of PCD [5,9,17,24]. Several recent studies of PhyCys transgenic plants have provided additional support for their positive involvement in abiotic stress responses. For example, overexpression of the *AtCYS4* gene confers thermotolerance [25] and overexpression of the *GsCPI14* gene enhances tolerance to alkali stress in *Arabidopsis* [19]. Thus,

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.08.002

PhyCys may play a crucial role in the regulation of various physiological and developmental processes, plant stress tolerance, and defense responses [2].

Jatropha curcas L. (Euphorbiaceae) is a multi-purpose bush/small tree native to Central America. The species grows well in arid and semi-arid environments and has been utilized as a source of biodiesel and traditional folk medicines [26]. *J. curcas* is highly tolerant to abiotic stresses, including salinity [27], heavy metal [28], drought [29], and high temperature [19], indicating that the species has an efficient molecular and physiological system to adapt to these adverse conditions. These characteristics make *J. curcas* potentially useful as a rich reservoir of genes for resistance to abiotic stresses. In this study, we present a molecular and functional characterization of the cysteine proteinase inhibitor (CPI) gene for *J. curcas*, *JcCPI*, which was significantly upregulated in response to salinity stresses. *JcCPI* function was determined in prokaryotic (*Escherichia coli*) and eukaryotic (*Nicotiana benthamiana*) model plant systems.

2. Materials and methods

2.1. Plant materials and treatments

J. curcas seeds were grown and treated as described by Zhang et al. [30]. After 20 d of growth, the seedlings were transferred to 100-mL beakers containing cotton soaked in half-strength Hoagland solution. A week later, half-strength Hoagland solution containing the desired solute was used for salinity treatments. The uniformly sized seedlings were treated with 300 mM NaCl for 2 d. Controls (kept in half-strength Hoagland solution) were grown in a greenhouse at 28 ± 1°C under 16/8 h (light/dark) photoperiod conditions adjusted to an intensity of 1000 μmol m^{-2} s^{-1}. Leaves from all of the treated seedlings were collected at the specific time points, and the materials were immediately frozen in liquid nitrogen and stored at -80°C. Tissue-specific expression assays were performed in the roots, stems, leaves, and petiole of 1-month-old control seedlings, as well as in mature seeds. All tissues examined were stored at -80°C after dissection. At least three independent replicates of each experiment were performed.

2.2. Amplification and sequence analysis of JcCPI

Total RNA was extracted using the RNeasy Plant Mini Kit (Tiangen, Beijing, China). The quality and the concentration of RNA were determined by 1.0% agarose gel electrophoresis and spectrophotometry (NanoVue; GE Healthcare, Little Chalfont, UK). First-strand cDNA synthesis was performed using 5 μg of total RNA as a template and a PrimeScript First Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's instructions. The full-length coding sequence of *JcCPI* was amplified from the cDNA using gene-specific primers *JcCPIf* and *JcCPIr* (Table 1) based on NCBI sequence data (GenBank accession no. FJ899657.1). The products were subcloned into pMD19-T vectors (Takara) and sequenced. Genomic analysis was based on data from the Jatropha Genome Databases (http://www.kazusa.or.jp/jatropha/). The protein physical property analysis used the On-line Analysis System (http://web.expasy.org/compute_pi/). Sequence similarity was examined using BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank database. The protein modification sites were predicted with Proscan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html).

The protein tertiary structure was predicted using the Swiss-model tool (http://swissmodel.expasy.org/interactive). The amino acid sequences of cloned cDNA fragments were deduced and protein sequences were aligned using the program DNAMAN 6.0. Phylogenetic relationships were analyzed by multiple alignments of CPI proteins using the MEGA 4.1 program. Analysis of transcription response elements was performed with the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) databases.

Table 1
Primers for PCR used in this paper.

Primers	Sequences (5′ to 3′)	Usage
JcCPIf	ATGGCAACCGTCGGCGGTATTA	Cloning of JcCPI ORF and semi-quantitative RT-PCR
JcCPIr	CTATGCGGTAGACTCTGTCGGGGTT	Cloning of JcCPI ORF and Semi-quantitative RT-PCR
JcCPI-qf	CTCGTTTCGCTGTTGATGACTA	Quantitative real-time PCR
JcCPI-qr	TTGACCACCATCCTCTACCTCC	Quantitative real-time PCR
Jc18s-qf	AGAAACGGCTACCACATC	Quantitative real-time PCR
Jc18s-qr	CCAAGGTCCAACTACGAG	Quantitative real-time PCR
JcCPI-32f	CGC*GGATCC*ATGGCAACCGTCGGCGGTATTA	Quantitative real-time PCR
JcCPI-32r	C*GAGCTC*CTATGCGGTAGACTCTGTCGGGGTT	Quantitative real-time PCR
NtActin-f	CCACACAGGTGTGATGGTTG	Semi-quantitative RT-PCR
NtActin-r	CACGTCGCACTTCATGATCG	Semi-quantitative RT-PCR

2.3. Expression analysis of JcCPI by real-time quantitative PCR

First-strand cDNA was synthesized using the method described above. Quantitative real-time PCR (qRT-PCR) was carried out using primers JcCPI-qf and JcCPI-qr (Table 1) and SYBR® Premix Ex Taq™ II (TaKaRa) and a CFX96 Real-time PCR machine (Bio-Rad, Hercules, CA, USA). The PCR conditions were 95°C for 10 s, followed by 40 cycles at 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s, then 3 min at 72°C. The 18S ribosomal RNA (18S rRNA) (GenBank accession no. AY823528) from *J. curcas* was used as the internal reference.

2.4. Expression of JcCPI in E. coli BL21

Primers JcCPI-32f and JcCPI-32r (Table 1) were designed and synthesized to amplify the coding sequence of JcCPI. The resulting fragment was digested with BamHI/SacI and ligated into the corresponding restriction sites of the expression vector pET32a. The recombinant plasmid pET32a-*JcCPI* was transformed into *E. coli* strain BL21(DE3) (Trans) for protein expression.

A single colony of *E. coli* strain BL21 cells harboring the recombinant plasmid pET32a-*JcCPI* and empty vector pET32a were inoculated at 37°C in LB medium containing ampicillin (100 mg L^{-1}), with shaking (180 rpm) until the cell cultures at OD600 value of 0.6. Protein expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and the cultures were grown for an additional 4 h. Overexpressed proteins were detected by analyzing the total protein using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue R250 staining.

2.5. Purification of JcCPI from E. coli and determination of cysteine proteinase inhibitory activity

Cells containing *JcCPI* were induced at 28°C for 8 h and harvested by centrifugation at 8000 × g for 10 min. Pellets were thawed and suspended in protein binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5–50 mM imidazole, pH 7.4). The cells were then sonicated for 6 s at 9-s intervals for 30 min. Cellular debris was removed by centrifugation at 4°C and 20,000 × g for 30 min. Supernatant containing JcCPI was subjected to a protein purification assay using BeaverBeads™ His-tag Protein Purification Kit (BeaverBeads, China) following the manufacturer's instructions. Protein concentration was measured using bicinchoninic acid (BCA) [31] and the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Bovine serum albumin was used as standard protein.

The effect of purified His-JcCPI protein on the activity of papain, which is the most widely studied cysteine protease, was determined

using azocasein (Sigma, St. Louis, MO, USA) as a proteinase substrate according to the method of Zhang et al. [21]. Enzyme activity was estimated by absorbance at 420 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). All assays were repeated at least three times.

2.6. Salinity stress tolerance assay using transformed E. coli cells

The effects of salinity on the growth of transformed *E. coli* BL21 (DE3) cells with pET32a (empty vector) and pET32a-JcCPI (recombinant plasmid) were examined. IPTG induction and cell cultures were prepared following the methods described above. For salinity stress, the concentration of all induced cultures in LB medium was adjusted to an OD600 value of 0.8 and 400 μL of culture samples was inoculated in 30 mL of LB medium containing 800 mM NaCl and grown at 37°C. OD600 values were recorded every hour for 48 h. The cell growth experiment was repeated three times, and the mean and standard deviation were calculated.

2.7. Construction of the JcCPI binary expression vector for plant transformation

To overexpress *JcCPI* in tobacco (*N. benthamiana*), cDNA was cloned in pYG8198. The resulting vector, pYG8198-*JcCPI*, was introduced into *Agrobacterium tumefaciens* strain GV3101, which was then used for plant transformation. The transformation of wild-type (WT) tobacco was performed as described by Horsch et al. [32], and the transformants were selected using 50 mg L^{-1} hygromycin (Amresco, Solon, OH, USA). Transgenic lines were screened via a semi-quantitative RT-PCR amplification analysis and western blots. Seeds of the homozygous T2 transgenic lines were harvested for subsequent salinity stress tolerance experiments.

2.8. Molecular analysis of the transgenic lines

2.8.1. Transcript analysis

The relative expression of the *JcCPI* gene was analyzed in three transgenic lines by semi-quantitative RT-PCR using the primers JcCPIf and JcCPIr with the following PCR protocol: 94°C for 5 min, followed by 28 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. The actin gene (GenBank accession no. X63603) from tobacco was used as the internal reference.

2.8.2. Protein expression analysis

Antibodies against the purified recombinant *JcCPI* were raised by injecting the protein into mouse and the anti-JcCPI antiserum was used for the western blotting analysis. Plant leaves were ground in liquid nitrogen and transferred to extraction buffer (100 mM Tris–HCl [pH 8.0], 1 mM EDTA, pH 8.0, 10 mM β-mercaptoethanol, and 200 mM sucrose) followed by centrifugation at 13,000 × g for 20 min to obtain the soluble protein. The protein extracts were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF) by electroblotting. After transfer, the PVDF was blocked in 5% milk in TPBS (0.1% Tween 20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4), then incubated with anti-JcCPI antiserum diluted 1:6400 in TPBS with 5% milk for 1 h at room temperature. Following three washes with TPBS at 10-min intervals, the membrane was incubated with goat anti-mouse horseradish peroxidase-conjugated antibodies (ZSGB-BIO, Beijing, China) diluted 1:10,000 for 1 h at 37°C. The blots were washed five times with TPBS

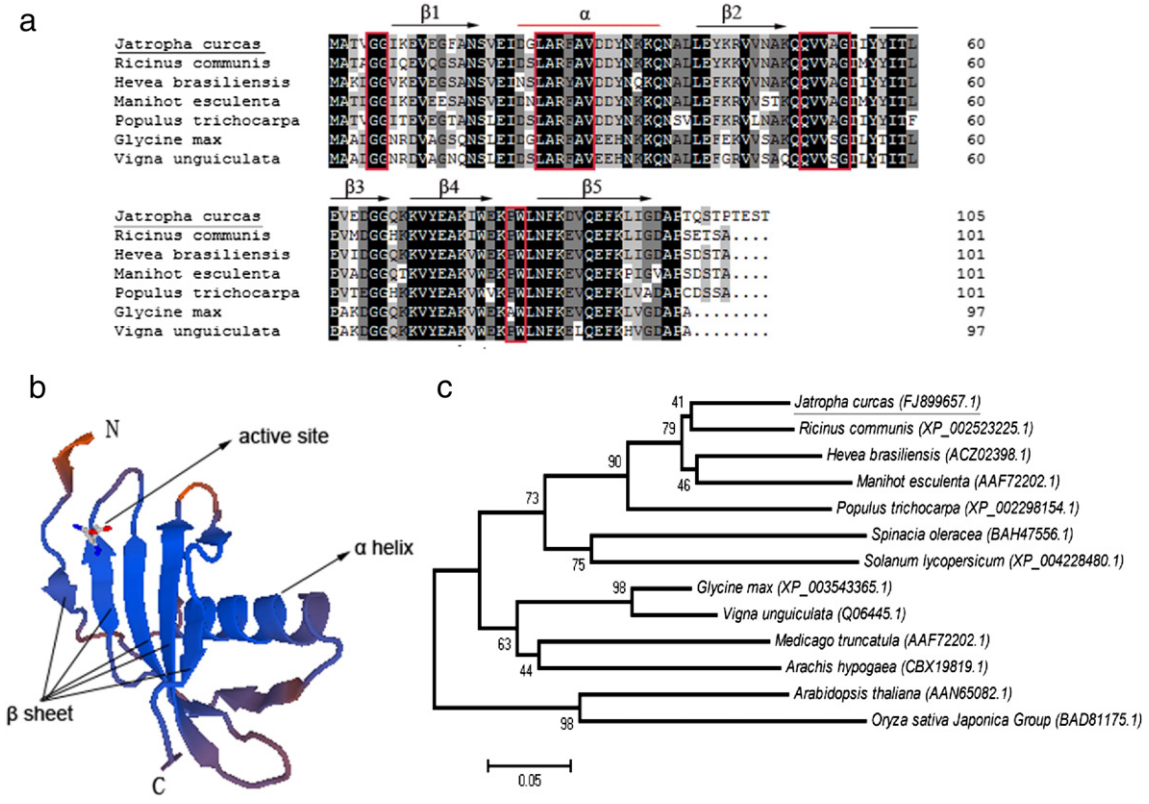

Fig. 1. JcCPI protein sequence alignment, tertiary structure and phylogenetic analysis. (a) Alignment of JcCPI with cystatins from other plant species based on the full-length amino acid sequence. The α-helix and five β-sheets were marked with red solid line and black arrows, respectively. The highly conserved motifs (GG, LARFAV, QxVxG and PW) were marked with red solid boxes. The highly conserved regions of the alignments have been shaded; (b) predicted protein tertiary structure analysis; (c) phylogenetic analysis of JcCPI with homologues from other plants.

and the signal was detected for a period of between 1 and 3 min using chemiluminescence (Bio-Rad).

2.9. Analysis of salinity stress tolerance of T2 transgenic tobacco

The 40-d-old WT and T2 transgenic seedlings grown in soil were watered with 0, 100, or 200 mM NaCl solution every 3 d for 2 weeks. The experiment was repeated at least three times and three independent lines were used. The seedling phenotypes were photographed after 2 weeks of growth, and the physiological and biochemical indices were measured. The amount of malondialdehyde (MDA) in the leaves was estimated as described previously [33]. Fresh material (0.5 g) was collected to determine the MDA levels. Antioxidant enzyme activity was determined following the method of Li et al. [34]. The single enzyme extraction system was used to assay three antioxidant enzymes (GR, glutathione reductase; CAT, catalase; APX, ascorbic acid peroxidase) simultaneously. Cell death was assayed 24 h after the indicated treatments by incubating tobacco leaf cells for 24 h with 0.05% (w/v) Evan's blue. Unbound dye was removed by extensive washing. The dye bound to dead cells was solubilized in 1% SDS for 24 h at 28°C and quantified by A600 [35].

3. Results

3.1. Isolation and bioinformatic analysis of JcCPI

We isolated the JcCPI gene from *J. curcas*. The *JcCPI* cDNA had an open reading frame of 321 bp encoding a protein of 106 amino acids with a predicted molecular mass of 11.84 kDa and a PI value of 4.97. Based on an analysis of JcCPI using the Jatropha Genome Database, the gene consisted of two exons and one intron. Querying the amino acid sequence against the NCBI database indicated homology to several cystatin-like proteins including RcCPI (86% identity), HbCPI (85% identity), MeCPI (84% identity), PtCPI (80% identity), GmCPI (74% identity), and VuCPI (71% identity). All of these proteins showed homology to *JcCPI* in the cystatin-like domains (Fig. 1a), which are common in the cystatin families. Such conserved regions included *Gly5*, the reactive site motif Q-X-V-X-G (Q49-V50-V51-A52-G53), and the *PW* motif (P79-W80). In addition, the *JcCPI* domain included the same consensus LARFAV sequence for PhyCys (red solid box in Fig. 1a). Another motif identified using the ProScan program was a putative casein kinase II phosphorylation site T-P-T-E (residues 100–103 in the C-terminal region numbering). Using the Swiss-model tool, a model for the *JcCPI* tertiary structure was generated using PDB entry 4lzi.1.A as a template. The generated structure (Fig. 1b) possess a sequence identity of 62% with the best modeling template PDB entry 4lzi.1, which reports the crystal structure of multicystatin from *Solanum tuberosum* [36], and shows the typical structural elements of plant cystatins, including an α-helix spanning the LARFAV motif and five antiparallel β-sheets (β1, β2, β3, β4 and β5). Based on a phylogenetic analysis, *JcCPI* was closely related to the *Ricinus communis* cystatin. Euphorbiaceae cystatins, including JcCPI and RcCPI, grouped together (Fig. 1c).

3.2. Transcription profile of JcCPI

We analyzed the expression patterns of *JcCPI* in the roots, stems, leaves, and petiole of one-month-old *J. curcas* seedlings, as well as in mature seeds. The transcription level of *JcCPI* was determined by qRT-PCR. *JcCPI* was constitutively expressed in all examined tissues, but was expressed most highly in the petiole. The expression levels of *JcCPI* in the leaf were 0.5-fold lower than in the petiole (Fig. 2a). This result indicated that JcCPI expression in vegetative tissues was non-tissue-specific.

Considering previous reports that the expression of *CPI* genes can be induced by high salinity [19], we used qRT-PCR to determine whether *JcCPI* expression was also induced by salinity stress. As shown in Fig. 2b, under salinity treatment, the transcript level of *JcCPI* showed an obvious increase and peaked at 3 h, after which the expression level subsided at 6 h and then slightly increased again at 24 h, suggesting that *JcCPI* plays a role in salinity stress. To gain a better understanding of the role of *JcCPI* in response to environmental stresses, we extracted the putative promoter region, 1.5-kb sequences upstream of the beginning of the *JcCPI* coding region, from the Jatropha Genome Database, and used it to search for homologous motifs in the PlantCARE and PLACE databases. Consistent with the qRT-PCR results, the *JcCPI* promoter also contained various cis-acting elements involved in defense and stress responses (Table 2), such as TC-rich repeats (defense and stress responsive elements), a long-terminal repeat (cis-acting element involved in low-temperature responsiveness), an abscisic acid-responsive element (cis-acting element involved in the abscisic acid responsiveness) and a WUN-motif (wounding responsiveness).

3.3. Expression, purification, and proteinase inhibition activity of recombinant JcCPI

The expression plasmid pET32a-*JcCPI*, which harbors the *JcCPI* coding sequence, and the empty vector pET32a as a control were introduced into *E. coli* strain BL21. The expressed proteins were analyzed by SDS-PAGE. Electrophoresis of the control protein pET32a and recombinant protein JcCPI clearly showed highly expressed proteins of approximately 21 kDa (left arrow in Fig. 3a) and 33 k Da (right arrow in Fig. 3a) in size, respectively. With the His-tag fusion,

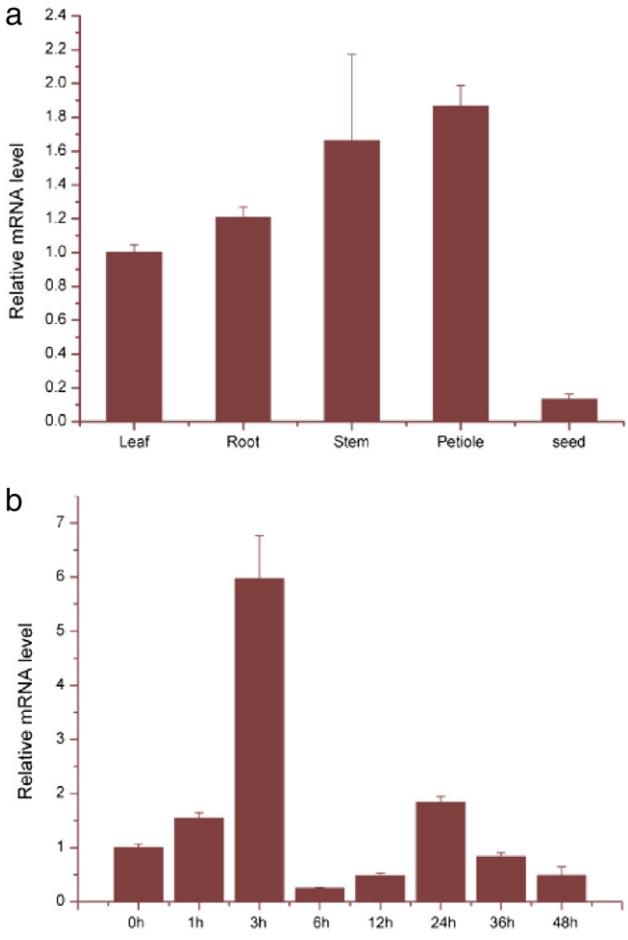

Fig. 2. Quantitative real-time PCR analysis of JcCPI gene expression. (a) Tissue specific expression. (b) Expression levels under salinity stress, total RNA was isolated from leaf samples.

Table 2
Details of *JcCPI* 5′ flanking region cis-elements involved in pathogen- and stress-responsive expression.

cis-element	Sequence	Function
TGACG-motif	TGACG	MeJA responsiveness
GARE-motif	AAACAGA	Gibberellins responsiveness
ABRE	TACGTG	ABA responsiveness
TCA-element	CCATCTTTTT	Salicylic acid responsiveness
G-Box	CACGTT	Light responsiveness
chs-CMA1a	TTACTTAA	Light responsiveness
TC-rich repeats	ATTTTCTCCA	Defense and stress responsiveness
LTR	CCGAAA	Low-temperature responsiveness
W Box	TTGACC	Pathogen-responsiveness
WUN-motif	TCATTACGAA	Wounding responsiveness

the size of the expressed recombinant protein was larger than the expected size of 11.8 kDa. The single band of the purified His-*JcCPI* with an apparent molecular mass of approximately 33 kDa (right arrow in Fig. 3a) in Coomassie Blue-stained SDS-PAGE was observed in an eluted solution from the Beaver beads.

To determine whether the recombinant JcCPI protein was a functional cysteine protease inhibitor, purified His-JcCPI protein was assayed against papain using azocasein as a substrate after pre-incubation with the inhibitor at various concentrations. As shown in Fig. 3b, the proteolytic inhibition activity of papain gradually increased as the His-JcCPI protein concentration increased. His-JcCPI protein (8 μg) reduced papain (20 μg) activity by 50%, indicating that the recombinant JcCPI protein exhibited total inhibition of papain activity in vitro.

3.4. Overexpression of JcCPI in E. coli increases growth during salinity stress

We tested the growth performance of transformed *E. coli* cells expressing pET32a-*JcCPI* under non-stress and stress conditions (i.e. high salinity). The growth curve of pET32a-*JcCPI* was compared with that of the strain transformed with pET32a as a control. The recombinant (pET32a-*JcCPI*) and control (pET32a) strains showed

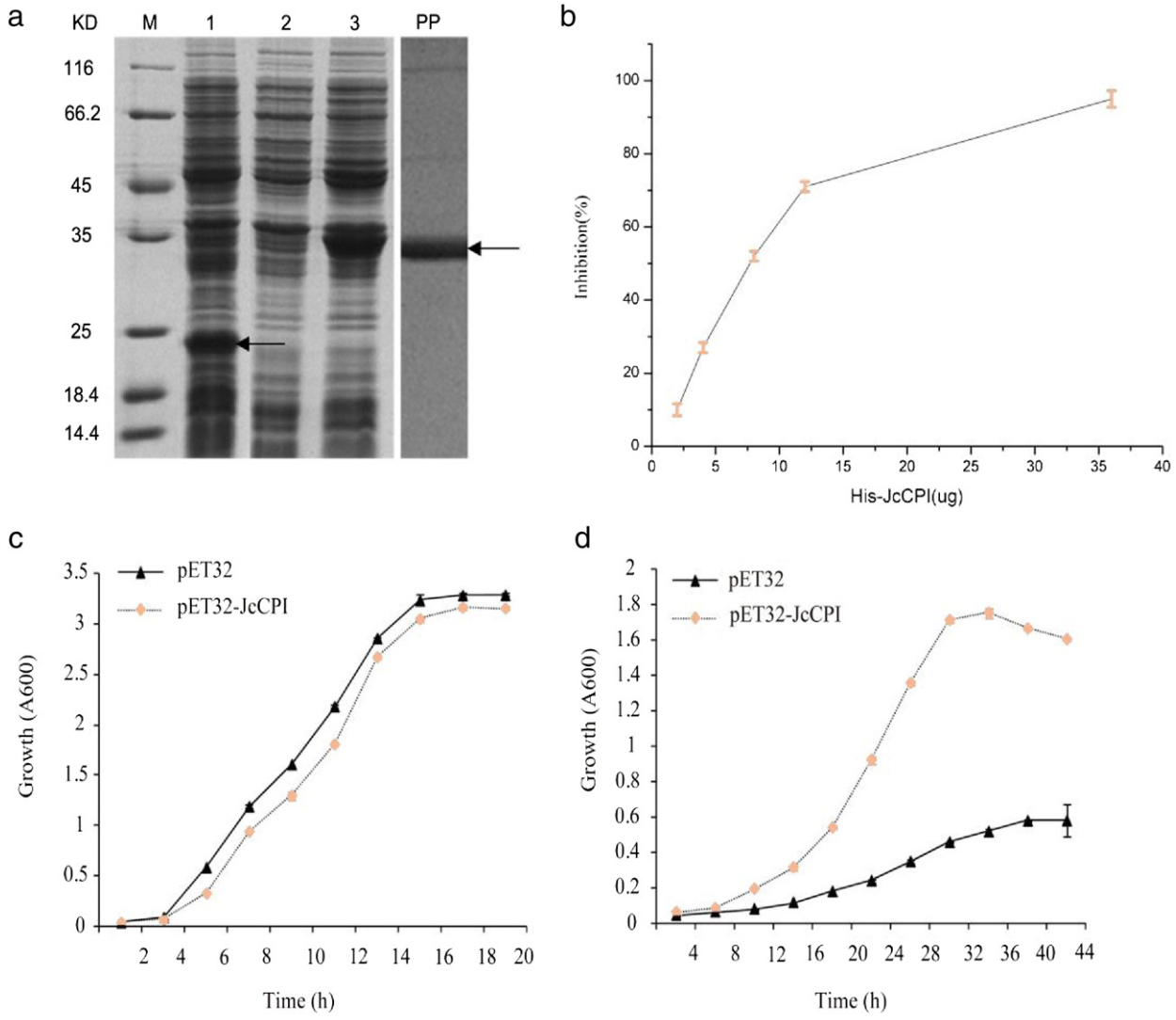

Fig. 3. Analysis of the recombinant His-JcCPI protein expression, purification, inhibition activity and salinity stress tolerance. (a) SDS-PAGE (12%) analysis of JcCPI over-expression in *E. coli* BL21. Lane M, protein marker (Fermenta 26610); lane 1, crude extracts of *E. coli* BL21 containing the empty vector pET32a with IPTG induction (left arrow); lane 2, crude extracts of *E. coli* BL21 containing the pET32a-JcCPI without IPTG induction; lane 3, crude extracts of *E. coli* BL21 containing the pET32a-JcCPI with IPTG induction (right arrow); lane PP, purified His-JcCPI fusion protein, the over-expressed His-JcCPI fusion protein is indicated by the right arrow. (b) Inhibition activity of His-JcCPI to papain is expressed as enzyme inhibition activity in the presence of increasing inhibitor concentrations. (c) and (d) Effect of NaCl on the growth of transformed *E. coli* cells containing a recombinant plasmid (pET32-JcCPI) or empty vector (pET32) under non-stress and 800 mM NaCl stress, respectively. Three replicate experiments were conducted, and the average activities ± standard errors calculated using Excel statistical function were presented.

similar growth under non-stress conditions (Fig. 3c). For high-salinity stress, 800 mM NaCl was used, which severely reduces the growth of *E. coli*, and the lag phases of the recombinant and control strains were approximately 9 and 16 h, respectively (Fig. 3d). The results suggested that *E. coli* cells producing *JcCPI* were more tolerant to salinity stress.

3.5. Overexpression of JcCPI in T2 transgenic plants improves tolerance to high salinity

To further evaluate the functional significance of *JcCPI* with respect to salinity stress, we generated transgenic tobacco plants that overexpressed *JcCPI* under the control of the 2XCaMV35S promoter. Gene expression in three independent homozygous T2 transgenic lines (lines T1, T2, and T3) was confirmed using different molecular approaches (Fig. 4a, Fig. 4b).

To investigate whether overexpression of *JcCPI* in plants increased salinity tolerance, 40-d-old seedlings from T2 homozygous and WT lines were exposed to continuous stress consisting of different concentrations of NaCl (0, 100, or 200 mM NaCl) added to the soil for 2 weeks. Based on a phenotypic analysis, the WT seedlings showed severe chlorosis and stunted phenotypes and ultimately died, whereas the T2 transgenic seedlings maintained continuous growth (Fig. 4c). As shown in Fig. 4d, Fig. 4e, Fig. 4f, and Fig. 4g, the enhanced salinity tolerance of the transgenic plants was further confirmed by estimating physiological changes. In comparison to WT plants, transgenic plants expressing JcCPI exhibited lower MDA contents and higher CAT, GR, and APX antioxidase activity.

To examine the mechanism that results in the salinity tolerance phenotype in lines overexpressing *JcCPI*, we used cell death assays of the T2 and WT plants before and after exposure to 200 mM NaCl stress because PCD is induced by NaCl stress [9]. Furthermore, PCD activates cysteine proteases, which can be inhibited by cystatins. As expected, the cell viability in T2 plants was significantly higher than that in WT plants (Fig. 5). These results indicated that the increased salinity tolerance of transgenic plants can be attributed to inhibition of cysteine protease activity.

4. Discussion

Cystatins are generally conserved among plant genera and play an important role in diverse physiological and defense processes, especially in stress tolerance [2]. In this study, we isolated a gene, *JcCPI*, encoding a cystatin-like protein from *J. curcas*. Homologous and phylogenetic analysis indicated that *JcCPI* belonged to a widely studied plant cystatin-like protein (PhyCys). These proteins have several common characteristics, including LARFAV motifs and the active site Q-X-V-X-G [37]. In addition, the main body of *JcCPI* consisted of an α-helix and a five-stranded antiparallel β-sheet similar to cystatins from other species [2]. Gene expression analyses are important for deciphering gene functions. Based on qRT-PCR, basal *JcCPI* mRNA levels were low in mature seeds (Fig. 2a), unlike most phytocystatins, which are stably expressed at high levels at seed maturity [20]. Moreover, *JcCPI* transcript accumulation was detected in response to salinity stress (Fig. 2b). Several stress-responsive cis-regulatory elements were predicted in the putative promoter region (Table 2). These results revealed that *JcCPI* might be involved in the plant response to salinity stress.

Recently, a cystatin, BvM14, has been reported to enhance plant tolerance to salinity stress in *Arabidopsis* [11]. In light of the present results for *JcCPI* and the expression profiles of other cystatins [19,22], we hypothesized that PhyCys members are involved in the response to multiple stresses and play important roles in abiotic stress tolerance. To our knowledge, PhyCys research focuses mainly on disease and pest management [2]; therefore, more studies that examine the function of PhyCys in response to abiotic stresses are needed. Here, we demonstrated the positive function of *JcCPI* in response to salinity stress in both prokaryotic and eukaryotic model systems.

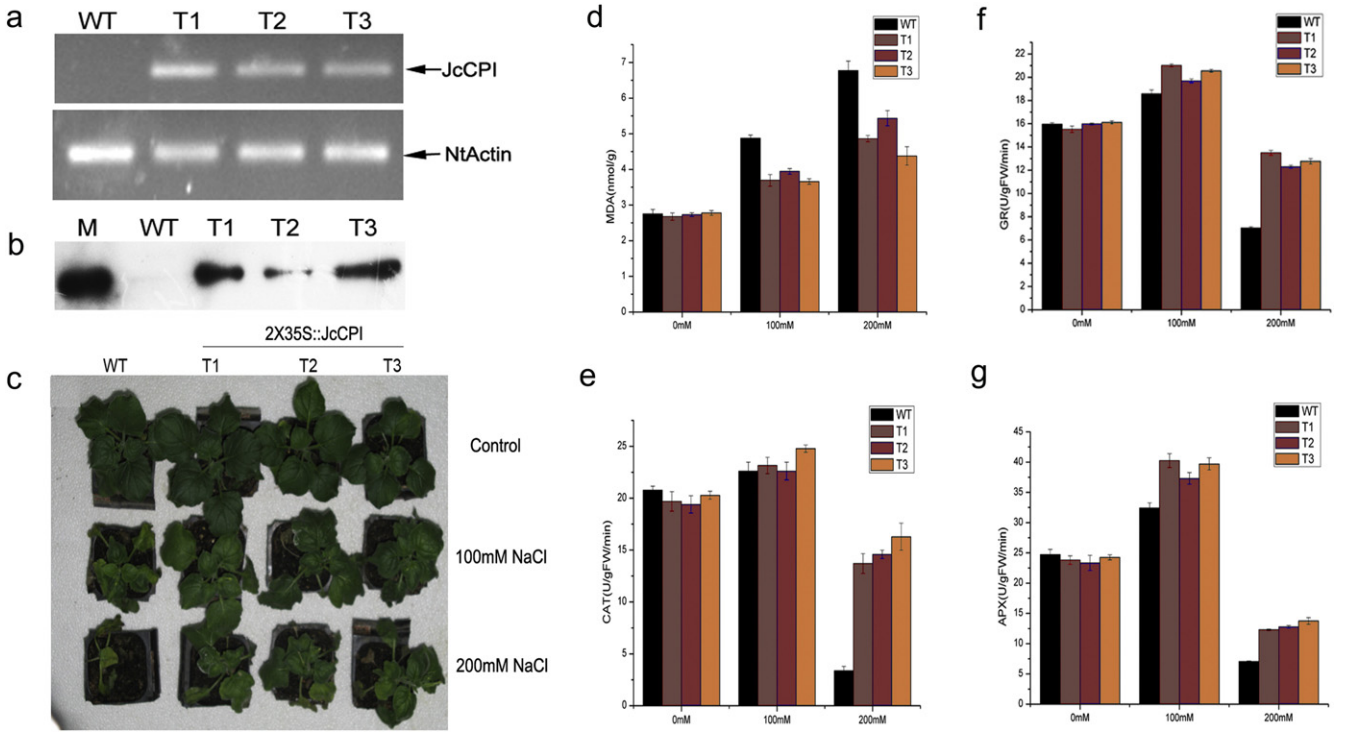

Fig. 4. Molecular, phenotype and physiological index analysis of soil-grown WT and JcCPI plants in response to salinity stress. Wild type (WT), Transgenic lines (T1, T2 and T3). Molecular analysis: (a) analysis of the accumulation of transcripts by semi-quantitative RT-PCR; (b) detection of JcCPI protein by western blot; (c) phenotypical analysis. Physiological index analysis: (d) MDA content; (e) CAT enzyme activity; (f) GR enzyme activity; and (g) APX enzyme activity.

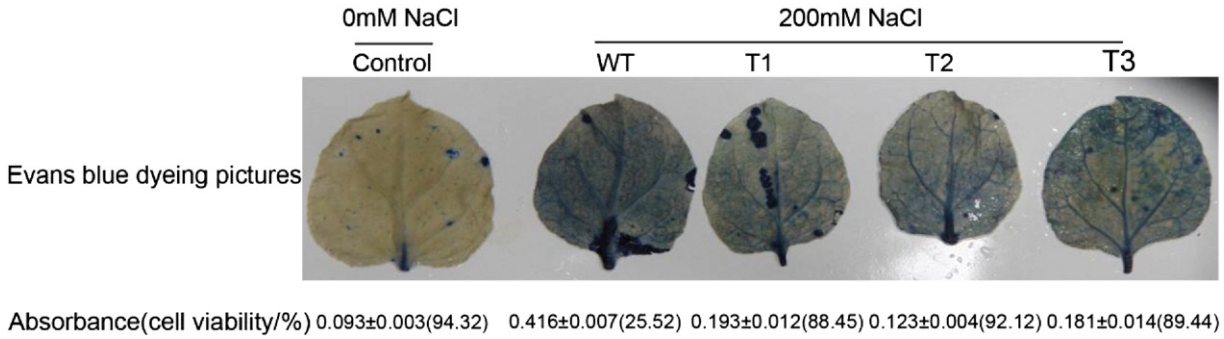

Fig. 5. Evans blue dyeing pictures and cell viability in leaves of wild-type and transgenic plants under high salt stress. Wild type (WT), Transgenic lines (T1, T2 and T3).

Many studies have shown that PhyCys are natural inhibitors of cysteine proteases; they regulate and balance responses in many biological, physiological, and cellular processes [2]. Papain, a well-characterized plant cysteine protease, plays an important role in the PCD process [17]. Here, in the prokaryotic expression assay, we confirmed the inhibitory activity of His-JcCPI on papain in vitro (Fig. 3b). Heterologous expression of *JcCPI* in *E. coli* cells demonstrated that pET32a-*JcCPI* recombinant *E. coli* cells are more tolerant to salinity than the control with the vector alone (Fig. 3d). However, there is still limited information about the mechanism by which PhyCys improves *E. coli* growth under stress conditions. According to a previous report, when abiotic stress is applied to bacterial cultures, abiotic stress causes the activation cascade of PCD-like events with higher caspase-like protease activity [38]. Therefore, we speculated that caspase-like protease is inhibited by the presence of caspase-like inhibitors to delay the PCD-like process of apoptosis in prokaryote cells, conferring stress tolerance.

PhyCys proteins inhibit the key processes involved in PCD [9,17] and can restrict the stress response by these mechanisms [9]. Aside from the functions mentioned above, cystatins play a regulatory role in photosynthesis and respiration [39]. Further experiments have found that overexpression of *JcCPI* in tobacco increases the tolerance to salinity stress. MDA is a byproduct of enzyme- and oxygen radical-induced lipid peroxidation, and is widely used as a biomarker of oxidative stress in plants [40]. In addition, it is clear that plants contain a series of antioxidant enzymes (such as CAT, GR, and APX) that function properly to scavenge toxic reactive oxygen species and protect plants from the associated damage [34]. Thus, low MDA contents and high antioxidant enzyme activity are linked with stress tolerance. As such, we assayed MDA content and CAT, GR, and APX activity in both types of tobacco under salinity conditions. Transgenic lines appeared much taller and stronger (Fig. 4c), and presented relatively lower levels of MDA (Fig. 4d) when treated with NaCl. Antioxidant enzyme (CAT, GR, and APX) activity increased significantly under 100 mM NaCl stress, but decreased to a low level for a higher NaCl concentration (200 mM) in both types of tobacco, indicating that antioxidant enzyme activity is suppressed in plants treated with higher NaCl concentrations (Fig. 4d, Fig. 4e, Fig. 4f, Fig. 4g). Transgenic lines exhibited higher antioxidant enzyme activity in the whole salinity stress process (Fig. 4d, Fig. 4e, Fig. 4f, Fig. 4g), suggesting a stronger tolerance to salinity stress. A hypothesis is that *JcCPI* enhanced salt stress tolerance by maintaining cellular membrane stability and antioxidant enzyme activity, thereby protecting vital cellular activities from stress damage.

In addition, transgenic lines showed higher cell viability (Fig. 5), indicating that the suppression of cell death was induced by salinity stimuli. The basic function of a cystatin is to regulate endogenous cysteine protease activity, which is activated at the onset of PCD [5]. Increased protease activity results in the degradation of many specific proteins that help the plant overcome stress conditions [9]. Thus, blocking the activity of cysteine protease, which does not occur in the WT plants, might reduce salinity stress-induced cell death. Based on these results and those of previous studies, we inferred that *JcCPI* mediates the response to salinity stress by interacting with papain or other cysteine proteases.

Although the exact mechanism is unknown, current data indicates that some of the protective mechanisms are the same or similar in prokaryotes and eukaryotes under stress conditions. We suggest that *JcCPI* is a gene involved in the response of transgenic plants to abiotic stresses. The specific physiological functions of *JcCPI* in abiotic stress tolerance will be the focus of future studies.

Conflict of interest

The authors declare that there are no conflict of interest.

Financial support

This research was supported by the National Key Technologies R & D Program of China (Code: 2011BAD22B08) and the National Science Fund of China No. 31070276 and No. 31270360.

Acknowledgments

We thank Palaniyandi Karuppaiya for helping with language processing.

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