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

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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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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