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Research article

JcZFP8, a C2H2 zinc finger protein gene from *Jatropha curcas*, influences plant development in transgenic tobacco

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ABSTRACT

Background: Jatropha curcas L., as an important strategic biofuel resource with considerable economic potential, has attracted worldwide attention. However, *J. curcas* has yet to be domesticated. Plant height, an important agronomic trait of *J. curcas*, has not been sufficiently improved, and the genetic regulation of this trait in *J. curcas* is not fully understood. Zinc finger proteins (ZFPs), a class of transcription factors, have previously been shown to play critical roles in regulating multiple aspects of plant growth and development and may accordingly be implicated in the genetic regulation of plant height in *J. curcas*.
Results: In this study, we cloned *JcZFP8*, a C2H2 ZFP gene in *J. curcas*. We found that the JcZFP8 protein was localized in the nucleus and contained a conserved QALGGH motif in its C2H2 structure. Furthermore, ectopic expression of *JcZFP8* under the control of the *35S* promoter in transgenic tobacco resulted in dwarf plants with malformed leaves. However, when *JcZFP8* was knocked out, the transgenic tobacco did not show the dwarf phenotype. After treatment with the gibberellic acid (GA) biosynthesis inhibitor paclobutrazol (PAC), the dwarf phenotype was more severe than plants that did not receive the PAC treatment, whereas application of exogenous gibberellin3 (GA3) reduced the dwarf phenotype in transgenic plants.
Conclusions: The results of this study indicate that *JcZFP8* plays a role in *J. curcas* plant phenotype through GA-related pathways. Our findings may help us to understand the genetic regulation of plant development in *J. curcas* and to accelerate breeding progress through engineering of the GA metabolic pathway in this plant.

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1. Introduction

Transcription factors are a class of proteins that regulate gene transcription in organisms and that can directly or indirectly act on specific nucleotide sequences to activate or inhibit expression of a target gene [1]. According to the location and number of cysteine and histidine residues, Zinc finger proteins (ZFPs) can be classified into a number of different types including C2H2, C2HC, and C4HC3 [2]. Many plant C2H2 ZFPs also harbor the highly conserved QALGGH sequence, which is unique to plant ZFPs [3]. Previous studies have shown that this structure is necessary for DNA-binding activity in plants [4].

Functionally, the C2H2 ZFPs in plants bind to DNAs, RNAs, and proteins that are involved in transcriptional regulation, RNA metabolism, and many other biological processes [1,5]. Studies have shown that ZFPs can regulate plant growth and stress resistance. A series of ZFPs related to stress were discovered and validated in *Arabidopsis* [6,7]. ZFPs are also involved in the stress responses of other dicotyledonous plants such as soybean [8] and tomato [9]. In addition to dicotyledons, ZFPs have also been found to be involved in abiotic stress response and tolerance in monocotyledons [10]. With regard to plant growth and development, C2H2 ZFPs in plants participate in multiple processes of plant development. The C2H2 ZFP gene *LIF*, for example, has been shown to increase the number of lateral branches and decrease plant height following overexpression in a petunia hybrid, with transgenic plants showing increase in the number and size of cells in stems, leaves, and flowers [11]. Recently, a series of C2H2 ZFPs in plants, including ZFP6 and GIS3, were confirmed to participate in the development of trichomes [12,13]. Moreover, when expressing *Arabidopsis* genes in tobacco, the same phenotypes were also found in heterologous transgenic tobacco [14]. In addition, previous studies have indicated that many C2H2 ZFPs can regulate plant development through hormone signal pathways.

The plant *Jatropha curcas* L. is a member of the Euphorbiaceae family and is well known mainly for its high biofuel content. Its oil content can exceed that of many cultivated oilseed crops and could be used as an alternative to petroleum-based diesel fuel [15]. The multipurpose

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Jatropha also has utility as a green manure and fertilizer as well as in the manufacture of soap, pesticides, and traditional medicines [16]. Furthermore, *J. curcas* has strong resistance to drought, is easily propagated, and shows adaptation to a wide range of environmental conditions [17]. As a tropical and subtropical plant, *J. curcas* is distributed in the central regions of Central and South America, Africa, and Asia [18]. Owing to limited availability of traditional fossil fuels and the secondary environmental problems associated with burning fossil fuels, such as in greenhouse, biodiesel, as a fossil fuel substitute, has recently attracted considerable attention. With regard to this, *J. curcas,* with its high oil content and fine environmental protection characteristics, represents an ideal renewable "green energy" crop.

To date, however, *J. curcas* has not been domesticated or improved and is still considered as a wild plant [19]. The application of dwarfing traits has greatly increased the yields of plants, thereby leading to the "green revolution" of crops, which occurred worldwide in the last century [20]. Dwarf plants are compact plant types with small crowns, which are convenient traits for cultivation management and are generally associated with high productivity. For example, dwarf rice is the most important agronomic trait closely related to photosynthetic efficiency and yield [21]. Exploring more dwarf resources can deepen our understanding of the genetic mechanisms of plant height and provide new germplasm for cultivating new varieties. In this study, we cloned and analyzed the function of a C2H2 ZFP gene (*JcZFP8*) from *J. curcas*, and we found that overexpression of this gene negatively influenced plant growth and leaf development in transgenic tobacco plants.

2. Materials and methods

2.1. Plant materials and treatment

J. curcas and *Nicotiana tabacum* L. plants were germinated from mature seeds and grown in culture chambers under cool, white fluorescent lights (16-h light/25°C; 8 h dark/20°C). To investigate the effect of plant hormone application, tobacco seedlings were treated with 10 μM gibberellin3 (GA3) and paclobutrazol (PAC: a gibberellic acid [GA] biosynthesis inhibitor) by cultivating in 1/2-strength Murashige and Skoog medium containing GA3 or PAC. Experiments were carried out with three independent biological replicates.

2.2. Cloning and sequence analysis of JcZFP8

Total RNA of *J. curcas* was isolated using an RNAprep Pure Plant Kit (Tiangen, China). First-strand cDNA was synthesized using a PrimeScript™ RT Reagent Kit (Takara, China) according to the manufacturer's instructions. On the basis of the cDNA sequence, gene-specific primers (sF1: 5′-ATGGATAAGAGCGAAAGAG-3′; sR1: 5′-CAGA TGAAGATCTAAACTCACA-3′) were designed, and these primers were used to amplify the full-length coding sequence. Subcellular localization of the protein was predicted using WoLF PSORT (http://wolfpsort.seq.cbrc.jp/). Multiple alignments of protein sequences were calculated using the ClustalX tool (http://www.eki.ac.uk/Tools/). Using the MEGA 7.0 software package (http://www.megasoftware.net/), a phylogenetic tree was constructed by the neighbor-joining (NJ) method.

2.3. Subcellular localization of JcZFP8

To examine the subcellular localization of JcZFP8, the full-length coding sequence of *JcZFP8* was amplified by PCR using the gene-specific primers with cleavage sites of *Xba*I and *Sal*I. The amplified product was double digested with *Xba*I and *Sal*I enzymes and inserted into a pBI221-GFP vector to generate a *35Sp:JcZFP8:GFP* construct. The recombinant vector was transformed into *N. tabacum* L. protoplast cells. After transformation, the cells were incubated in dark at 20–25°C for 14 h and subsequently examined under a fluorescence microscope (Leica TCS SP5 II system), using green fluorescent protein (GFP) fluorescence signal excitation.

2.4. Construction of expression vectors and development of transgenic tobacco lines

To generate transgenic tobacco plants overexpressing the gene of interest, the full-length coding sequence of the *J. curcas JcZFP8* gene was inserted into a pBI121 vector driven by the *CaMV35S* promoter. The construct was verified by sequencing and transformed into the *Agrobacterium tumefaciens* strain GV3101 by the freeze–thaw method. Finally, the GV3101 strain was used to transform wild-type (WT) tobacco by the leaf disc method, as described by Horsch et al. [22]. The kanamycin-resistant transgenic plantlets were identified by PCR and real-time PCR (qPCR) analyses using gene-specific primers for the *JcZFP8* gene. All further experiments were performed using homozygous lines in T_2 generation. WT and *JcZFP8*-overexpressing (OE) tobacco plants were collected to measure morphological indices including plant height and leaf length. For knockout (KO) of *JcZFP8* in OE transgenic tobacco plants, we used the CRISPR-Cas9 system by constructing vectors containing gRNA, according to the method described by Xing et al. [23].

2.5. Quantitative real-time PCR (qPCR)

RNA extraction and first-strand cDNA synthesis were performed by the methods described above. Reactions were carried out in a Bio-Rad CFX96 Real-Time PCR machine (Bio-Rad, USA) using 20-μL reaction volumes containing 2 μL of appropriate cDNA from each sample, 10 μL of SsoFast™ EvaGreen Supermix (Bio-Rad, USA), and 1 μL of each primer (qF: 5′-ATCAGCAACCTATCAATGG-3′; qR: 5′-TCACGATGAAG AGTAGCA-3′), and this reaction mixture was made to the final volume with double-distilled H_2O. The PCR profile included one cycle at 95°C for 20 s, followed by 40 cycles at 95°C for 5 s, and Tm °C for 20 s and with a final melting curve profile of 65–95°C and 0.5°C/s. Quantification was done by the $^{\Delta\Delta}$Ct method. qRT-PCR was performed in triplicate, and the actin gene was used as an internal control.

3. Results

3.1. Gene isolation and sequence analysis

The complete sequence of *JcZFP8* was 931 bp in length, with a 5′-UTR and 3′-UTR of 186 bp and 10 bp, respectively (Fig. 1a; Fig. S1). The amino acid sequence was predicted on the basis of the *JcZFP8* sequence. The open reading frame of this gene encodes a putative protein of 244 amino acids. It is a small neutral protein with a predicted molecular mass of 27.38 kD and pI of 6.55. On the basis of WoLF PSORT program analysis, the protein was predicted to be localized in the nucleus.

The deduced JcZFP8 protein sequence contains a conserved C2H2-type zinc finger–like motif (CHYCCRNFPTSQALGGHQNAH) at the C terminus. Alignment analysis of the JcZFP8 protein sequence with related sequences revealed that these proteins contain a highly conserved QALGGH sequence (Fig. 1a), which is a feature that has a specific function in plants. Subsequently, a phylogenetic tree was constructed by the neighbor-joining method based on amino acid sequences from monocots and dicots (Fig. 1b). The constructed phylogenetic tree showed that JcZFP8 was more closely related to similar proteins in dicots such as *Ricinus communis* (XP_002531176.1) and *Vitis vinifera* (XP_003632719.1) as well as to other C2H2 ZFPs in dicots. These results are consistent with the established evolutionary relationships of these species.

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