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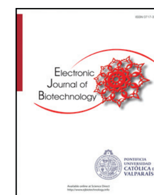

Research article

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

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A B S T R A C T

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* may play 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.

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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 *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/25°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′-CAGATGAAGATCTAAACTCACA-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, 55°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 $2^{-\Delta\Delta Ct}$ 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

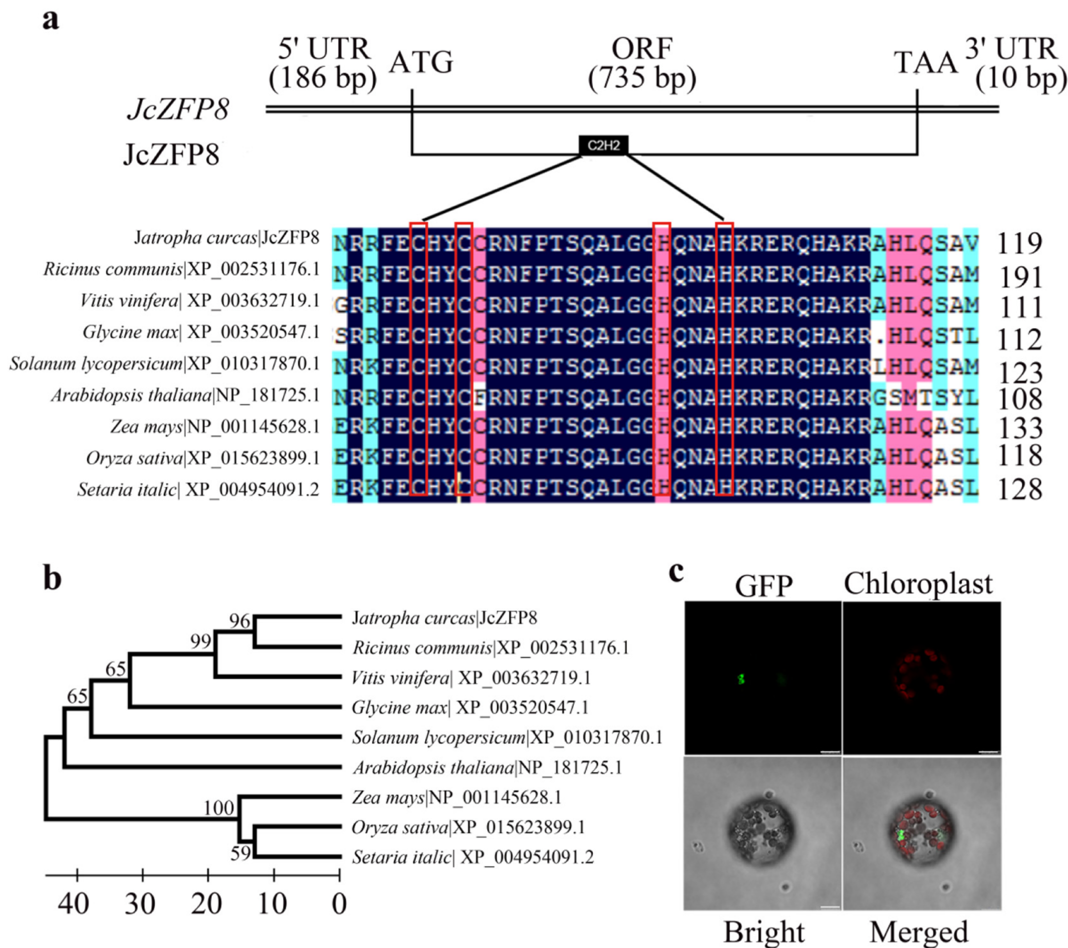

Fig. 1. Sequence characteristics of *JcZFP8*. (a) Sequence structure *of JcZFP8* and amino acid alignments of the JcZFP8 protein with eight homologs from other species. The boxes indicate the C2H2 zinc finger domains. UTR, untranslated region; ORF, open reading frame. (b) Phylogenetic tree of JcZFP8 and different model plant species constructed with MEGA 7.0 by the neighbor-joining method. (c) Subcellular localization of JcZFP8. GFP, fluorescence of the green fluorescent protein; Merged, digitally merged images of GFP and chloroplast.

dicots. These results are consistent with the established evolutionary relationships of these species.

3.2. Subcellular localization of JcZFP8

Most of the previously discovered C2H2-type ZFPs in plants have been shown to be localized in the nucleus. Similarly, software predictions also indicated that JcZFP8 is located in the nucleus. To further demonstrate that JcZFP8 is indeed localized in the nucleus, we examined its subcellular localization using a plant transient expression vector (pBI221-GFP). The full sequence of *JcZFP8* was fused in frame to the GFP reporter gene under control of the *CaMV35S* promoter. The *35Sp:JcZFP8:GFP* recombinant plasmid was then injected into tobacco protoplast cells followed by observation under a fluorescence microscope. Confocal microscope imaging showed that the fluorescence signals of the *35Sp:JcZFP8:GFP* fusion protein were clearly and exclusively located in the nuclear regions of tobacco cells, thereby confirming that JcZFP8 localizes in the nucleus (Fig. 1c). This observation is consistent with the findings of previous research on ZFPs in other plants.

3.3. Identification of transgenic plants

To identify the role of *JcZFP8* in plants, we introduced a *JcZFP8*-OE construct (Fig. 2a) into *N. tabacum*, which is a good model and a commercially bred plant, by an *Agrobacterium*-mediated method. The OE tobacco plants were identified by PCR and qRT-PCR using *JcZFP8*-specific primers. The gene expression of *JcZFP8* was analyzed by qRT-PCR. qRT-PCR results showed that *JcZFP8* was highly expressed in the OE lines, whereas there was no expression of this gene in WT tobacco plants. High expression of *JcZFP8* was detected in the OE lines 1, 4, 8, and 9, and relatively low expression of this gene was observed in the OE lines 2 and 3 (Fig. 2b). The expression of *JcZFP8* further confirmed that *JcZFP8* was successfully introduced into the tobacco plant genome, with stable inheritance and expression in the transgenic lines. To verify the function of *JcZFP8*, the OE lines displaying relatively high expression levels were selected for further investigation.

To identify alterations in OE plants caused by the overexpression of *JcZFP8*, the gene was knocked out in OE tobacco lines by the Crispr-cas9 method, as previously described [23]. A diagram of the KO vector, containing a tagged protospacer adjacent motif (PAM) and a target, is shown in Fig. 2c. To confirm mutation events in OE tobacco lines, the open reading frame of *JcZFP8* was also cloned and sequenced. The results indicated that there was a single-nucleotide insert mutation per site in the targeted mutation of OE plants (Fig. 2d).

3.4. Effects of JcZFP8 on plant height and leaf size

Three representative transgenic lines displaying relatively high expression levels were selected for further investigation. At the seedling stage, differences in the heights of transgenic and WT plants were observed, and these differences became more apparent with an

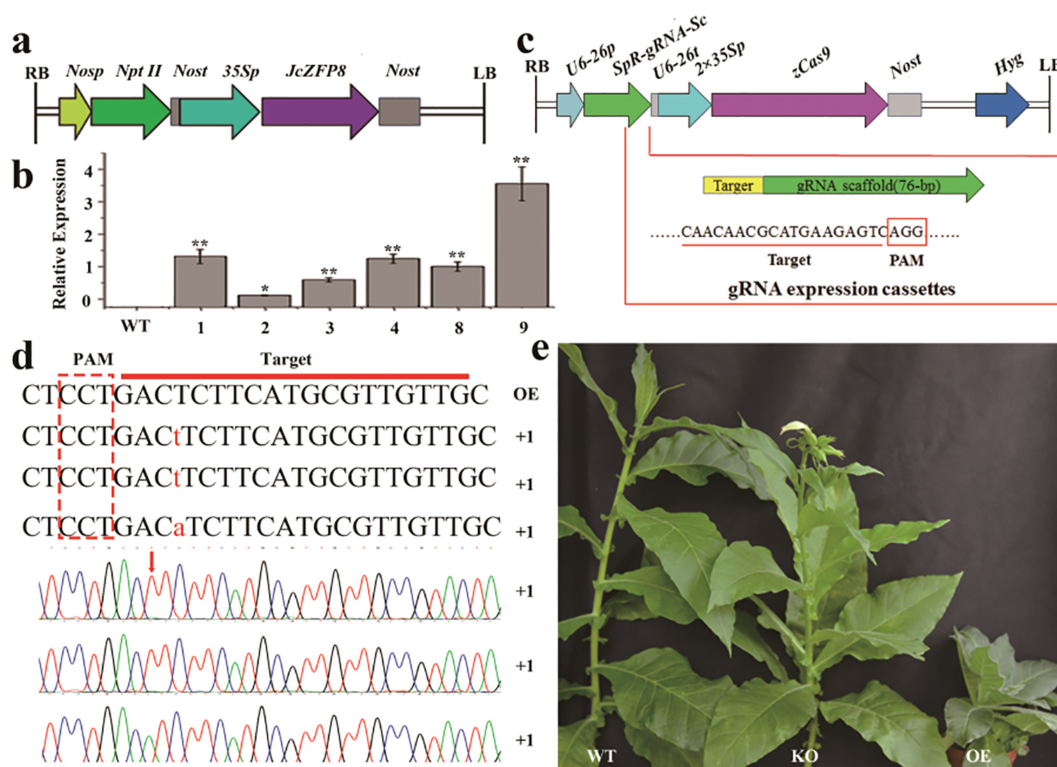

Fig. 2. Identification of transgenic plants and *JcZFP8* knockout by the Crispr-cas9 method. (a) Map of the vector used for overexpression of *JcZFP8*. LB/RB, left/right border; *35Sp*, *35S* promoter; *Nosp*, *NOS* promoter; *Npt II*, neomycin phosphotransferase gene; *Nost*, *NOS* terminator. (b) Transcription levels of *JcZFP8* in transgenic tobacco lines (OE). (c) Crispr-cas9 vector carrying a gRNA targeting the *JcZFP8* gene. PAM, the putative cleavage site (red box); *U6-26p*, Arabidopsis *U6* gene promoter; *U6-26t, U6-26* terminator with downstream sequence; *zCas9, Zea mays* codon-optimized *Cas9*; *gRNA-Sc*, gRNA scaffold. (d) Sequence confirmation of target gene mutations of a representative *JcZFP8* gene. Red arrow and lowercase letters indicate the mutation sites. (e) Phenotypes of the wild type (WT), knockout tobacco line (KO), and overexpressing transgenic tobacco line (OE).

increase in plant growth (Fig. 3a and b). Compared with the WT plant, transgenic plants had an obvious dwarf phenotype and shortened internode. After 135 days, the height of WT plants was approximately threefold higher than that of the transgenic plants. When we observed the leaf morphology of WT and transgenic plants, we found that the leaves of transgenic plants were smaller and narrower than those of the WT plants (Fig. 3c and d) and also had a higher density of trichomes (data not shown).

After *JcZFP8* KO in OE plants, the KO tobacco showed no obvious alterations in plant development compared to WT plants, which further indicated that overexpression of *JcZFP8* leads to significant changes in tobacco phenotypic traits (Fig. 2e).

3.5. Plant height in response to GA

Because *JcZFP8*-OE lines displayed a dwarf phenotype, which is similar to that of GA mutants, we considered the possibility that *JcZFP8* may regulate the expression of GA-related genes controlling morphogenesis. This prompted us to investigate whether the overexpression of *JcZFP8* is associated with GA synthesis and signal transduction.

We therefore assessed the effects of GA and PAC on the growth of transgenic and WT plants (Fig. 4a and b). The effectiveness of PAC treatment was evident from the significant decrease in plant height. In the presence of PAC, the development of both WT and transgenic plants was almost completely inhibited. Moreover, growth inhibition in the transgenic tobacco plants was observed to be more pronounced. After GA3 application, the height of transgenic lines was fourfold greater than that of plants without GA treatment. The phenotypes of the transgenic lines were partially restored and similar to those of the control. These results demonstrated that GA signaling pathways might function normally in transgenic plants.

4. Discussion

Plant height is an important plant trait that is closely related to yield. Therefore, reducing plant height is of considerable significance in the production of *J. curcas* [24]. Breeding dwarf varieties is an effective approach to adapting crops to mechanization and reducing production costs for oilseed plants [25]. In this study, *JcZFP8* from *J. curcas* was identified and characterized, and we demonstrated that this gene can cause plant dwarfism in transgenic tobacco plants. The findings of this study could therefore help us to guide the theory of plant development in *J. curcas* and accelerate the bioengineering of this plant.

In plants, C2H2 ZFPs share a similar structure. In addition to containing a conserved zinc finger structure, most of these proteins also contain the QALGGH sequences that involved in DNA binding [3, 4]. The QALGGH sequence is located inside the zinc finger helices, whereas animal and yeast C2H2 ZFPs do not contain this motif. This element was originally identified in petunia and was subsequently found in *Arabidopsis*. On the basis of the tandem or dispersed nature of ZFPs in *Arabidopsis*, 176 C2H2 ZFPs can be classified into A, B, and C sets, of which 81% contain the QALGGH motif, which is consistent with petunia [26]. QALGGH elements are also found in the ZFPs of other dicots such as poplar [27,28]. This element also appears in monocots such as millet and wheat [29,30]. Research shows that among millet C2H2 ZFPs, 97% harbor the QALGGH motif, and these ZFPs are accordingly designated as Q-type zinc fingers [30]. Through structural analysis, we found that JcZFP8 contains the QALGGH motif in the C2H2 structure, which is similar to that in the C2H2 ZFPs of other plants.

ZFPs are one of the largest gene families in plants and play an important regulatory role in plant-specific biological processes such as flower development, epidermal hair formation, morphogenesis, seed development, and apoptosis [3]. Previous studies have shown that ZFPs

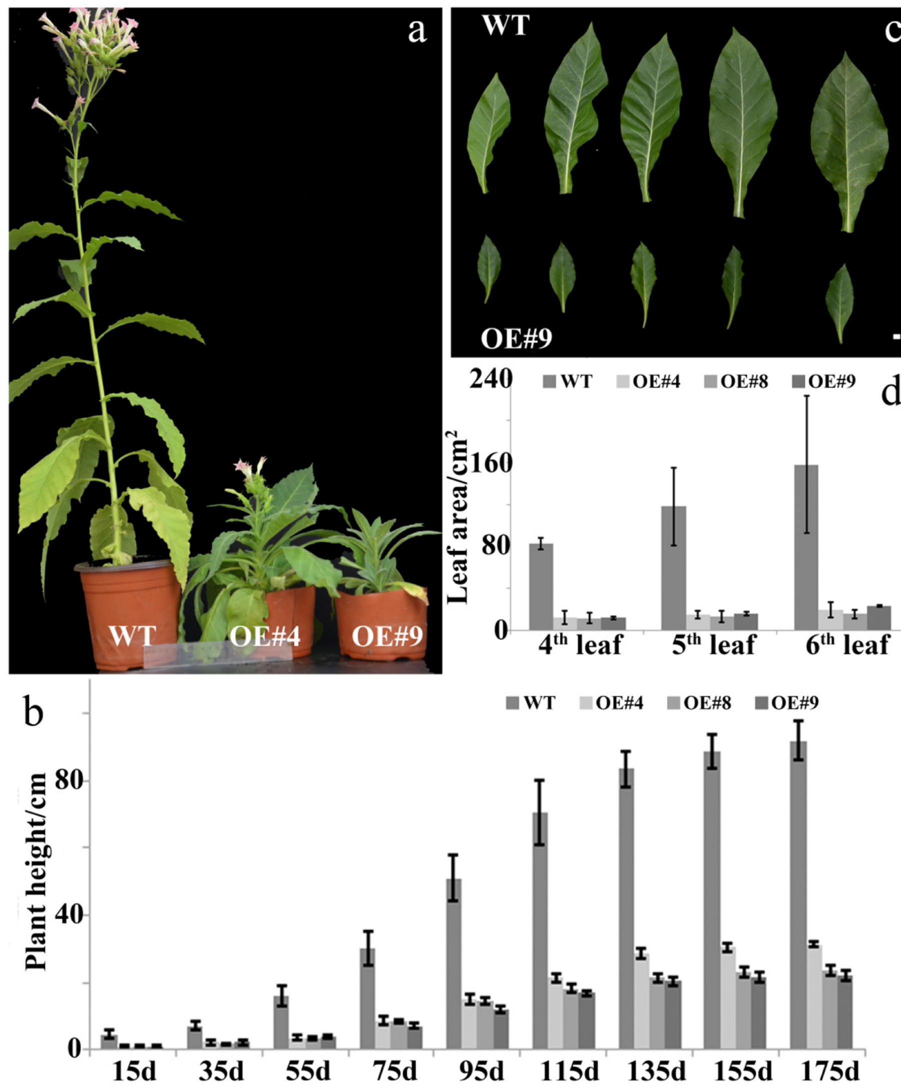

Fig. 3. Effects of *JcZFP8* overexpression on the height of tobacco plants. (a) Plant phenotype of *JcZFP8*-overexpressing OE#4 and OE#9 lines and wild-type (WT) plants. (b) Height of transgenic line seedlings compared to that of WT seedlings from day 15 to 175 under glasshouse conditions. (c) Leaves from transgenic lines and WT plants at 175 days. (d). Comparison of leaf sizes. The error bars represent the standard deviation from three independent experiments.

can play a regulatory role by binding to DNA, RNA, and proteins [1]. Most studies have indicated that plant ZFPs with a single zinc finger structure are mainly involved in various stages of plant growth and development. However, the C2H2-type ZFPs associated with various abiotic stresses are generally typical, double zinc finger structures. JcZFP8 contains a single zinc finger motif and may thus be involved in plant development. The findings of the present study are consistent with this assumption. Nevertheless, previous studies have also demonstrated that regulation by ZFPs with a single zinc finger structure plays a role in stress resistance. For example, when *ZAT10* is overexpressed, transgenic plants

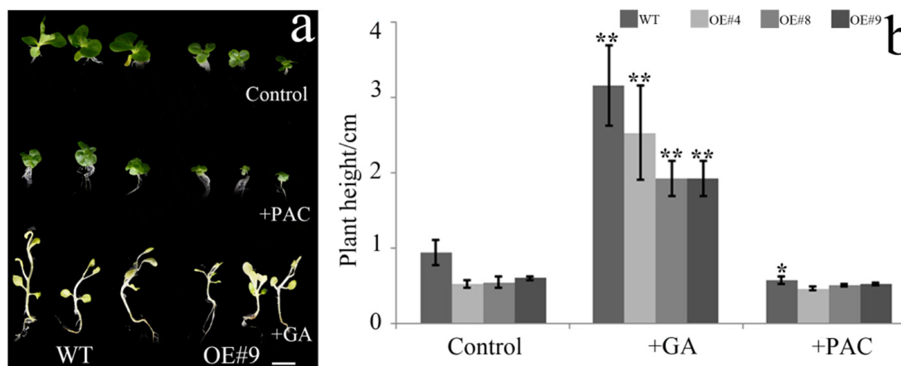

Fig. 4. Application of endogenous gibberellin3 (GA3) and paclobutrazol (PAC) in *JcZFP8*-overexpressing lines. (a) Plant phenotype of *JcZFP8*-overexpressing lines and wild-type (WT) plants treated with 10 μM GA3 and PAC. (b) Comparison of plant heights. The error bars represent the standard deviation from three independent experiments.

show enhanced tolerance to osmotic stress, for which the regulation of MAP kinases and the phosphorylation of ZAT10 are required [31]. However, whether *JcZFP8* can enhance plant stress resistance needs to be further verified.

Studies in model plants show that plant C2H2 ZFPs are involved in the regulation of plant height. Overexpression of petunia *PhSUP1* leads to dwarfing in transgenic plants, accompanied by changes in petals and leaves [32]. Petunia *LIF* genes affect plant development by reducing cell number and size, thus resulting in plant dwarfing [11]. This indicates that the C2H2 ZFPs can have a significant influence on plant development, which is consistent with the tobacco traits resulting from the heterologous expression of *JcZFP8* observed in the present study. In addition to plant dwarfing, transgenic plants also showed abnormal growth of other organs, thus indicating that the signaling network is complex and may involve the regulation of one or more types of genes. A similar phenomenon was also found in *JcZFP8* transgenic tobacco plant (data not shown).

Many ZFPs can affect the growth and development of plants through hormone signaling pathways. When a single *Arabidopsis* ZFP gene, *ZFP10*, was overexpressed in tobacco, transgenic plants displayed dwarfing, abnormal leaf phenotypes, and early flowering, and although exogenous application of GA3 reduced the dwarf phenotype, it did not restore normal plant height [33]. Sun et al. [34] found that overexpression of the C2H2 ZFP gene *URO* could alter the balance of auxin (IAA) and influence plant phenotypic changes in *Arabidopsis*. Furthermore, the C2H2 transcription factors *ZFP5* and *ZFP6* have been shown to regulate trichome initiation and development by integrating GA and cytokinin signaling [12]. GA plays an important role in plant height, and mutations of GA synthesis and signal transduction processes can cause plant dwarfism. Overexpression of *JcZFP8* in tobacco resulted in traits similar to those seen in GA mutants, and plant height could be partially restored through the application of GA.

In conclusion, *JcZFP8*, a member of the C2H2 ZFP gene family from *J. curcas*, was isolated and characterized. We found that JcZFP8 was localized in the nucleus and contained the characteristic plant-specific QALGGH conserved sequence. Overexpression of *JcZFP8* in tobacco resulted in dwarfing and development of smaller leaves, thereby indicating that JcZFP8 plays a role in plant development. In addition, application of exogenous GA rescued the dwarf phenotype in transgenic plants. Future studies will focus on investigating the mechanisms underlying the role of *JcZFP8* in developmental control.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2018.05.008

Conflict of interest

The authors declare that they have no conflict of interests.

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