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ARTICLE

Brassinosteroid Biosynthetic Gene *CmDWF4* Regulates Bud Outgrowth in *Chrysanthemum morifolium*

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ABSTRACT

Brassinosteroids (BRs), a class of steroid phytohormones, play a critical role in plant growth and development. The *DWF4* gene encodes a cytochrome P450 enzyme (CYP90B1), which is considered a rate-limiting enzyme in BR biosynthesis. Here, we identified a homologous gene of *DWF4* in chrysanthemum, *CmDWF4*. This gene was predicted to encode 491 amino acid residues with a molecular weight of 56.2 kDa and an isoelectric point (pI) of 9.10. Overexpression of *CmDWF4* in chrysanthemum was found to significantly increase growth rate, number, and length of lateral buds. Transcriptome analysis showed that multiple xyloglucan endotransglycosylase/hydrolase (XTH) family encoding genes associated with cell wall modification were up-regulated in *CmDWF4*-overexpressing lines. qRT-PCR assay confirmed the up-regulation of *CmXTH6*, *CmXTH23*, and *CmXTH28* in *CmDWF4*-overexpression line. Overall, this work establishes a mechanism by which BR biosynthetic gene *CmDWF4* promotes lateral bud outgrowth in chrysanthemum, possibly through regulating cell elongation and expansion.

KEYWORDS

BR; axillary bud; shoot branching; XTHs; cell elongation

1 Introduction

Shoot branching is a major determinant of plant morphogenesis and a key trait that determines the yield potential of agricultural, horticultural, and forestry crops [1]. Optimizing shoot architecture significantly improves the ornamental value of cut flowers, especially that of chrysanthemums [2]. Shoot branching can be divided into two important stages, namely the formation of the axillary meristem (AM) and the outgrowth of axillary buds. The formation of AM is regulated by internal factors [3], while the outgrowth of axillary buds is controlled by both internal and external factors, including environment, phytohormones, developmental stage, and key genes [3–5].

Among a range of factors that regulate bud outgrowth, hormonal signaling plays a principal role. Auxin was the first phytohormone discovered to be involved in the regulation of shoot branching by maintaining

apical dominance and inhibiting the growth of axillary buds [6,7]. In addition, strigolactone (SL) was found to inhibit bud outgrowth [8,9]. Cytokinin (CK) was reported to antagonize auxin and SL to promote bud outgrowth [10–12]. Auxin-CK-SL is a classic model to explain the control of bud outgrowth, which is summarized as 'the second messenger model' and/or 'the auxin transport canalization-based model' [13–15]. Apart from these three hormones, recent reports suggested that BR is also involved in the regulation of shoot branching development [16,17].

BRs, a class of steroid plant hormones, were originally characterized for their function in cell elongation [18]. They were discovered to have a crucial function in plant growth and development [19,20]. In rice, BR deficient mutants as well as BR signal transduction blocked mutants showed significantly reduced tiller numbers compared with the wild type [16,21]. Conversely, transgenic rice with enhanced BR signaling developed more tillers compared with the wild type [16]. In *Arabidopsis*, BES1, a positive regulator of the BR signaling pathway, regulates shoot branching through SLs-mediated signaling [22–24]. This leaves doubts about the branching regulation function of BRs in dicotyledonous plants. However, in the latest study, BRs have been found as the major signal integrating multiple pathways to control bud outgrowth in tomato [17]. In general, the function of BR in the regulation of bud outgrowth is still rarely reported. More evidence is needed to understand the role of BR in the regulation of shoot branching in dicots.

The biosynthesis of BR requires a variety of enzymes, which are encoded by the genes *STE*/*DWF7*, *DWF5*, *DWF1*/*DIM*, *DWF4*/*CYP90B1*, *CPD*/*CYP90A1*, *DET2*/*DWF6*, *ROT3*/*CYP90C1*, and *CYP85A1*/*2*/*BR6OX1*/*2* [25,26]. In the process of steroid biosynthesis, these genes were obviously up-regulated [27]. The *DWF4* gene encodes a cytochrome P450 enzyme (CYP90B1) that mediates multiple 22α-hydroxylation steps, which is a rate-limiting step in BR biosynthesis [28,29]. CYP90B1 forms a complex with uniconazole or brassinazole, further inhibits BR biosynthesis [30]. Overexpression of *DWF4* leads to increased vegetative development, seed number, and seed yield in *Arabidopsis* and maize [31,32], as well as improved stress tolerance in *Brassica napus* and potato [33,34] and enhanced fruit quality in tomato [35]. However, the mechanism by which *DWF4* regulates shoot branching remains unclear.

Chrysanthemum (*Chrysanthemum morifolium*) is a commercially important ornamental species with various branching types. However, remove lateral buds in chrysanthemum cultivation is labor and time consuming. Therefore, controlling chrysanthemum branch number is essential and has long been attracted the attention of breeders. Here, we identified a BR biosynthetic gene *CmDWF4*, and generated transgenic chrysanthemum, we found that *CmDWF4* regulates shoot branching in chrysanthemum. Then, the regulatory mechanism of *CmDWF4* was explored by transcriptome sequencing and quantitative analysis of transgenic lines. This study provides a further understanding of the function of the *DWF4* gene and adds evidence for the role of BR in regulating shoot branching in chrysanthemum.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

The chrysanthemum cultivar 'Jinba' was used as the wild type (WT). Transgenic plants OX-2, OX-10, and OX-23 were obtained via the *Agrobacterium*-mediated leaf disc method in the background of WT. Chrysanthemum was propagated asexually by cuttings. The cuttings were cultivated in 12 × 6 aperture disks with a mixture of vermiculite and nutritional soil (1:1, vol/vol). After rooting, the consistent plants were transferred to pots containing the same substrate. The plants were cultivated in a controlled environment (22°C ± 2°C, 16 h of light and 8 h of darkness, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, 70% relative humidity) [36]. When the plants were relocated to the pots for 30 days, water was supplied once a week using a nitrogen-rich nutrient solution.

2.2 Isolation of CmDWF4 and Sequence Analysis

The sequence information of the BR biosynthetic gene *CmDWF4* was obtained from chrysanthemum transcriptome and TAIR website (https://www.arabidopsis.org/Blast/index.jsp). The full-length ORF

sequence of *CmDWF4* (1467 bp) was cloned with the primer pair *CmDWF4*-F and *CmDWF4*-R from the cDNA of 'Jinba'. All primers used in this study (Table S1) were designed using the Primer Premier 5 software. Amino acid sequence alignment with functionally reported genes *AtDWF4* [31], *StDWF4* [34], *GhDWF4* [35], and *ZmDWF4* [32] was performed using DNAMAN software. The construction of a phylogenetic tree was carried out using the MEGA-X software [37].

2.3 Vector Construction and Chrysanthemum Transformation of pORE-R4-CmDWF4

Restriction enzyme sites *Bam*H I and *Eco*R I were inserted into the cloned *CmDWF4* gene by primers *CmDWF4-Bam*H I-F and *CmDWF4-Eco*R I-R (Table S1). The PCR products and pORE-R4 vector were digested with 2.5 μL *Bam*H I and 2.5 μL *Eco*R I (Takara, Japan). To join the target gene fragment with the target vector, 5.0 μL Solution I (Takara, Japan) was utilized. The generated vector pORE-R4-*CmDWF4* was transferred into *Agrobacterium tumefaciens EHA105* using the electro-transformation approach. The *Agrobacterium*-mediated leaf disc transformation method [36] was used to infect pORE-R4-*CmDWF4* into chrysanthemum 'Jinba'. MS solid medium with 7 $mg{\cdot}L^{-1}$ kanamycin was used to screen for normal rooting lines. DNA was extracted from rooted seedlings for identification to obtain *CmDWF4*-overexpressing lines.

2.4 Treatment and Measurement of Lateral Buds

When the plants were 45 days old, the leaves and shoot apex above the third node were removed with a disinfection blade as a decapitation treatment. Nodes were numbered from top to bottom and the first fully expanded leaf was the first node. After decapitation for 6 days, the length of the first three lateral buds from the stem to the top of the bud was measured with a 20 cm measuring ruler as the total elongation. As we have found that the first three lateral buds elongated significantly after decapitation in chrysanthemum 'Jinba'. Lateral bud length of intact plants was measured starting from the stem and was simulated from the 6^{th} to the 39^{th} node by HeatMap of TBtools software [38].

2.5 Quantitative Real-Time PCR (qRT-PCR) Analysis

After decapitation treatment in WT, the first three lateral buds were collected from each plant at 0, 12, 24, 36, 60, 84, and 108 h, respectively, and three plants were used as one biological replicate. The root, stem, leaf, top bud, and axillary bud of WT were also sampled for analysis of the expression pattern of *CmDWF4*, using three plants as a biological replicate. Transgenic plants OX-2, OX-10, and OX-23 were extracted from the leaves, with three plants used as a biological replicate. Total RNA was extracted using the Quick RNA isolation Kit (Huayueyang, China) according to the operation manual. The chrysanthemum *CmUbiquitin* [39] gene was used as the reference sequence. The primer pair *CmDWF4*-RT-F and *CmDWF4*-RT-R and other quantitative primers used in this study are listed in Table S1. The qRT-PCR reaction system was as follows: 10 μL SYBR Premix Ex Taq™ II (Takara, Japan), 2 μL primer F/R (1 μM), 0.5 μL template cDNA (50 $ng{\cdot}\mu L^{-1}$), and 5.5 μL ddH_2O. The $2^{-\Delta\Delta Ct}$ method [40] was used to calculate relative expression levels. The results are shown as the average of three biological replicates.

2.6 Transcriptome Analysis

Quantitatively validated transgenic lines OX-2, OX-10, OX-23, and WT were sent to the Annoroad Gene Tech. (Beijing) Co., Ltd. (China) for RNA sequencing. RNA was extracted from 30-day-old chrysanthemum leaves. To predict unigene expression levels in the four samples, we used the fragments per kilobase of exon model per million mapped fragments (FPKM) method. FPKM of different samples was visualized by HeatMap of TBtools software [38]. The RNA-seq raw data of 12 samples generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) under accession number PRJNA915417.

2.7 Statistical Analysis

The expression of *CmDWF4* in different tissues and qRT-PCR analysis of *CmDWF4* with the 20 mM sucrose treatment was performed by statistical variance analysis using the IBM SPSS Statistics 25 software. Other data were analyzed for significance using Student's *t*-test. The differences between mean values were separated at a level of $p < 0.05$ or $p < 0.01$.

3 Results

3.1 CmDWF4 Potentially Regulates Lateral Bud Outgrowth in Chrysanthemum

According to an effective culturing system using two-node stems [41], the BR biosynthetic gene *CmDWF4* was up-regulated with the 20 mM sucrose treatment, which promoted the growth of the upper bud [42]. Our qRT-PCR verification showed that *CmDWF4* was up-regulated by 4.01-folds in the upper buds treated with 20 mM sucrose (Fig. S1).

To further identify the potential role of *CmDWF4* in lateral bud growth, 45-day-old chrysanthemum seedlings were used for decapitation treatment. We then analyzed *CmDWF4* expression using the RNAs extracted from the axillary buds at 0, 12, 24, 36, 60, 84, and 108 h. Results showed that decapitation led to a rapid increase in the transcriptional level of *CmDWF4* within 12 to 24 h (Fig. 1), indicating that *CmDWF4* may play an important role in lateral bud outgrowth in chrysanthemum.

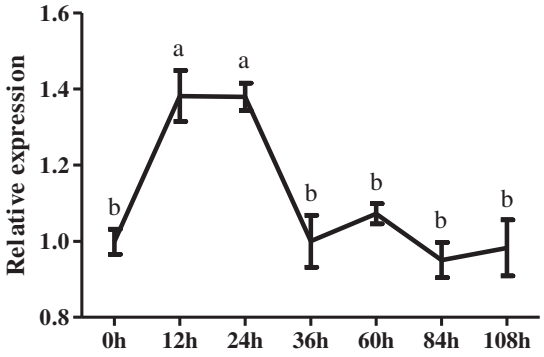

Figure 1: Expression pattern of the *CmDWF4* gene in axillary buds in response to decapitation. Data are presented as means ± SE (n = 3). Significant differences are indicated by different letters ($p < 0.05$)

3.2 Expression Pattern of CmDWF4

Next, we examineed the expression of *CmDWF4* in root, stem, leaf, top bud, and axillary bud of the 45-day-old chrysanthemum seedlings. We found highest expression of *CmDWF4* in leaves, which was 14.93 times than that in roots. Moreover, we found that the transcriptional level of *CmDWF4* in axillary buds was 1.43 times higher than that in the top buds (Fig. 2).

3.3 Cloning and Characterization of CmDWF4

The full-length *CmDWF4* sequence was cloned by specific primers (*CmDWF4*-F and *CmDWF4*-R, Table S1) from the cultivar 'Jinba' cDNA. Sequence analysis revealed that the open reading frame (ORF) of *CmDWF4* was 1476 bp, which was predicted to encode 491 amino acid residues with a molecular weight of 56.2 kDa and an isoelectric point (pI) of 9.10. The predicted amino acid sequence of *CmDWF4* contained four conserved features of the CYP450 family: domains A, B, and C and heme-binding domain (Fig. 3A). A phylogenetic analysis implied that CmDWF4 was most closely related to *Artemisia annua* protein AaDWF4 (Fig. 3B).

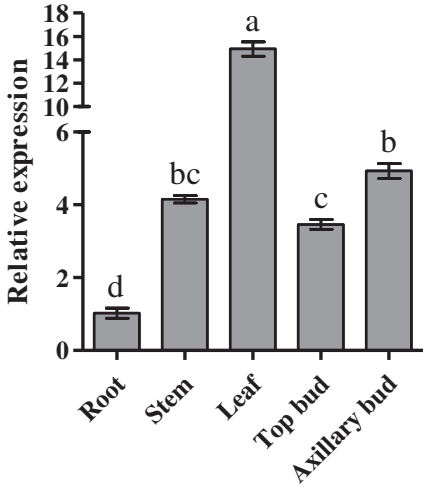

Figure 2: Expression profiles of *CmDWF4* in different tissues of chrysanthemum. Data are presented as means ± SE (n = 3). Significant differences are indicated by different letters ($p < 0.05$)

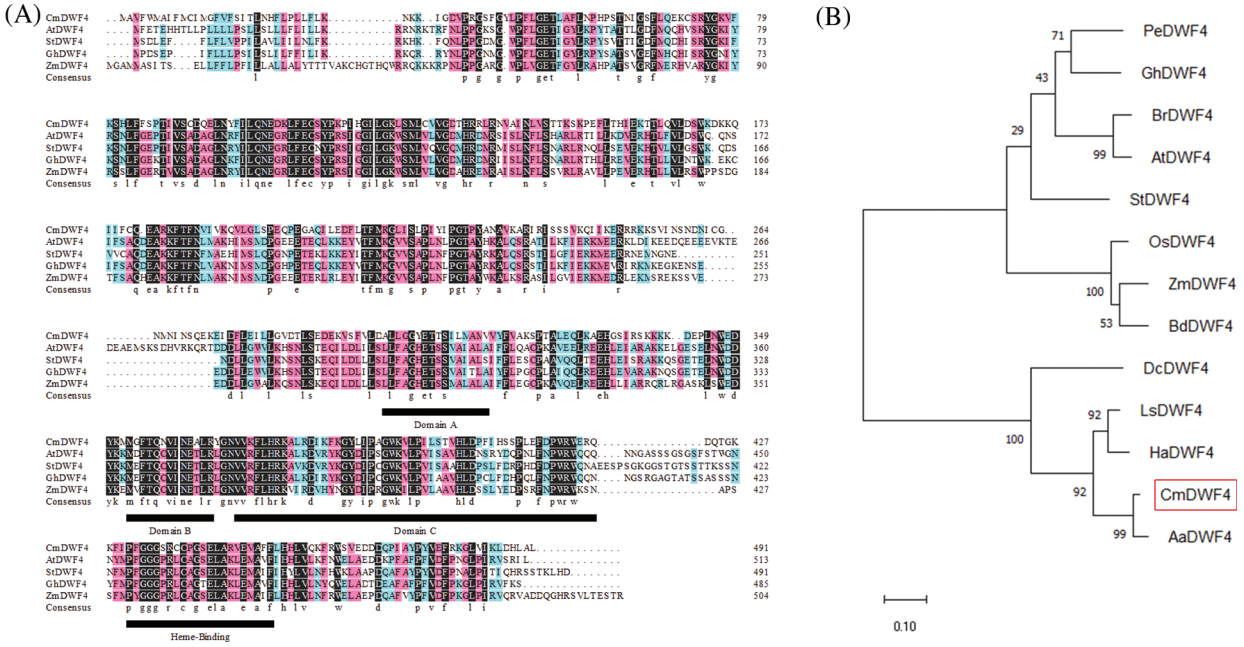

Figure 3: Sequence analysis of CmDWF4 and related DWF4s. (A) Polypeptide alignment of CmDWF4 with DWF4s from *Arabidopsis*, potato, cotton, and maize. The conserved features of the CYP450, domains A, B, and C, and heme-binding domain were indicated by underlining. (B) Phylogenetic tree of *CmDWF4* and related DWF4s. The GenBank accession numbers of P450s are as follows: PeDWF4 (*Populus euphratica*, HQ452827.1), GhDWF4 (*Gossypium hirsutum*, NP_001313765.1), BrDWF4 (*Brassica pekinensis Rupr.*, Bra030023.1), AtDWF4 (*Arabidopsis thaliana*, NP_190635.1), StDWF4 (*Solanum tuberosum*, XM_006340546.1), OsDWF4 (*Oryza sativa*, AB206579.1), ZmDWF4 (*Zea mays*, EF519871.1), BdDWF4 (*Brachypodium distachyon*, KQK22730.1), DcDWF4 (*Daucus carota var. sativa Hoffm.*, DCAR_017553), LsDWF4 (*Lactuca sativa*, XP_023770054.1), HaDWF4 (*Helianthus annuus*, XP_021998202.1), and AaDWF4 (*Artemisia annua*, PWA81420.1)

3.4 Effects of CmDWF4 on Lateral Bud Initiation and Outgrowth

To further investigate the function of *CmDWF4* in chrysanthemum, we generated the overexpression lines of *CmDWF4* in the cultivar 'Jinba' by introducing the plasmid pORE-R4-*CmDWF4*. Positive transgenic lines were identified by conducting a genomic PCR assay using primers *pORE-R4-F* and *CmDWF4-R2* (Table S1). Finally, three independent transgenic lines were obtained: OX-2, OX-10, and OX-23 (Fig. S2). qRT-PCR analysis revealed that the transcriptional level of *CmDWF4* in OX-2, OX-10, and OX-23 was 3.02, 5.19, and 8.19 times than that in WT, respectively (Fig. 4C).

The 45-day-old *CmDWF4* transgenic lines OX-2, OX-10, OX-23, and WT were decapitated at the same time. The growth of three lateral buds at the top of the plants was observed after 6 days in the long-day culture chamber. It was found that the first three lateral buds of the overexpression lines OX-2, OX-10, and OX-23 were significantly longer than those in WT (Fig. 4A). Statistical analysis showed that the length of the total three lateral buds of OX-2, OX-10, and OX-23 was 5.17, 5.00, and 5.20 cm, respectively, which were significantly greater than the 3.13 cm length of lateral buds in WT (Fig. 4B), indicating an essential role of *CmDWF4* in lateral bud initiation and outgrowth in chrysanthemum.

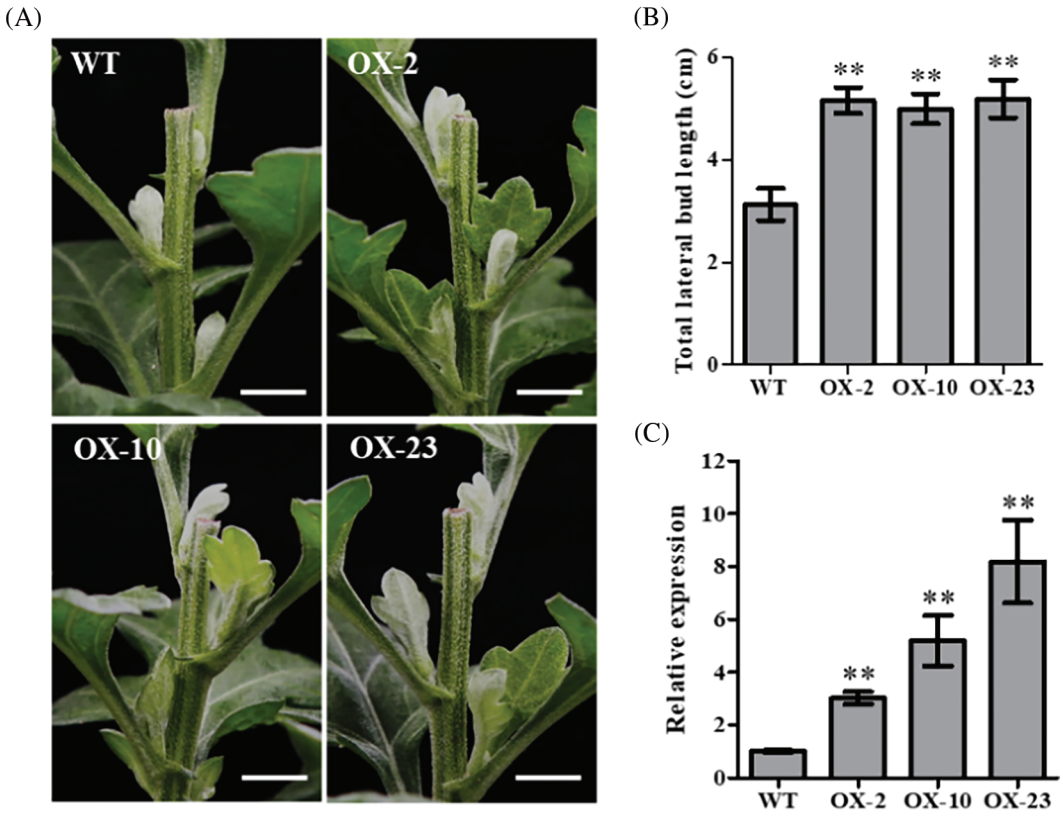

Figure 4: Effects of *CmDWF4* on lateral bud initiation and outgrowth after decapitation. (A) Lateral bud outgrowth phenotypes of overexpressed *CmDWF4* lines in the 'Jinba' background after decapitation for 6 days. Scale bars represent 1.0 cm. (B) Statistics of the total length of the first three lateral buds. Data are presented as means ± SE (n = 6). (C) qRT-PCR analysis of relative expression of *CmDWF4* in transgenic lines. Data are presented as means ± SE (n = 3). Significant variations from WT are indicated with asterisks (**$p < 0.01$)

3.5 Effects of CmDWF4 on the Number and Length of Lateral Buds

The phenotype of 65-day-old *CmDWF4* transgenic lines was identified. It was found that the overexpressed lines OX-2, OX-10, and OX-23 had more and longer buds compared with WT (Fig. 5A). The length of lateral buds at the same node (from node 6 to node 39, S6–S39) was compared by a color scale. The results showed that 73.52% of the buds of OX-2, 85.29% of the buds of OX-10, and 70.59% of the buds of OX-23 were longer than the buds of WT (Fig. 5B). Statistical analysis showed that the number of lateral buds greater than 0.5 cm in OX-2, OX-10, and OX-23 was 36.0, 35.5, and 38.0, respectively, which was significantly larger than 30.0 in WT (Fig. 5C). The total lateral bud length of OX-2, OX-10, and OX-23 was 68.95, 69.33, and 58.30 cm, respectively, which was also significantly greater than 43.73 cm in WT (Fig. 5D). These results indicated that *CmDWF4* positively regulates the growth of lateral bud in chrysanthemum plants.

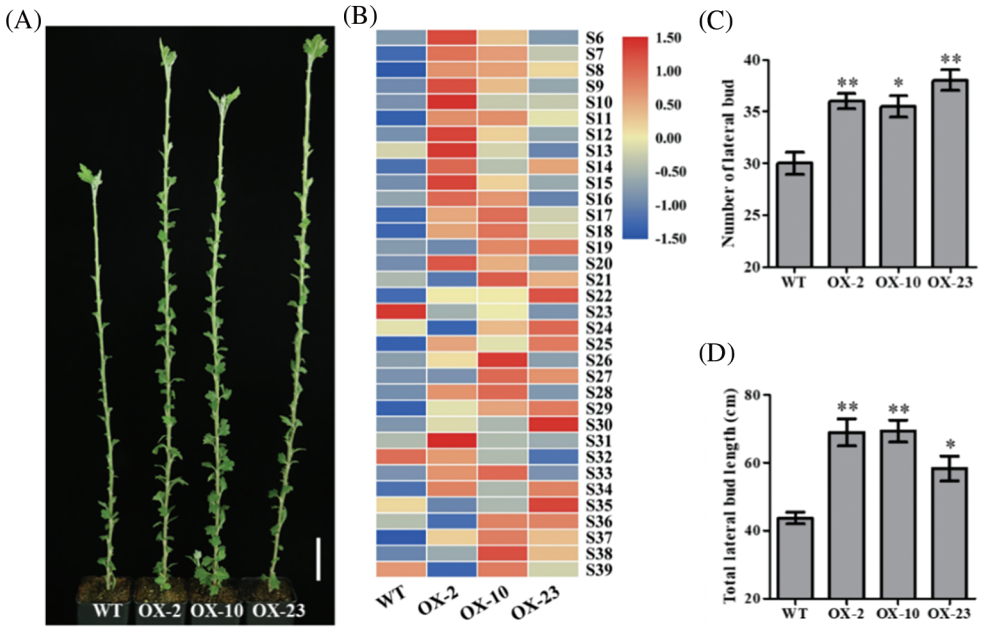

Figure 5: Effects of *CmDWF4* on the number and length of lateral buds in intact chrysanthemum plants. (A) Lateral bud outgrowth phenotypes of overexpressed *CmDWF4* lines. Scale bar represents 5.0 cm. (B) Comparison of lateral bud length at the same node with the color scale. S6–S39 represents the 6th to the 39th node. (C) The number of lateral buds longer than 0.5 cm in (A). (D) Total lateral bud length of the plants in (A). Data are presented as means ± SE (n = 4). Significant variations from WT are indicated with asterisks (*$p < 0.05$, **$p < 0.01$)

3.6 CmDWF4 Promotes Lateral Bud Outgrowth through Regulating Cell Elongation and Expansion

To explore the mechanism of *CmDWF4* in regulating chrysanthemum bud outgrowth, we performed transcriptome sequencing using *CmDWF4*-overexpressing lines OX-2, OX-10, and OX-23. Five differentially expressed genes related to BR signaling were identified, and their expression levels were up-regulated in *CmDWF4*-overexpressing lines (Fig. 6B). In addition, multiple xyloglucan endotransglycosylase/hydrolase (XTH) family encoding genes were up-regulated in OX-2, OX-10, and OX-23 compared to WT (Fig. 6A). XTHs modify a major structural component of the plant cell wall, xyloglucan, and therefore may influence cell elongation and expansion [43–45]. qRT-PCR was used to verify the transcript levels of *XTHs* in *CmDWF4*-overexpressing transgenic lines. Result showed that three *XTHs* genes in OX-2, OX-10, and OX-23 lines were significantly up-regulated compared with WT

plants (Figs. 6C–6E). The expression levels of *CmXTH6*/*CmXTR10* in OX-2, OX-10, and OX-23 were 1.74, 1.47, and 1.89 times that of WT, respectively (Fig. 6C). The expression levels of *CmXTH23*/*CmXTR6* in OX-2, OX-10, and OX-23 were 1.82, 1.78, and 2.87 times that of WT, respectively (Fig. 6D). The expression levels of *CmXTH28*/*CmEXGT-A2* in OX-2, OX-10, and OX-23 were 2.05, 1.87, and 1.86 times that of WT, respectively (Fig. 6E). The data implied that overexpression of *CmDWF4* might regulate the expression of cell wall-related genes to stimulate cell elongation and expansion to promote lateral bud outgrowth.

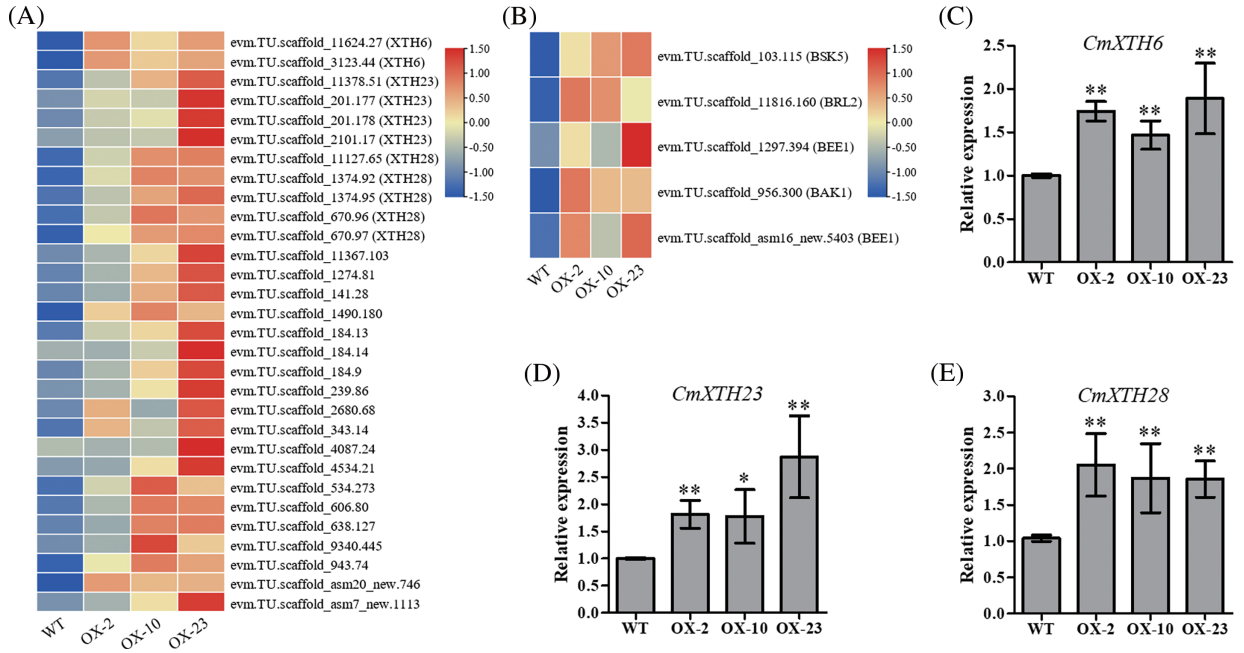

Figure 6: Transcriptome analysis and quantitative validation of *CmDWF4* overexpressing lines. (A) Expression levels of *XTHs* genes in WT, OX-2, OX-10, and OX-23 RNA-seq data. (B) BR signaling-related genes expression levels in WT, OX-2, OX-10, and OX-23 RNA-seq data. Data are presented as means (n = 3) of FPKM by $\log_2$ scale and row scale. (C) The relative expression level of *CmXTH6* by qRT-PCR analysis. (D) The relative expression level of *CmXTH23* by qRT-PCR analysis. (E) The relative expression level of *CmXTH28* by qRT-PCR analysis. Data are presented as means ± SE (n = 3). Significant variations from WT are indicated with asterisks (*$p < 0.05$, **$p < 0.01$)

4 Discussion

Plant growth and development are aided by BRs, a class of plant-specific steroid hormones. BRs promote tillering in rice [16] and bud outgrowth in tomato [17]. The cytochrome P450 enzyme (CYP90B1) encoded by the *DWF4* gene is a rate-limiting step in BR biosynthesis [28–30]. Previous studies have demonstrated that the BR biosynthetic gene *CmDWF4* was up-regulated in the growing bud with the 20 mM sucrose treatment (Fig. S1) [41,42]. The role of sucrose in promoting plant bud outgrowth has been widely reported [46,47]. In this study, the expression of *CmDWF4* increased in lateral buds of WT within 12 to 24 h in response to decapitation treatment (Fig. 1). Therefore, we speculated that *CmDWF4* was involved in the regulation of lateral bud outgrowth in chrysanthemum.

Transgenic experiments were conducted to verify whether *CmDWF4* was involved in the regulation of lateral bud outgrowth. After obtaining *CmDWF4* overexpressing lines OX-2, OX-10, and OX-23, the growth rate, number, and length of the lateral buds were observed in the intact plants or decapitated plants. The

results showed that the initiation and sustained growth of lateral buds were promoted in OX-2, OX-10, and OX-23 lines (Figs. 4 and 5). In *Arabidopsis*, *AtDWF4* functions to increase vegetative growth and seed yield [31]. In maize, *ZmDWF4* improves photosynthetic ability and enhances yield [32], indicating that *CmDWF4* has functional similarity with *AtDWF4* in *Arabidopsis* and *ZmDWF4* in maize in shoot branching regulation. However, in *Arabidopsis* and maize, no reports showed how *DWF4* regulates the lateral bud outgrowth. In other species, the function of *DWF4* in regulating lateral bud outgrowth has not been reported. This study provides new clues on the shoot branching regulation of the *DWF4* gene in different species by the homologous transformation of *CmDWF4* in chrysanthemum.

Transcriptome analysis of *CmDWF4* overexpressing lines showed that multiple xyloglucan endotransglucosylases/hydrolases (XTHs) encoding genes were up-regulated (Fig. 6A). Enzymologically, xyloglucan endotransglucosylase (XET) and xyloglucan endohydrolase (XEH) activities are used to describe the XTHs family of enzymes. XTHs modify a major structural component of the plant cell wall, xyloglucan, and thus may affect cell elongation and expansion [43–45]. It has been reported that exogenous BL promotes the expression of *XTHs* genes [48,49]. The key brassinosteroid signaling pathway transcription factor *BES1* acts directly upstream of *XTH19* and *XTH23* to control their expression [50]. However, the regulatory relationship of *DWF4* on *XTHs* is rarely reported. The family of 33 *Arabidopsis XTHs* genes is divided into three major groups according to the genetic structure. *XTH6* [51], *XTH23* [50], and *XTH28* [44] belong to group 1, group 2, and group 3, respectively [45]. Quantitative validation in our study showed that the expression of *CmXTH6*, *CmXTH23*, and *CmXTH28* was significantly increased in OX-2, OX-10, and OX-23 lines (Figs. 6C–6E), suggesting that overexpression of *CmDWF4* might result in cell elongation and expansion by upregulating the expression of the *XTHs* gene. Eventually promoted chrysanthemum shoot branching.

BR is widely known to promote cell elongation [18,52]. The response of *XTH23* to BR regulation has been recorded [50]. In this study, the transcriptional level of *CmDWF4* was significantly increased in OX-2, OX-10, and OX-23 lines (Fig. 4C). The expression levels of BR signaling-related genes were up-regulated in *CmDWF4*-overexpressing lines, including *BAK1* (BRI1 associated receptor kinase 1) [53,54], *BSK5* (brassinosteroid-signaling kinase 5) [55], *BEE1* (BR enhanced expression 1) [56], and *BRL2* (BRI1-like receptor kinase 2) [57] (Fig. 6B). This indicated that BR content in *CmDWF4*-overexpressing lines was indeed increased. Together, these data provide strong evidence that the lateral bud outgrowth phenotypes and associated gene expression level changes in transgenic lines are due to elevated BR levels.

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Author Contributions: Xianrong Fu performed most of the experiments and wrote the manuscript. Aiping Song and Fadi Chen directed experiments and revised the manuscript. Bo Peng, Song Li, Weixin Liu and Lingling Zhang assisted in the experiments. Fadi Chen, Jiafu Jiang and Sumei Chen supervised the research.

Ethics Approval: The authors declare that this article is in compliance with ethical standards of the journal.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

Table S1: Primers used in this study

Primer name	Sequence (5′ to 3′)
CmDWF4-F	ATGGCTGTTTTTTGGATGGCA
CmDWF4-R	TTAAAGGGCCAAATGGTCCAG
pORE-R4-F	GACGCACAATCCCACTATCC
CmDWF4-R2	TGATGATATTCTGATTCTAGCCTTAACT
*CmDWF4-Bam*H I-F	CGGGATCCATGGCTGTTTTTTGGATGGCAATC
*CmDWF4-Eco*R I-R	CGGAATTCTAAGGGCCAAATGGTCCAGCTTGA
CmUbiquitin-RT-F	AGCTGAGCAGACTCCCGATG
CmUbiquitin-RT-R	AGGCGATTCATCAGTACCAAGTG
CmDWF4-RT-F	TGATACCAGCAGGTTGGAAGG
CmDWF4-RT-R	GGACTCGAATGGATAAATGGGT
CmXTH6-RT-F	TGGGAAGCAGATGATTGGGC
CmXTH6-RT-R	CAGTTGGTTGGGTTGGACGC
CmXTH23-RT-F	AAAGAACCAACCAATGAGGATACAC
CmXTH23-RT-R	CTGAGCCACTGCCACAAGATG
CmXTH28-RT-F	CCCCTTACATTGCTGAGTTTTCC
CmXTH28-RT-R	TTTCGTCCTTTGTGATGGCGT

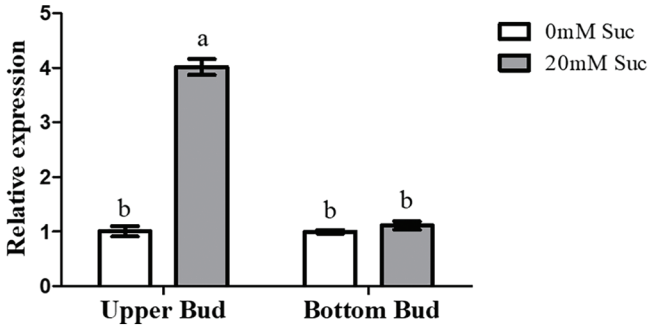

Figure S1: qRT-PCR analysis of *CmDWF4* with the 20 mM sucrose treatment. Data are presented as the means ± SE (n = 3). Significant differences are indicated by different letters ($p < 0.05$)

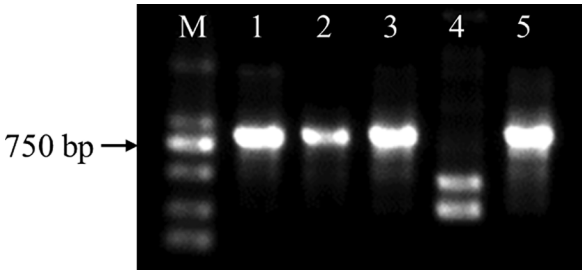

Figure S2: Identification of *CmDWF4* transgenic lines at the DNA level. M: DL2000 DNA Marker (TaKaRa); 1: OX-2; 2: OX-10; 3: OX-23; 4: WT; 5: plasmid pORE-R4-*CmDWF4*