Development of semi-dwarf Echinacea purpurea by inducing silencing of the endogenous brassinosteroid- biosynthetic gene DWF4

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Abstract

Brassinosteroids (BRs) are plant steroidal hormones that are crucial for plant growth and development, with dwarfism being a hallmark of BR deficiency. DWARF4 (DWF4) is a key enzyme that catalyzes the rate-limiting C22 oxidation step in the BR biosynthetic pathway. Understanding its role in ornamental plants is crucial for developing dwarf varieties that can significantly enhance their aesthetics and broaden their applications. This study aimed to provide the functional analysis of the *DWF4* gene isolated from *Echinacea purpurea*. We found that the *EpDWF4* gene comprised a 1458 bp open reading frame that encodes 486 amino acids and is highly expressed in leaves. EpDWF4 showed high homology to AtCYP90B1, a steroid 22-alpha-hydroxylase involved in the BR biosynthetic pathway in *Arabidopsis thaliana*. Transgenic *E. purpurea* plants with suppressed *EpDWF4* expression were generated using an RNA interference vector that produced hairpin-looped double-stranded RNAs, leading to sequence-specific RNA silencing of *EpDWF4*. This resulted in a dwarf phenotype. The physiological function of *EpDWF4* was evaluated by complementation in *Arabidopsis* plants. *EpDWF4* completely rescued the extremely dwarf phenotype of the *A. thaliana* dwf4-102 mutant. In addition, transgenic homozygous *A. thaliana* plants containing a copy of *35S: EpDWF4* in the intergenic region significantly increased in height. Moreover, steroid 22-alpha-hydroxylase activity, which converts campestanol to 6-deoxocathasterone, increased by 43% in these transgenic plants compared to wild-type *A. thaliana*. Our results indicate that the knockdown of BR biosynthesis-related genes by RNA interference (RNAi) supports valuable dwarf plant production for commercial horticultural or agricultural applications.

Introduction

In 1979, the structure of the first plant brassinosteroids (BRs) with physiological functions similar to those of auxins was obtained from bee-collected pollen of *Brassica napus L.*(Grove et al. 1979). The biosynthetic genes of BRs control various processes, including cell expansion, division, and differentiation; seed formation and germination; root growth and development; vascular differentiation; flowering time; photomorphogenesis; and tolerance to various abiotic stressors (Anfang and Shani 2021; Chen et al. 2024; Guo et al. 2024; He et al. 2024). Significant progress in understanding the action and biosynthesis of BR has been made through extensive research conducted on the model plant species *Arabidopsis thaliana*.(He et al. 2024; Planas-Riverola et al. 2019). Several *A. thaliana* mutants associated with BR biosynthesis and perception have been constructed, including *Atdwf4* (Choe et al. 1998), *Atdwf1* (Best et al. 2016; Klahre et al. 1998), *Atdwf7/Atste1* (Choe et al. 1999), *Atdet2* (Chory et al. 1991), *Atdwf11* (Tanabe et al. 2005), and *Atbri1* (Clouse et al. 1996). Mutants that disrupt the various stages of BR biosynthesis or signal transduction typically exhibit distinct dwarf phenotypes. These phenotypes are characterized by severely stunted growth, extended lifespan, delayed flowering and senescence, dark-green and small curled leaves, reduced fertility, and altered photomorphogenesis in darkness (Best et al. 2016; Mori et al. 2002; Nolan et al. 2020). In BR-deficient mutants, these phenotypes could be rescued to the wild type (WT) by the external application of BRs. Endogenous BR levels in plant tissues are significantly lower than those of other plant hormones and are tightly regulated by BR signaling (Kim and Russinova 2020).

The biosynthetic pathway of BRs is complex and highly interconnected. It involves two parallel pathways, including sterol- and BR-specific biosynthesis pathways. These pathways are further categorized into early and late C-6 oxidation pathways (Fujioka et al. 1998). Initially, early and late C-6 oxidation pathways were considered essential components of BR biosynthetic pathways. The presence of 6-deoxocastasterone suggests the existence of common rate-limiting steps in BR biosynthesis (Bishop et al. 1999). Subsequently, an early C-22 hydroxylation pathway distinguishable at the campesterol stage was identified. However, this understanding was revised after it was found that DWF4 acts on multiple biosynthetic intermediates during the upstream steps of BR biosynthesis.

DWF4 catalyzes C-22 hydroxylation, including an early C-22 oxidation pathway, and acts on multiple substrates such as CR, CN, and 6-oxo CN (Fujiyama et al. 2019). Plant sterols can also undergo hydroxylation, displaying different alkyl groups at C-24 of the 5-acholestane skeleton, such as C-27 (cholesterol), C-28 (campesterol), and C-29 (sitosterol), which are converted to 28-nobrassinolide, brassinolide, and 28-homobrassinolide, respectively (Nomura et al. 2001). The catalytic activity of DWF4 can be modulated by the quantity of enzymes present, post-translational modifications, or steric regulation by the substrate or final product. DWF4 mutants have been characterized in several plant species. In the woody plant apple, *MdDWF4* gene knockdown resulted in dwarfism (Zheng et al. 2018a, Zheng et al. 2018b). In rice, *Osdwarf4-1* mutants show typical traits, including erect leaves in mature stages, shortening of the second internode in the culm, and reduced grain length (Sakamoto et al. 2006). In contrast, ectopic overexpression of *DWF4* increased hypocotyl and inflorescence height, prolonged flowering, and increased the number of silique-bearing branches, seed yield, and BR levels (Choe et al. 2001). The function of *DWF4* has been elucidated in Brassicaceae (Choe et al. 1998), Rosaceae (Zheng et al. 2018b), Poaceae (Castorina and Consonni 2020), Solanaceae (Zhou et al. 2018), and Vitaceae (Parada et al. 2022).; however, it is poorly understood in Asteraceae plants.

Echinacea purpurea is an upright perennial herb belonging to the genus *Echinacea* in Asteraceae. Recent studies have focused on evaluating the pharmaceutical value of *E. purpurea*, which can be used as a dietary supplement to enhance immune function and treat upper respiratory tract infections (Gancitano et al, 2024). Furthermore, *E. purpurea* has significant economic value owing to its potential as an ornamental plant with extensive applications. It possesses large, showy flower heads and blooms from early to late summer months. However, the height of *E. purpurea*, which ranges from 100 to 150 cm, affects agricultural and horticultural outcomes, including poor growth and reduced resistance to wind and rain damage. BR controls plant height; however, genomic information related to BR biosynthesis in *E. purpurea* remains underexplored.

Dwarfism is a valuable agronomic and horticultural trait in flower breeding. It enhances lodging resistance and is ideal for indoor viewing. Most dwarfism genes, such as *CmDRP* in *Chrysanthemum morifolium*, have been cloned, functionally characterized, and used in plant breeding programs (Zhang et al. 2022). Plant hormones significantly influence plant height, and the role of BRs in determining plant height has been elucidated (Nolan et al. 2020). To develop dwarf ornamental *E. purpurea* plants, we used RNA interference to downregulate BR biosynthetic genes. However, BR-related genes in *E. purpurea*

remain unidentified. In the present study, we isolated *EpDWF4*, which is orthologous to *A. thaliana CYP90B1*, from *E. purpurea* and characterized its function in transgenic *E. purpurea* and *A. thaliana* plants. Dwarf *E. purpurea* plants with suppressed *EpDWF4* expression were successfully generated via transformation with an RNA interference vector that induced sequence-specific RNA silencing of *EpDWF4*. Our results suggest that a *DWF4* gene in BR biosynthesis pathway can be used to breed ornamental dwarf *E. purpurea* plant via RNAi-mediated gene silencing. These findings enhance our understanding of the molecular and genetic regulations of *E. purpurea* plant height.

Materials and methods

Plant materials and growth condition

Seeds of *Echinacea purpurea* were purchased from ACC KA Seeds & Seedling Co., Ltd. (Gunposi, Korea). The WT *E. purpurea* eastern purple coneflower ecotype was used in this study. *E. purpurea* seeds were surface-sterilized in a centrifuge tube by treating with 70% ethanol for 5 min, then five times with a bleach solution containing 1.2% (w/v) NaClO solution, and rinsing for 20 min with sterile water. The seeds were poured onto plates containing nutrient agar plates containing 0.44% (w/v) Murashige and Skoog salts (Murashige and Skoog, 1962), 3% (w/v) sucrose, 100 mg/l myo-inositol (Sigma, USA), 2 mg/L glycine (BioShop, Canada), 3 mL/L of Plant Preservative Mixture (PPM™, Plant Cell Technology, USA), 0.8% (w/v) agar, and pH 5.7. For vernalization, the plates were cold-treated at 4°C in the dark for 1 week. They were subsequently grown under light conditions as light-grown plants [16: 8 light (200 μmol m^{-2} sec^{-1}), dark, 23°C, respectively, and 70 – 90% humidity].

WT *Arabidopsis thaliana* ecotype *Columbia-0* (*Col-0*) was provided by the College of Agriculture, Guizhou University. The dwf4-102 mutant *A. thaliana* seeds (SALK_020761) supplied by the Arabidopsis Biological Resource Center (ABRC) were used in the complementation experiment (Zhang et al. 2012). Transgenic *A. thaliana* plants were generated by *Agrobacterium*-mediated transformation using the floral dipping method (Clough and Bent, 1998). All seeds of *A. thaliana* were surface-sterilized using 70% ethanol for 5 min, treated with a bleach solution containing 20% NaClO and 0.05% Triton X 100 five times, and rinsed with sterile water for 20 min. The seeds were sown on 0.8% agar-solidified medium containing half-strength Murashige and Skoog salts and 2% sucrose (w/v) at a pH of 5.7 on plastic plates. After 72 h, vernalization was cold-treated in the dark at 4°C. The plates grew under light conditions as light-grown plants [16:8 light (200μmol m^{-2} sec^{-1}): dark, 23°C, respectively, and 65% humidity]. Ten-day-old seedlings were transferred to the soil.

Cloning of EpDWF4 by rapid-amplification of cDNA ends (RACE) method

A Total RNA Isolation Kit (Vazyme, Nanjing, China) was used to extract total RNA from 100 mg of frozen plant leaves. RNA concentration was measured using a NanoDrop 2000/2000c spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, USA), and the integrity was assessed using agarose gel electrophoresis. A GeneRacer™ Kit (Invitrogen, USA) was used to synthesize cDNA. All primers used in this study are listed in Table S1. An LA Taq Reaction Kit (Takara, Japan) was used for RACE PCR. Modified thermal asymmetric interlaced PCR (tail-PCR) programs were used as previously described (Choi et al. 2015). For detection and purification, the PCR products were then subjected to electrophoresis on a 1.2% agarose gel. The amplified putative fragments were cloned into the pGEM-T vector (Promega, Madison, WI, USA). The recombinant plasmids were transformed into *Escherichia coli*. The identity of the PCR fragment was confirmed using restriction enzyme mapping and DNA sequence analysis.

Bioinformatics analysis of EpDWF4 gene and protein

Multiple protein sequence alignments (MSA) of EpDWF4 and other DWF4-like proteins were performed using DNAMAN 7.0 software. *EpDWF4* sequences and previously reported *DWF4/CYP90B1s* from other plants were used for phylogenetic analyses. In the MEGA program (ver. 7.0), a phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm with 1000 bootstrap replications (Tamura et al. 2011). SWISS-MODEL (https://www.expasy.org/resources/swiss-model) was used for structural analysis of the EpDWF4 protein.

Genomic DNA isolation and southern blot analysis

GenElute™ Plant Genomic DNA Mini Kit (Sigma, USA) was used to extract genomic DNA from plant leaf tissues. 40 μg of the genomic DNA samples were analyzed via gel blot hybridization. The samples were electrophoresed on 0.8% agarose gel in 0.5 × TBE buffer, transferred to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech, Buckinghamshire, UK) by vacuum blotter (Bio-Rad, U.S.), and then UV-crosslinked. The remaining steps were performed following the manufacturer's instructions for the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland). The DNA probes were prepared *via* specific *EpDWF4* primers and hygromycin (HPT) primer labels in the presence of DIG.

Semi-quantitative RT-PCR

The total *E. purpurea* RNA samples were isolated from leaves, stems, and roots. The samples were ground into a powder in liquid nitrogen, and total RNA was extracted. The DNase I-treated (Takara, Japan) total RNA was reverse-transcribed using the ImProm-II™ Reverse Transcription System (Promega, USA) and oligo-dT primer following the manufacturer's instructions. *A. thaliana* cDNA was synthesized from the entire plant tissues using the same method. The relative expression of *EpDWF4* was determined using CFX96 Touch™ (Bio-Rad, Hercules, CA, USA), Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), and specific primers (Table S1). *E. purpurea Actin* gene was used for normalization. The gene expressions were quantified using the $2^{-\triangle\triangle CT}$ method.

Vector construction for EpDWF4

Sense and antisense EpDWF4 DNA fragments (233 bp) corresponding to 1,110-1,343 nucleotides of the amino acid-coding region were amplified by PCR from *E. purpurea*. The primers used are listed in Table S1. The identity of the PCR fragment was confirmed using restriction enzyme mapping and DNA sequence analysis. To generate the pFGC5941-*EpDWF4* RNAi construct, sense and antisense *Ep*DWF4 fragments were inserted upstream and downstream of the *chalcone synthase A (CHSA)* intron in sense and antisense orientations using *Asc I* / *Swa I* and *Xba I* / *Sma I* restriction sites of the pFGC5941 vector (http://www.chromdb.org/rnai/vector_info.html). This is a binary vector designed to produce double-stranded RNA in plants. The proper orientation and reading frame of the insertion in pFGC5941-*EpDWF4* were confirmed using restriction enzyme mapping and DNA sequence analysis. The recombinant plant expression vector pFGC5941-*EpDWF4* was introduced into *Agrobacterium tumefaciens* LBA4404 *via* electroporation.

EpDWF4 DNA fragments were amplified by RT-PCR from the total RNA extracts isolated from *E. purpurea* plants. The primers used are listed in Table S1. The *EpDWF4* fragment was inserted into the pPZP2Ha3 vector using *Xho* I and *Xba* I restriction sites. The proper orientation and reading frames of the insertion in pPZP2Ha3-*EpDWF4* (35S: *EpDWF4*) were confirmed using restriction enzyme mapping, DNA sequencing, and plasmid DNA PCR. The recombinant plant expression vector 35S: *EpDWF4* was introduced into *A. tumefaciens* GV3101 *via* electroporation.

Agrobacterium-mediated transformation

E. purpurea leaf disks (approximately 1 cm^2) were prepared from young sterile seedlings. Recombinant *A. tumefaciens* LBA4404 harboring pFGC5941-*Ep*DWF4 RNAi construct was diluted to OD_{600} = 0.8 with MS salt solution containing 2% sucrose. Leaf disks were immersed for 10 min in the diluted recombinant *Agrobacterium* suspension. Leaf disks were blotted onto sterile filter paper to remove excess recombinant *Agrobactium* suspension. The leaf disks were placed on MS agar medium (2.2 g/L MS salt, 2% sucrose, 0.7% microagar) and incubated for 2 days under dark conditions. The leaf disks were transferred to E8 medium [2.2 g/L MS salt, 100 mg/L myo-inositol, 1mg/L glycine, 3 mL/L PPM™, 1.0 mg/L benzylaminopurine, 0.01 mg/L naphthalene acetic acid, 0.6 mg/L phosphinothricin, and 250 mg/L cefotaxime], and incubated in a 25℃-culture room under a 16-h illumination (200 μmol/m/s) and 8-h darkness cycle. The regenerated shoots were excised, transferred to a rooting medium [2.5 g/L woody plant medium, 1.5% sucrose, 1.5 mg/L acid], and incubated under the same growth conditions. The rooted plantlets were then transferred to potting soil.

A. thaliana was transformed with recombinant *A. tumefaciens* GV3101 harboring the recombinant plant expression vector, *pPZP2Ha3-EpDWF4*. Briefly, overnight cultured recombinant *Agrobacterium* was dispersed to OD_{600} = 0.8 in a 10% sucrose solution with the addition of Silwet L-77 (Lehle seed, Round Rock, TX, USA) and mixed homogeneously at a concentration of 0.05%. Recombinant *Agrobacterium*

solutions were sprayed onto WT and dwf4-102 *A. thaliana* plants thrice every other day. *A. thaliana* plants were grown in growth chambers at 22℃ with 16 h of daylight. Recombinant *Agrobactium*-transformed seeds were harvested and germinated on an agar medium to select putative transgenic plants. After germination, activation-tagged mutants were selected on basic agar medium plates containing 25 mg/L HPT and 250 mg/L carbenicillin.

Flanking sequence analysis

To identify the flanking sequences around the genomic inserts, an inverse PCR method was used, involving a series of DNA digestions and self-ligations. 5 μg genomic DNA was extracted from WT and EpDWF4-TL plants and digested by 30 U restriction enzyme *Xba I* (Takara, Japan), respectively. Purification followed sufficient digestion, and the same volume of isopropanol was added to product mixtures and stored at -20℃ for 2 h. Then, the mixtures were returned to room temperature and centrifuged at 12,000 rpm for 10 min. Pellets were washed with 70% ice EtOH twice after supernatant removal and dissolved by 20 μl ddH_2O. Lug-purified digest products were used in self-ligation by the T4 DNA ligase kit (Roche, USA). The final volume was adjusted to 100 μL and incubated for 48 h at 4℃. The purification step was continued after self-ligation. To minimize the loss, the final volume was increased to 500 μL with double distilled water. The final pellets dissolved in 20 μL ddH_2O and used as template DNA in the first PCR with FSF1 and FSR1 primers. The 1 μL PCR products were used as a template in nested PCR and amplified by FSF2 and FSR2 primers. All of the PCR products were separated on a 1% agarose gel with ethidium bromide staining. The PCR primers are listed in Table S1.

Western blot analysis

Protein samples were separated by electrophoresis on 8 − 12% polyacrylamide-sodium dodecyl sulfate (SDS) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (PALL Life Science). The membranes were pre-incubated with blocking solution [5% (w/v) non-fat dried milk in TBS-T (TBS with 0.05% (v/v) Tween-20)] for 1h at room temperature and then incubated overnight at 4℃ with mouse anti-His antibody (1:5000 dilution in blocking solution; GeneTex, Irvine, CA, USA). The membranes were washed thrice with TBS-T and incubated for 2.5 h with anti-mouse IgG (1:5000 dilution in blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBS-T, protein bands were detected using the ECL Pico Western blotting detection reagent (Thermo Fisher Scientific Inc.).

Statistical analysis

The pepper and transgenic lines were analyzed at least thrice with separate biological replicates. Data were analyzed using a one-way ANOVA and SPSS version 21 (IBM, Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

Results

Bioinformatic analysis of EpDWF4

The total *EpDWF4* sequence was 1,717 bp, consisting of a 1,458 bp open reading frame and 65 bp 5' and 194 bp 3'-untranslated regions. This sequence was deposited in GenBank (accession number KJ833768). *EpDWF4* encodes 486 amino acids. The relative molecular mass of the protein was 55.6 kDa, and its theoretical isoelectric point was 8.68. Multiple sequence alignment analysis showed that it shares the typical structures of CYP90/DWF4 proteins, such as AtCYP90B1, ZeDWF4, and ZmDWF4, had sequence identities of > 72%, > 85%, and > 74%, respectively. The EpDWF4 protein showed high homology with AtCYP90B1, and conserved amino acids were present at the expected positions in the deduced *EpDWF4* sequence (Fig. 1a). A phylogenetic tree of *EpDWF4* was constructed using amino acid sequences of several other CYP90B1/DWF4 orthologs (Fig. 1b). The tertiary structure of *EpDWF4* was identified with 91% accuracy. The α-helix constitutes 47.22%, the random coil 42.68%, and the extended chain 10.10%. (Fig. 1c). *EpDWF4* is closely related toAtCYP90B1, which belongs to CYP gene family of the plant cytochrome P450 superfamily. Based on these results, the identified gene may function as a steroid 22-alpha hydroxylase similar to the *AtCYP90B1* gene.

Suppressing the expression of EpDWF4 in E. purpure caused a dwarf phenotype

Gene expression patterns are key indicators of gene function. The *EpDWF4* gene was analyzed at the transcriptional level using real-time quantitative (qRT-PCR) in four different tissues: roots, stalks, leaves, and inflorescences. The leaf exhibited the highest expression of *EpDWF4*, followed by the inflorescence and stalk. On the contrary, the root exhibited the lowest expression (Fig. S1a). To explore the function of *EpDWF4* in *E. purpurea*, RNAi-*EpDWF4* transgenic plants were generated to downregulate *Ep*DWF4 by plant tissue culture. Several RNAi-*EpDWF4* transgenic plants were generated by *Agrobacterium*-mediated transformation (Fig. S1b). PCR analysis of genomic DNA confirmed that all RNAi-*EpDWF4* transgenic plants contained the RNAi construct (Fig. S1c). *EpDWF4* transcript levels were measured by qRT-PCR using RNA isolated from each transformant line. The mRNA levels of *EpDWF4* in RNAi-*EpDWF4* transgenic plants were significantly lower than those in WT plants (Fig. 2b). Morphological differences were observed between the WT and RNAi-*EpDWF4* transgenic plants at the adult stage (Table S2). Transgenic *E. purpurea* plants exhibited phenotypic alterations, displaying a dwarf phenotype approximately 45% of the height of the WT plants (Fig. 2a and c).

Overexpression of EpDWF4 functionally complements null dwf4 mutations

To investigate the potential role of EpDWF4 as a C-22 hydroxylase in BRs, we used transgenic plants of the null mutant dwf4-102 that ectopically expresses the *EpDWF4* gene. The *EpDWF4* coding sequence, controlled by the CaMV 35S promoter, was transformed into a dwf4-102 mutant. The dwf4-102 mutant exhibited severe dwarfism, with short, rounded leaves and very short internodes along the inflorescence.

EpDWF4 supplementation resulted in enhanced leaf expansion and elongated petioles in seedlings grown under light conditions compared to the dwf4-102 mutant (Fig. 3a). It also exhibited increased hypocotyl elongation in dark-grown seedlings (Fig. 3b). Phenotypic analysis of the transformed lines showed that dwf4-102 plants carrying the *p35S: EpDWF4* T-DNA did not exhibit dwarfism and displayed growth habits similar to those of WT plants (Fig. 3c and d). This suggests that *EpDWF4* can fully rescue dwf4-102 phenotypes. PCR analysis showed that the *EpDWF4* gene could only be detected in *A. thaliana* dwf4-102 transgenic plants; however, they were undetected in the WT or dwf4-102 mutant plants (Fig. 3e). This result indicated that *EpDWF4* is functionally equivalent to *A. thaliana* AtDWF4/AtCYP90B1.

Heterologous overexpression of DWF4 in A. thaliana results in a significant increase in height

To further investigate the function of *EpDWF4*, *35S: EpDWF4* transgenic *A. thaliana* plants were generated using *Agrobacterium*-mediated transformation beginning with WT *A. thaliana* plants. Nine independent T1 transgenic lines were confirmed by medium selection and PCR analysis (Fig. S2a and b). We selected and propagated three distinct *EpDWF4* overexpression lines, including OE1, OE2, and OE3 (Fig. S2c) to obtain homozygous plants for subsequent experiments, using WT plants as controls.

Assessing the copy number of the 35S: *DWF4* transgene in *A. thaliana* overexpression lines confirmed the efficacy of gene overexpression, reduced the risk of gene expression instability or silencing, and provided precise data for validating gene function. Southern blot hybridization analysis with a HPT probe revealed the presence of a copy of *EpDWF4* in the OE-1 homozygous transgenic *A. thaliana* plant (Fig. 4a). To further verify the southern blot hybridization results, an *EpDWF4*-specific probe was employed to verify the single copy number in OE-1 transgenic *A. thaliana* plants (Fig. 4b). These findings indicate that the 35S: *EpDWF4* transgene was integrated into the *A. thaliana* genome as a single copy.

Following the above results, an in-depth analysis of the T-DNA-flanking genome sequence provides insights into the unique molecular characteristics of OE-1 transgenic plants. The location of the *35S: EpDWF4* T-DNA in the transgenic *A. thaliana* chromosome was verified by flanking sequence analysis using Hi TAIL-PCR with specific flanking PCR primers (Fig. 4c and d). A genomic DNA fragment of 250 bp was identified downstream of the T-DNA insertion site. BLAST analysis revealed that the T-DNA was inserted into a non-coding region of *A. thaliana* chromosome 1 (from 4587511 bp to 4587760 bp) (Fig. 4d). This finding indicates that the morphological and physiological changes observed in OE-1 transgenic plants can be attributed to the functions of *EpDWF4*, independent of the functions of endogenous coding genes in *A. thaliana.* Furthermore, flanking sequence analysis confirmed that the OE-1 transgenic plants were homozygous.

Protein synthesis is influenced by various factors, and detecting gene expression at the protein level using western blot analysis provides a direct insight into gene function and activity. The recombinant EpDWF4 protein was detected in transgenic *A. thaliana* plants by western blot analysis. The results showed that recombinant EpDWF4 was predominantly localized to the intracellular fraction and had a

molecular weight of approximately 56 kDa (Fig. 4e), which closely matched the molecular weight predicted from the cDNA sequence.

To investigate *EpDWF4* function, the morphological features of the OE-1 transgenic plants were analyzed (Table S3). The height of OE-1 increased by approximately 27% compared to WT *A. thaliana* (Fig. 5a and b). In contrast to the WT, the rosette leaves of OE-1 exhibited extended petioles and narrow, long, curled blades (Fig S3a and b). The lengths of the transgenic plant siliques significantly increased; however, the number of seeds per silique exhibited no significant change relative to the WT. This was due to the enhanced silique length and seed yield and the deformation and abortion of small siliques in transgenic plants (Fig. S3c). The siliques exhibited varying degrees of bending, had few seeds, and were thin and short. Abortive siliques appeared as small dots without seeds. To understand the response of the transgenic plants to light, their seeds were cultured on half-strength MS agar plates and grown in light. After seedling phenotypes for 10 days, the hypocotyls of transgenic plants were significantly lengthened, and the development of primary and lateral roots dramatically increased, corresponding to the inflorescence stem of the WT under light conditions (Fig. 5c).

Discussion

EpDWF4 is a key gene involved in steroid biosynthesis. Detailed analysis of the complete cDNA sequence of *EpDWF4* revealed an open reading frame of 1458 bp, encoding a protein with 486 amino acids. This structure showed significant homology with other known CYP90B1/DWF4 homologous proteins, such as AtCYP90B1 from *A. thaliana*, ZeDWF4 from Z. elegans, and ZmDWF4 from *Z. mays*, indicating a high degree of inter-specie conservation. The key conserved residues in the amino acid sequence further support the role of EpDWF4 as a steroid 22-alpha-hydroxylase, which is critical for understanding its role in plant growth and development. The sequence alignment of *EpDWF4* with AtCYP90B1, ZeDWF4, and ZmDWF4 showed > 72% similarity between *EpDWF4* and AtCYP90B1, reflecting a high level of sequence conservation. This conservation suggests that the gene likely performs similar functions across different plant species. Phylogenetic analysis further confirmed that *EpDWF4* was most closely related to AtCYP90B1, indicating that it may function in *E. purpurea*, similarly to AtCYP90B1 in *A. thaliana*. The *CYP90B1/DWF4* gene family is primarily involved in the steroid biosynthesis pathway, especially in the synthesis of plant growth regulators, such as BRs (Bancoi et al. 2002). The conservation of amino acid residues associated with enzymatic catalytic function supports the role of EpDWF4 as a steroid 22-alpha-hydroxylase. This enzyme is crucial in steroid synthesis, influencing the production and regulation of plant hormones essential for plant growth and development (Kim et al. 2006).

qRT-PCR analysis revealed significant variations in *EpDWF4* expression levels across different plant tissues. The highest expression was observed in the leaves, followed by the inflorescences and stems, with the roots exhibiting the lowest levels of expression. This tissue-specific expression pattern suggests that *EpDWF4* may have specialized roles depending on the developmental stage or physiological processes of the plant (Yang et al. 2022). Leaves are the main sites of photosynthesis and

secondary metabolite production. The high expression of *EpDWF4* suggests its crucial role in energy production, metabolism, and regulation (Milner et al. 2022). In contrast, the expression of *EpDWF4* in the roots was relatively low, suggesting that the activity of *EpDWF4* may be less critical for root function and more important for the development of the aboveground plant parts. This differential expression pattern aligns with developmental and physiological needs, emphasizing the role of *EpDWF4* in regulating growth and development through the modulation of hormonal and metabolic pathways across different tissues. Understanding these expression dynamics provides valuable insights into the function of EpDWF4 and underscores its importance in plant growth regulation.

To elucidate the function of *EpDWF4*, we generated RNAi-EpDWF4 transgenic *E. purpurea* plants. It exhibited a dwarf phenotype, reaching only approximately 50% of the height of WT plants. This stature reduction suggests that *EpDWF4* is crucial for regulating plant growth, further emphasizing its importance in plant development (Liu et al. 2007; Manghwar et al. 2022), potentially through its role in modulating steroid biosynthesis (Zhan et al. 2022). We also introduced this gene into the *A. thaliana* dwf4-102 mutant, which exhibited severe dwarfism due to disruptions in the BR biosynthesis pathway. Our results demonstrate that *EpDWF4* can fully restore the mutant phenotype, indicating its functional equivalence with AtCYP90B1. Transgenic plants exhibited WT-like morphology, including normal plant height and inflorescence structure, confirming that *EpDWF4* can perform enzymatic functions essential for BR biosynthesis.

EpDWF4 overexpression in *A. thaliana* resulted in significant phenotypic changes. The homozygous transgenic plants overexpressing *EpDWF4* grew normally and were characterized by larger organs compared to WT plants, such as larger rosettes, longer leaves, taller inflorescences, and longer petioles (Table 3). Plants that overexpress known genes for BR C-22 hydroxylases, such as tobacco plants overexpressing *DWF4*, typically exhibit larger organ sizes than control plants (Choe et al. 2001).

The increased silique length and seed yield in these plants indicate elevated BR biosynthesis, supporting the positive role of *EpDWF4* in growth regulation (Vukašinović, N. et al. 2021). PCR and southern blot analyses confirmed the presence of *EpDWF4* in OE-1 plants, with a T-DNA insertion in the non-coding region of *A. thaliana* chromosome 1. This insertion minimizes interference with endogenous gene function, indicating the role of *EpDWF4* in steroid biosynthesis and its effects on plant growth and development (Gao et al. 2024).

EpDWF4 plays a crucial role in steroid biosynthesis and has potential for breeding applications. *EpDWF4* is a vital gene for developing semidwarf cultivars, regulating plant height, and improving crop management and production efficiency (Zebosi et al. 2024). Semi-dwarf plants offer advantages, such as higher planting density, enhanced photosynthetic efficiency, and reduced water consumption, which are valuable in modern agriculture (Li et al. 2023; Song et al. 2023). By silencing *EpDWF4*, we obtained semi-dwarf phenotypes in *E. purpurea*, providing new insights for crop management and breeding research.

Conclusion

This study provides insights into the molecular functions of the *EpDWF4* gene in *E. purpurea* and confirms its functional equivalence with AtCYP90B1. Our study described the critical role of *EpDWF4* in BR biosynthesis and its significant effect on plant growth and development, thus enhancing our understanding of plant steroid biosynthesis mechanisms. These findings highlight the potential of *EpDWF4* in regulating plant growth, secondary metabolite production, and its value in breeding applications. Future studies should investigate the function of *EpDWF4* under various environmental conditions to better understand its role in plant adaptation and stress responses. These investigations may help develop crops that are resilient and well-adapted to diverse agricultural challenges.

Declarations

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests The authors declare that they have no competing interests

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Authors' contributions Writing original draft, performing the experiment and analyzing the data: J Z. and YY F. Preparing the materials: J L. and YH H.

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Figures

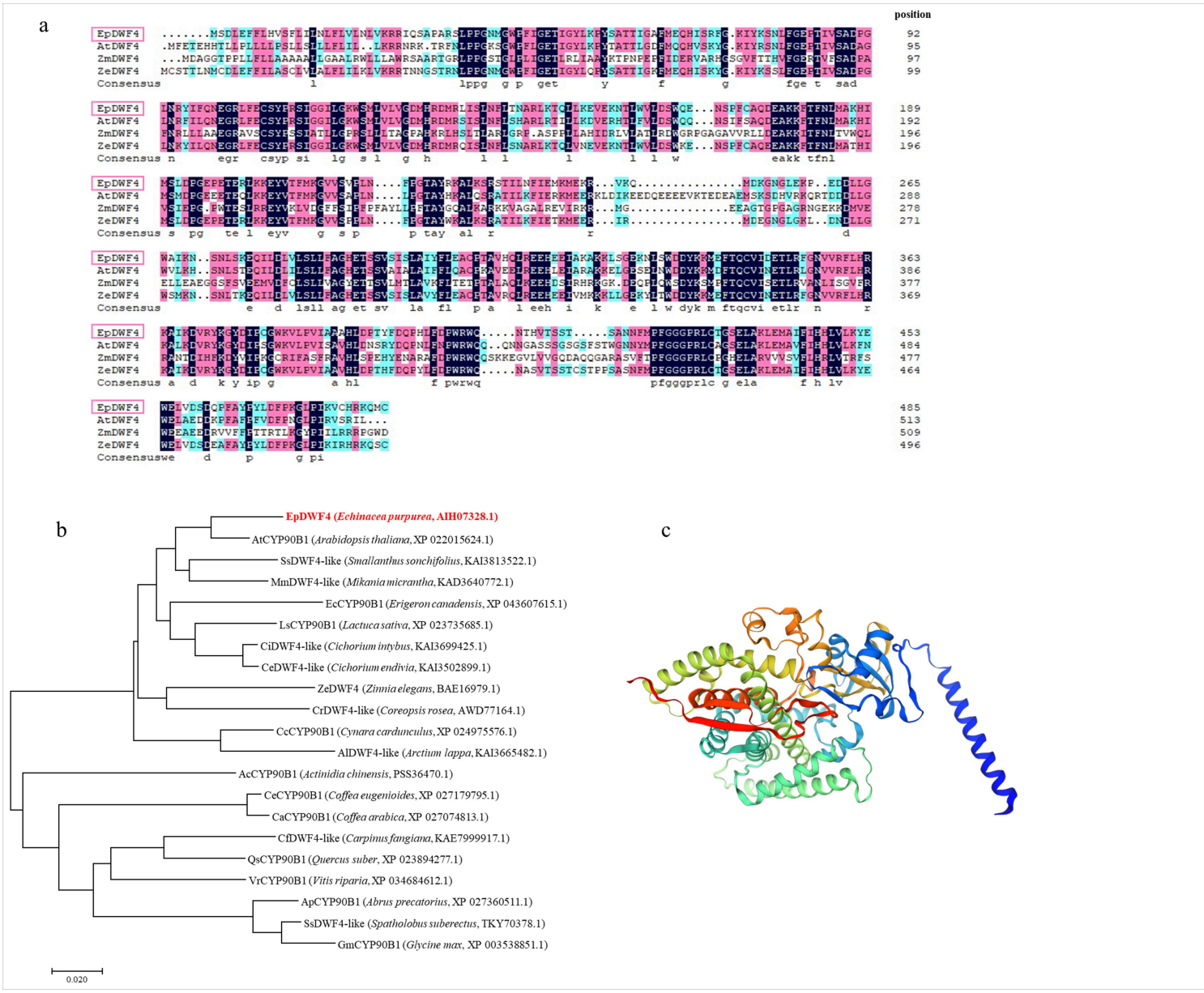

Figure 1

Bioinformatics analysis of *EpDWF4* **a** *EpDWF4* amino acid alignment in contrast to *AtDWF4*, *ZmDWF4* and *ZeDWF4*. **b**Phylogenetic analysis of EpDWF4 with DWF4 proteins from other species constructed by the neighbor-joining method. The bootstrap values (%) presented at the branches were calculated from 1,000 replicates. The scale bar indicates the sequence divergence is 0.02/unit bar, which represents 10% substitutions per nucleotide position. EpDWF4 is identified by a red frame. **c** The predicted tertiary structures of EpDWF4.

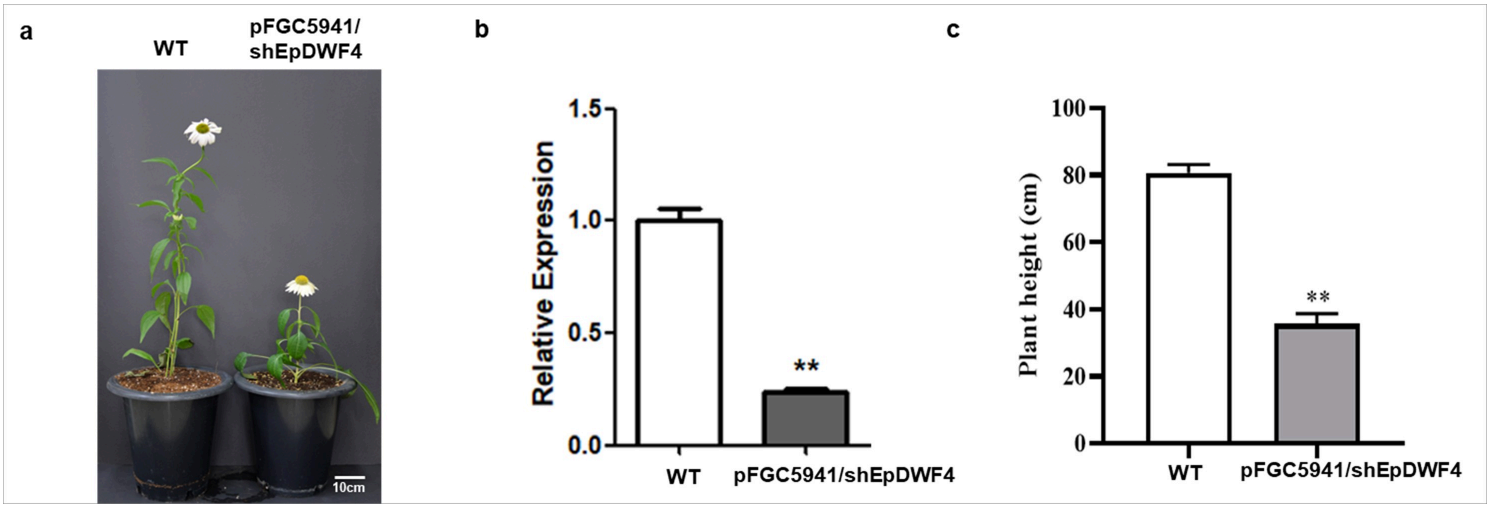

Figure 2

a Wild-type (WT) plant and transgenic *E. purpurea* plant suppression *EpDWF4* (pFGC5941/sh*EpDWF4*) in adult stage, scale bar = 10 cm. **b** qRT-PCR to determine the down-regulation of *EpDWF4* expression from transgenic *E. purpurea* plant. Expression levels were normalized to the actin gene, and the values shown are the means ± SD of 3 biological replicates. **c** Plant height of WT and pFGC5941/sh*EpDWF4* at flowering stage.Asterisks indicate significant differences compared to the WT (**P<0.01).

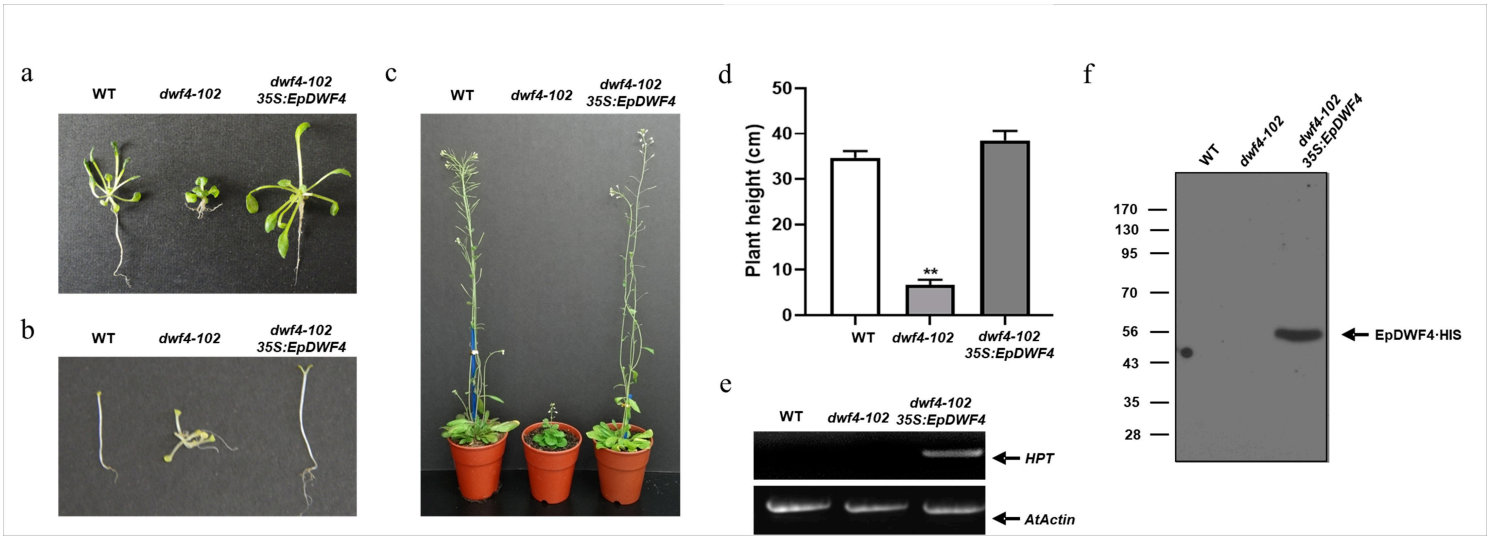

Figure 3

Phenotypic comparison between *A. thaliana* WT, a heterozygous *A. thaliana* dwf4-102 mutant (dwf4-102), and T1 generations of *A. thaliana* dwf4-102 mutant carrying *p35S: EpDWF4* (dwf4-102*/35S: EpDWF4*) Seeds from a WT, dwf4-102 and dwf4-102*/35S: EpDWF4*were germinated in standard growth media. At day 7 post-germination, all seedlings were transferred to vertical plates of control media under light conditions (**a**) and seedlings grown in the dark (**b**), scale bar = 1cm. The phenotype and plant height of WT, dwf4-102 and dwf4-102*/35S: EpDWF4* transgenic line in adult stage **(c and d)**, scale bar = 7cm. RT-PCR assay (**e**) and Western blot analysis (**f**) of *EpDWF4*expression in WT, dwf4-102, and dwf4-102*/35S:*

EpDWF4 transgenic line. Asterisks indicate significant differences as assessed by one-way ANOVA (Tukey's test) (**$P < 0.01$).

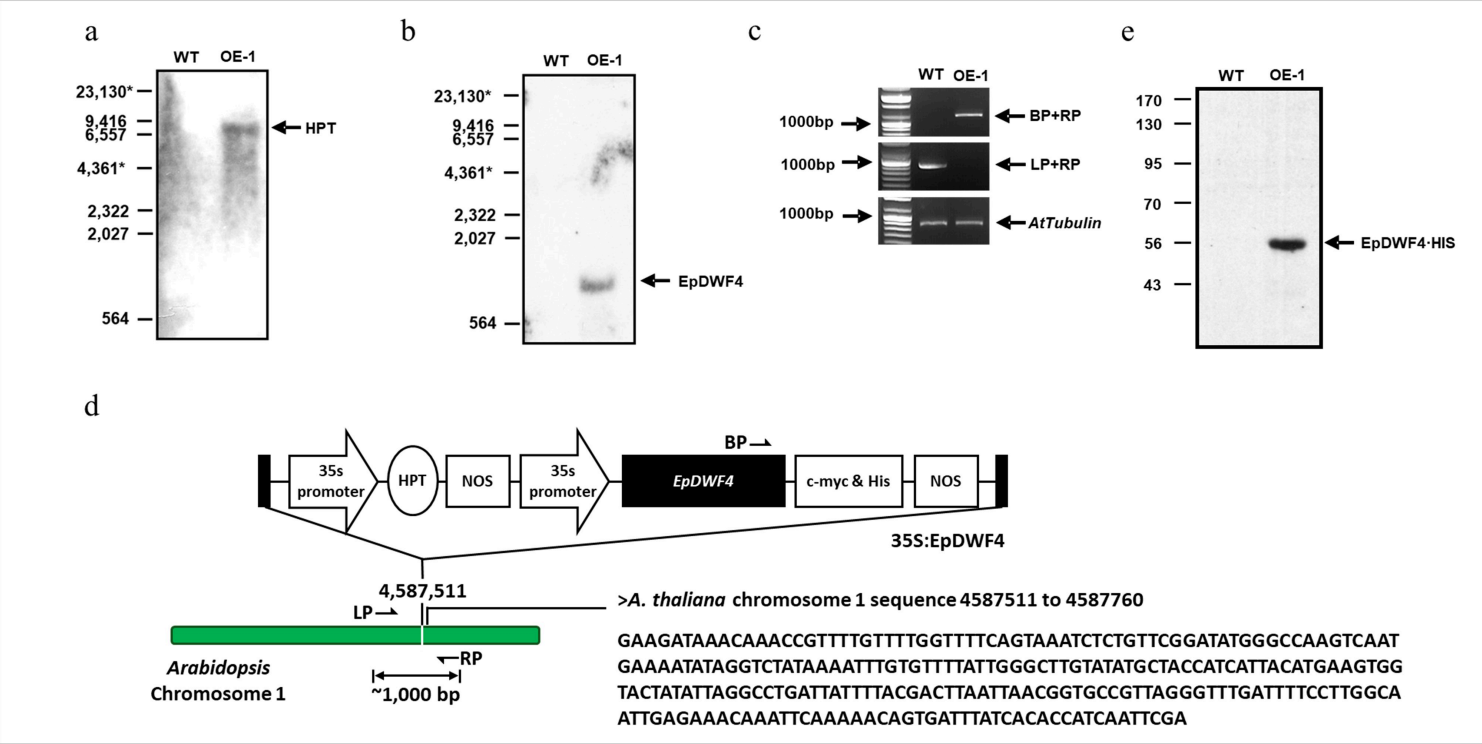

Figure 4

Southern blot analysis of OE-1 transgenic line The DNA probes were prepared *via* specific HPT primers (**a**) and *EpDWF4* primers (**b**) labeling in the presence of DIG. **c** Flanking sequences were obtained to determine the T-DNA locus of genomic DNA of homozygous OE-1 transgenic *A. thaliana*plants overexpressing *EpDWF4*. For a more detailed investigation, homogenous T3 lines of transformants were selected by genomic DNA PCR. BP primer was designed on the T-DNA portion, and LP and RP primers were located upstream and downstream of the T-DNA insertion area. LP and RP primers amplified approximately 900bp PCR products in WT *A. thaliana* genomic DNA compared to transgenic *A. thaliana* genomic DNA. However, about 1,100 bp fragments were present in transgenic *A. thaliana* genomic DNA, which is absent in WT, indicating that OE-1 was a homozygote. **d** T-DNA location area and information of the flanking part. **e** Western blot analysis to determine the expression of *EpDWF4* from homozygous OE-1 transgenic *A. thaliana*plants overexpressing *EpDWF4*.

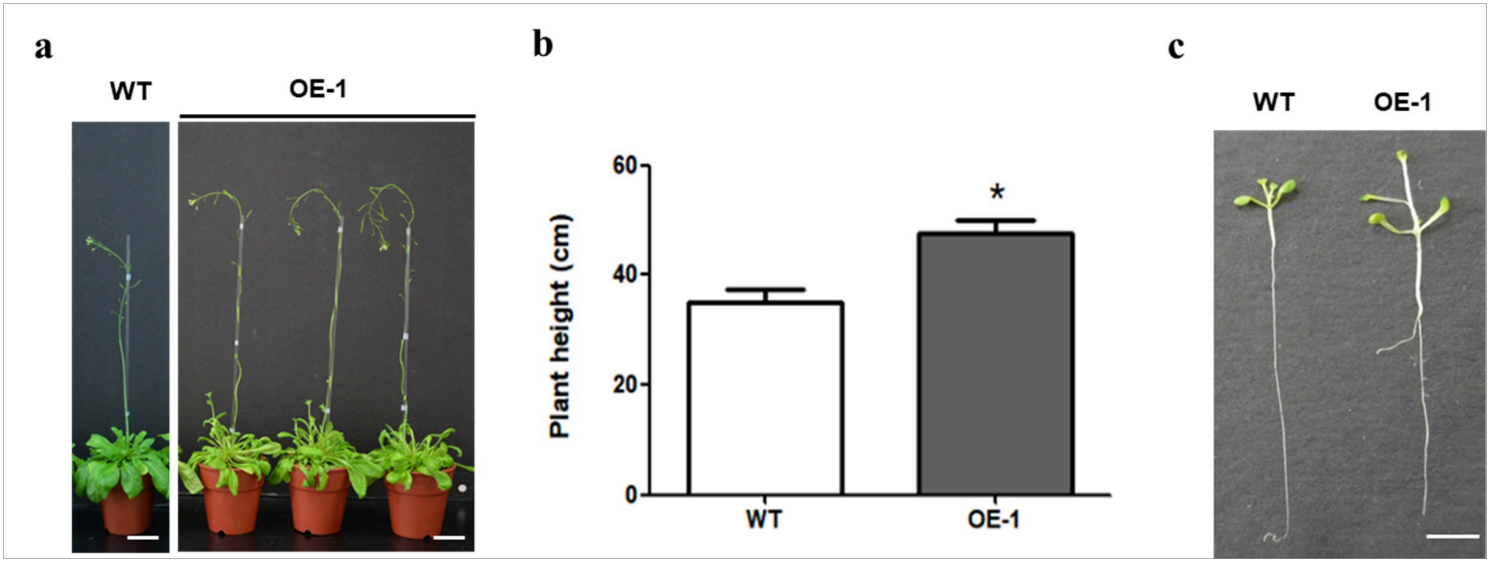

Figure 5

The phenotype of the WT *A. thaliana*plant and the homozygous transgenic line harboring one copy *35S: EpDWF4* structure (OE-1) **a** and **b** A phenotype and plant height of WT and OE-1 transgenic line in adult stage, scale bar = 7cm. **c** Seeds from the WT and OE-1 were germinated in standard growth media, scale bar = 0.5cm. At day 10 post-germination, all seedlings were transferred to vertical plates of control media under light conditions. Asterisks indicate significant differences compared to the WT (*P<0.05)

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