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# Roles of *DgD14* in regulation of shoot branching in chrysanthemum (*Dendranthema grandiflorum* 'Jinba')

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ABSTRACT

Shoot branching plays an important role in determining plant architecture. Strigolactones (SLs) negatively regulate shoot branching, and can respond to conditions of low or absent phosphate or nitrogen. The *D14* gene is a probable candidate as an SL receptor in rice, petunia, and *Arabidopsis*. To investigate the roles of *D14* in shoot branching of chrysanthemum, we isolated the *D14* homolog *DgD14*. Functional analysis showed that DgD14 was a nuclear-localized protein, and restored the phenotype of *Arabidopsis d14-1*. Exogenous SL (GR24) could down-regulate *DgD14* expression, but this effect could be overridden by apical auxin application. Decapitation could down-regulate *DgD14* expression, but this effect could be restored by exogenous auxin. In addition, *DgD14* transcripts produced rapid responses in shoot and root under conditions of phosphate absence, but only a mild variation in bud and stem with low nitrogen treatment. Indistinct reductions of P levels in shoot were observed in plants grown under low nitrogen conditions. The absence of phosphate and low levels of nitrogen negatively affected plant growth. These results demonstrate that P levels in shoot had a close relationship with phosphate, whereas nitrogen did not directly regulate *DgD14* expression in shoot. Taken together, these results demonstrated that *DgD14* was the functional strigolactone signaling component in chrysanthemum.



## 1. Introduction

Shoot branching is a ubiquitous phenomenon in plant growth, and is also one of the basic characteristics of plants that plays an important role in determining plant architecture. Branching is a highly plastic determinant of plant shape to allow plants to respond to environmental stresses (Evers et al., 2011). The axillary buds grow into shoots regulated by genetic, hormonal, developmental, and environmental factors (Schmitz and Theres, 1999; Beveridge et al., 2003; McSteen and Leyser, 2005; Ongaro et al., 2008). Plant hormones, such as auxins and strigolactones (SLs), inhibit bud outgrowth, while cytokinins (CKs) promote branching, and thus these hormones interact to regulate bud breaking and branching (Beveridge, 2006; Leyser, 2009).

SLs were recognized relatively recently as a new class of carotenoid-derived signal that can directly regulate shoot branching (Beveridge, 2006; Gomez-Roldan et al., 2008; Umehara et al., 2008). A series of increased branching mutants in diverse plant species have been identified, including the more axillary growth (*max*) mutants of *Arabidopsis thaliana*, the dwarf (*d*) and high tillering dwarf (*htd*) mutants of *Oryza sativa*, the decreased apical dominance (*dad*) mutants of *Petunia hybrida*, and the ramosus (*rms*) mutants of *Pisum sativum* (Domagalska and Leyser, 2011). They have been bred to allow investigation of the molecular mechanisms of SL biosynthesis, transport, and signaling. Through analysis of these mutants, seven genes have been identified. *D27* encodes a novel chloroplast-located iron-containing protein, which is the first enzyme in the SL pathway (Lin et al., 2009; Alder et al., 2012; Waters et al., 2012a); *CCD7* and *CCD8* encode divergent plastidic carotenoid cleavage dioxygenases that function in the chloroplast (Sorefan et al., 2003; Booker et al., 2004; Snowden et al., 2005; Ledger et al., 2010; Pasare et al., 2013); while *MAX1* encodes a cytochrome P450 monooxygenase and catalyzed carlactone to carlactonoic acid in *Arabidopsis*, it works downstream of *CCD7* and *CCD8*, and is a particularly strong candidate for contributing to diversification of inputs upstream of *MAX2* (Booker et al., 2005; Lazar and Goodman, 2006; Challis et al., 2013; Abe et al., 2014). In addition, *MAX2* encodes an F-box protein, function in signaling pathways downstream of SLs and is required for response to SLs.

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*D14* encodes an α/β-fold hydrolase, which is proposed to act in signaling or in the hydrolysis of SLs to an active compound and provides specificity to signaling via *MAX2* mediates both SLs and karrikins signaling (Stirnberg et al., 2002; Umehara et al., 2008; Hamiaux et al., 2012; Waters et al., 2012b; Challis et al., 2013). Finally, *D53* encodes a protein that shares predicted features with the class I Clp ATPase proteins, and acts as a repressor of SL signaling (Jiang et al., 2013; Zhou et al., 2013).

Classic decapitation studies, grafting, application, and one/two-node assays (Chatfield et al., 2000; Brewer et al., 2013; Chen et al., 2013) have shown that auxins, CKs, and SLs play a major role in regulating bud outgrowth. Auxins act indirectly on entering the bud through two mechanisms, the auxin transport canalization model and the second messenger model. The auxin transport canalization model works as follows: if it is assumed that active growth, buds establish their own polar auxin transport stream (PATS) into the main stem, then high auxin concentrations in the main stem can prevent bud activation by reducing the sink strength of the main stem for auxin, thereby preventing the canalization of auxin transport out of the bud. In the second messenger model, auxins in the main stem regulate the synthesis of CKs and SLs that act as auxin second messengers within the bud and regulate branching (Booker, 2003; Ward et al., 2013). Auxins moving down the plant can increase the expression of SL biosynthesis genes and can also negatively regulate CK content (Tanaka et al., 2006). CKs and SLs are directly transported into axillary buds to regulate outgrowth (Kohlen et al., 2011). SLs regulate xylem sap CK (X–CK) levels through a feedback signal (Beveridge et al., 2000; Morris et al., 2001; Foo et al., 2005), but the CK levels do not account for the more branching phenotypes in the *rms* or *d10* mutants (Zhang et al., 2010). SLs and CKs can also act antagonistically on pea bud growth (Dun et al., 2012). Auxins, CKs, and SLs interact in multiple feedback loops, and provide a robust balance to regulate shoot branching (Ferguson and Beveridge, 2009; Domagalska and Leyser, 2011; Ward et al., 2013).

The biosynthesis of SLs can respond to conditions of low or absent phosphate (P) and/or nitrogen (N) to regulate plant architecture. P or N limitations in plants cause reduction in shoot:root ratio, increased SL levels in the roots, regulation of lateral root formation, and stimulation of arbuscular mycorrhizal symbiosis (Umehara et al., 2010; Kapulnik et al., 2011; Ruyter-Spira et al., 2011; Bonneau et al., 2013). The different responses of SL expression to P or N deficiency are related to the nutrient acquisition strategies of plants (Yoneyama et al., 2012). In red clover, tomato, and alfalfa, P deficiency enhances SL exudation (Yoneyama et al., 2007; Lopez-Raez et al., 2008; Yoneyama et al., 2012), while in sorghum, rice, Chinese milk vetch, lettuce, and marigold, deficiency of either N or P promotes SL exudation (Yoneyama et al., 2007, 2012; Jamil et al., 2011).

*D14* encodes a protein of the α/β-fold hydrolase superfamily in rice, which has homologs in petunia (*DAD2* gene) and *Arabidopsis* (*AtD14* gene) (Arite et al., 2009; Hamiaux et al., 2012; Waters et al., 2012b). In petunia, SL-mediated interaction of DAD2 with PhMAX2A may trigger the SCF E3 ligase to target an unknown substrate for ubiquitination and degradation (Hamiaux et al., 2012). In *Arabidopsis*, the D14 protein family confers distinct responses to either SLs (D14) or karrikins (Kai2) (Waters et al., 2012b). In rice, *OsMADS57* interacts with *OsTB1* and control the outgrowth of axillary buds through *OsD14* (Guo et al., 2013). SL promotes D14-$SCF^{D3}$-mediated degradation of *D53* to regulate shoot branching (Jiang et al., 2013; Zhou et al., 2013).

Chrysanthemum (*Dendranthema grandiflorum*) is one of the most important commercial cut flowers, but requires manual decapitation or removal of lateral branches to maintain its architecture in commercial production. In chrysanthemum, the ability of SLs to inhibit bud activity depends on the presence of a competing auxin source, as auxins locally down-regulate biosynthesis of CKs in nodes. SLs also down-regulate the biosynthesis of CKs (Chen et al., 2013). The expression of *DgCCD8*, the SL biosynthesis gene, can be down-regulated by exogenous SL, but can be overridden by apical auxin application (Liang et al., 2010). *DgMAX2*, a key regulatory gene in SL signal transduction, is able to restore *max2-1* mutant branching to wild-type (WT) *Arabidopsis* (Dong et al., 2013). *DgBRC1* complemented the multiple branches phenotype of *Arabidopsis brc1-1*, its transcripts could respond to apical auxin supply and polar auxin transport (Chen et al., 2013).

In this study, we identified *DgD14*, a *D14* ortholog gene of the α/β-fold hydrolase superfamily, in chrysanthemum (*D. grandiflorum* 'Jinba'), and found that the expression of *DgD14* was inhibited by decapitation and induced by auxins. Furthermore, *DgD14* could produce a rapid response to absent phosphate or low N (LN) treatments. These findings provide new insights into the dynamics of the putative SL signaling component *DgD14* in chrysanthemum.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Chrysanthemum plantlets were propagated under sterile conditions in jars containing MS agar medium (Murashige and Skoog, 1962), and then grown in a tissue culture room at 22–25 °C with a photoperiod of 16/8 h light/dark and a light intensity of 100–120 μmol $m^{-2}$ $s^{-1}$.

For the decapitation assay and the sprayed hormone assay, the chrysanthemum cuttings (7 cm in height) were initially grown in vermiculite for 10 days, then plants were transferred into pots containing peat soil and vermiculite (1:1) in a greenhouse at 25/23 °C with a 16/8 h light/dark photoperiod.

### 2.2. Hormone stocks

Naphthaleneacetic acid (NAA; Sigma–Aldrich, Shanghai, China) stock solution was dissolved in 70% ethanol, and GR24 (LeadGen Labs, Orange CT USA) was dissolved in acetone. 6-benzylaminopurine (6-BA; Sigma–Aldrich, Shanghai, China) was dissolved in NaOH, while indole-3-acetic acid (IAA; Sigma–Aldrich, Shanghai, China) and gibberellin acid (GA; Sigma–Aldrich, Shanghai, China) were dissolved in ethanol.

### 2.3. Isolation of the full-length coding sequence for DgD14

Total RNA was extracted from stems with TRIzol Reagent (15596-026; Life Technologies/Invitrogen) and cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (#K1621; Thermo Scientific). Primers were designed for *DgD14* cloning based on the sequence regions of *D14* genes that are conserved among *Arabidopsis AtD14*, *Petunia DAD2*, and rice *D14* genes. After obtaining a conserved domain fragment using forward primer P_for and reverse primer P_rev, the 3′ fragment of *DgD14* was amplified by the rapid amplification of cDNA Ends (RACE) method, using the 3′ RACE primers 3′-race1 and 3′-race2, and the 5′ fragment of *DgD14* amplified in the same way using the 5′ RACE primers 5′-race1 and 5′-race2. The products, amplified using PrimeStarHS DNA Polymerase (TaKaRa, Dalian, China), were cloned into pMD18-T vector (TaKaRa) and verified by sequencing (Zhongke Xilin Biotechnology, Beijing, China). Sequence alignment and phylogenic analysis were performed using the ClustalW and ESPript programs (http://www.genome.jp/tools/clustalw/) and the MEGA 5.0 program (http://www.megasoftware.net/) respectively. Phylogenetic trees were generated with MEGA 5.0 using the Neighbor-

Joining (NJ) algorithm. Bootstrap analysis with 1000 replicates was used to evaluate the significance of the nodes. Pairwise gap deletion mode was used to ensure that the divergent domains could contribute to the topology of the NJ tree. Genomic DNA was extracted from shoot apex and young leaves using the CTAB method. The primers used were gD14_for and gD14_rev. Primer sequences were listed in Table 1.

### 2.4. Subcellular localization

For construction of the *35S::DgD14-GFP* reporter vectors, the open reading frame (ORF) of *DgD14* was amplified with primers *Sal*I_for and *Sma*I_rev and was cloned into the binary vector pEZS-NL. Transformation into onion (*Allium cepa*) was performed as described previously (Varagona et al., 1992). Onion peels were unfolded in water, and then viewed under a confocal laser scanning microscope (Nikon); images were acquired using the EZ-C1 Free-Viewer software (Nikon). Primer sequences were listed in Table 1.

### 2.5. Vector construction and plant transformation

For complementation experiments, the ORF of *DgD14* was amplified with primers *Xba*I_for and *Spe*I_rev and was cloned into vector p-Super1300+.The resulting constructs were transformed into *A. thaliana* mutant *Atd14-1* plants via *Agrobacterium* according to the floral-dip method (Clough and Bent, 1998). Independent transformants were screened on MS medium containing 70 mg/L Hygromycin B. Independent homozygous T3 lines with single insertion sites were used for the branching phenotype analysis. Amplification of *Ubiquitin* gene was performed using 28 cycles as a normalization control, and the expression of *DgD14* was performed using 30 cycles. Primers used for semi-quantitative reverse transcription PCR (RT-PCR) analysis of *DgD14* or *Ubiquitin* were *DgD14*_for and *DgD14*_rev; *UBQ*_for and *UBQ*_rev. Primer sequences were listed in Table 1.

### 2.6. Split-plate and two-bud section system

The split-plate system was modified from that described previously for *Arabidopsis* (Chatfield et al., 2000). We removed a 10-mm wide strip of medium from the centre of a plate containing 30 mL solidified MS medium. Using a micro-pipette, we then injected 5 μM NAA or an equal volume of ethanol into upper side of the block, and 5 μM GR24 or an equal volume of acetone into basal side. Chrysanthemum seedlings were grown to 10–12 cm high in sterile conditions; they had two nodes (node n and node n+1) and a stem after decapitation or had an intact shoot apex. After hormones had diffused evenly throughout the media, two-bud sections were cut from the chrysanthemum seedlings, and then inserted into media. The petri dishes were then held vertically in the culture room. The "control" chrysanthemum seedlings had two nodes and a stem after decapitation; those with an intact shoot apex were named "intact." Topical buds (are equal to node n), basal buds (are equal to node n+1), and stems were harvested separately 6 h after treatment for analysis of *DgD14* transcripts. For each sample, 10–12 plants were collected. All experiments were repeated for 3 biological replicates. Samples were snap-frozen in liquid nitrogen, and stored at −80 °C.

**Table 1**
List of primers used in this study.

| Primers | Sequences (5′-3′) |
|---|---|
| P_for | 5′-HCACTCYGTYTCCGCYATGATCG-3′ |
| P_rev | 5′-GTWATATCKGGYCTCATRTTGAAA-3′ |
| 3′-race1 | 5′-GTTTTGTGGGTCATTCGGTTTC-3′ |
| 3′-race2 | 5′-TGCTTCACCTAGGTTCCTAAATG-3′ |
| 5′-race1 | 5′-TTCTCAAACAACTCAGGCCTCCT-3′ |
| 5′-race2 | 5′-GCATATGCATCAAGACACGTGTACT-3′ |
| gD14_for | 5′-ATGGTGGAAACCCTTTTAAACG-3′ |
| gD14_rev | 5′-TCATCTTGAAATTGCTCTGTTG-3′ |
| SalI_for | 5′-GTCGACATGGTGGAAACCCTTTTAAACGC-3′ |
| SmaI_rev | 5′-CCCGGGTCTTGAAATTGCTCTGTTGAGGTG-3′ |
| XbaI_for | 5′-TCTAGAATGGTGGAAACCCTTTTAAACG-3′ |
| SpeI_rev | 5′-ACTAGTTCATCTTGAAATTGCTCTGTTG-3′ |
| DgD14_for | 5′-GGTGGAAACCCTTTTAAAC-3′ (RT-PCR) |
| DgD14_rev | 5′-CATCTTGAAATTGCTCTGTT-3′ (RT-PCR) |
| UBQ_for | 5′-AACCCTTGAGGTTGAATCATC-3′ |
| UBQ_rev | 5′-GTCCTTCTTTCTGGTAAACGT-3′ |
| DgD14_for | 5′-TACGAGGCATGGGTGTGTGGATC-3′ |
| DgD14_rev | 5′-GCACGGCGCCTTCACTAACCCT-3′ |
| 18S rRNA_for | 5′-AAACGGCTACCACATCCAAG-3′ |
| 18S rRNA_rev | 5′-ACTCGAAAGAGCCCGGTATT-3′ |

### 2.7. Hormone treatments

The chrysanthemum cuttings (7 cm in height) were initially grown in vermiculite for 10 days, then plants were transferred into pots in a greenhouse, and hormone treatments began after 2 weeks (when plants were about 10 cm in height). Once every 2 days, 20 mL solution per 12 plants was sprayed over whole plants including leaves, buds, and stems. The solution contained 50 mg/L IAA, GA, or 6-BA with 0.5% Tween-20 respectively, and water containing 0.5% Tween-20 was used as control. The upper one-third section of plant buds and stems were harvested 0, 1, 6, 24, and 48 h after treatments began, each containing tissue originating from 10 to 12 plants. Tissues were snap-frozen in liquid nitrogen, and stored at −80 °C. Phenotype observation was performed after 10 days, and measured bud burst rate, stem diameter and internode length. All experiments had 3 biological replicates.

### 2.8. Plant decapitation and exogenous auxin expression assay

The chrysanthemum cuttings (7 cm in height) were grown initially in vermiculite for 10 days, then plants were transferred into pots in a greenhouse, and the plant decapitation assay and exogenous auxin assay were performed once the cuttings reached 15 cm in height (about 3.5 weeks). The plant shoot apex was either decapitated or left intact. For the decapitated stems, Eppendorf tubes containing MS agar medium with 5 μM NAA or an equal volume of ethanol were placed over the residual stem after decapitation. Bud 1 was adjacent to the cut site with 2, 3, 4 progressively further away, stem has same order below the numbered bud. Buds and stems were harvested at 0, 1, 6, 24, 48 h after treatments start, snap-frozen in liquid nitrogen, and stored at −80 °C. Three replicates were used for each sample, with 12–15 plants used per replicate.

### 2.9. RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Plant samples of 100–150 mg, each containing tissue originating from 10 to 15 plants, were harvested and total RNA was isolated using TRIzol Reagent (Invitrogen, USA). The RNA (1 μg) of each tissue was reverse transcribed using FastQuant RT Kit (with gDNase; KR106; Tiangen, China). qRT-PCR was performed on a StepOnePlus™ Real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) using KAPA™ SYBR Fast qPCR Master Mix (Microread, Beijing, China). The reaction procedure was as follows: denaturation at 95 °C for 20 s, followed by 40 cycles of 5 s at 95 °C and 20 s at 58 °C. The chrysanthemum *18S*

*rRNA* gene was used as an internal control for normalization, and the data were analyzed by OneStepPlus™ software (Applied Biosystems). The relative expression of the detected genes was calculated using the relative $2^{-\Delta\Delta CT}$ method. Primers used for qRT-PCR were *DgD14*_for and *DgD14*_rev; *18S rRNA*_for and *18S rRNA*_rev. Primer sequences were listed in Table 1.

### 2.10. Hydroponic culture

Chrysanthemum seedlings were grown to 8–10 cm high in sterile conditions in three weeks after, their roots were washed free after open the caps 1 day and the plants were transferred to a hydroponic solution consisting of an improved Hoagland's solution. P

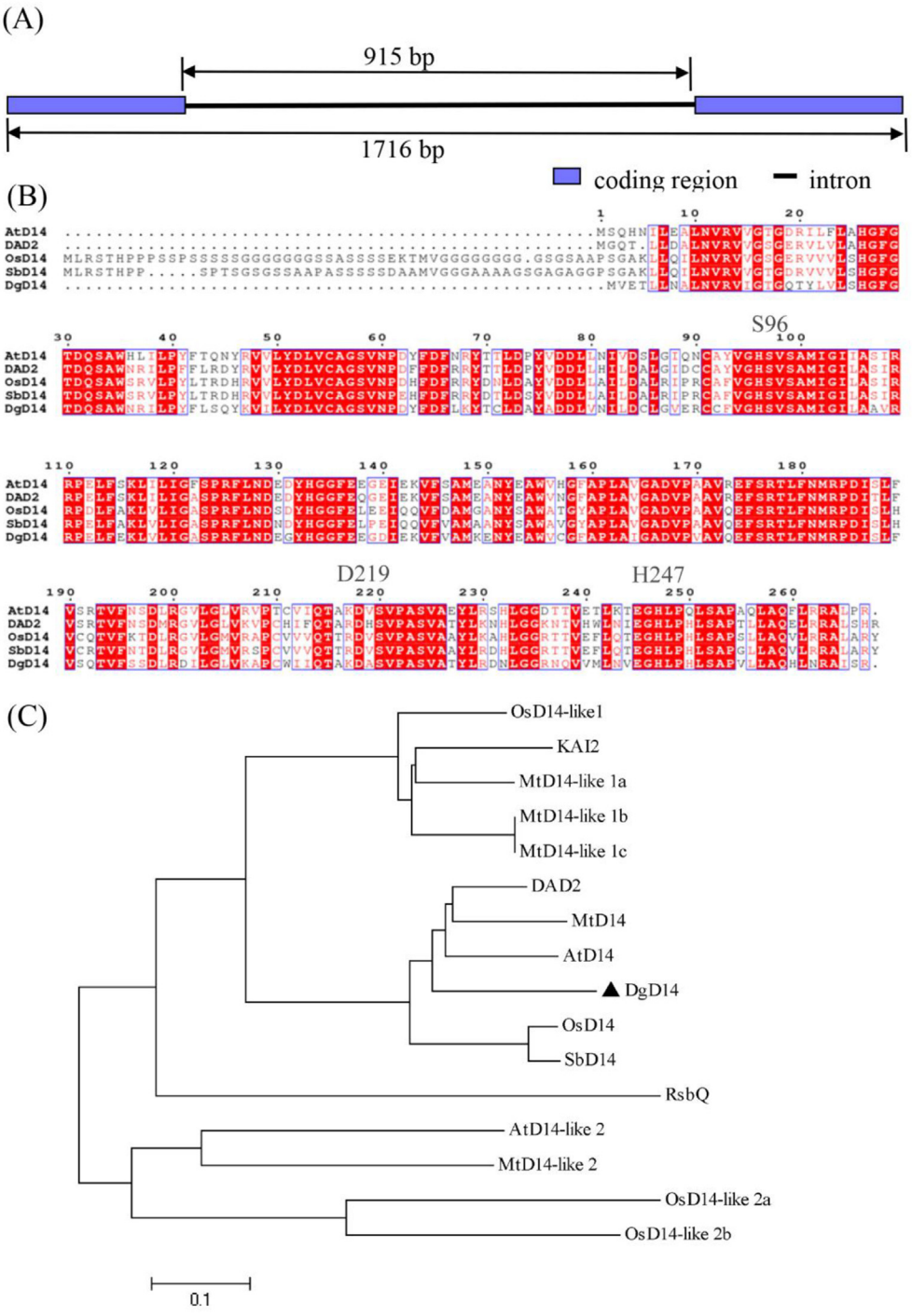


**Fig. 1.** Sequence analysis of the full-length cDNA named *DgD14*. **a** Structure of the *DgD14* gene. **b** Alignment of the predicted amino acid sequences of *DgD14* compared with homologs in *Arabidopsis* (AtD14, accession number NP_566220), petunia (DAD2, accession number XM_009775236), rice (OsD14, accession number NP_001049306), and sorghum (SbD14, accession number XP_002468316). The alignment was generated using ClustalW and ESPript (http://espript.ibcp.fr/ESPript/ESPript/). **c** Phylogenetic analysis of response regulators from a range of plant species. The aligned sequences were used to construct a phylogenetic tree using MEGA5.0. Accession numbers of genes used for phylogenetic analysis were listed in Supplementary Table S1.

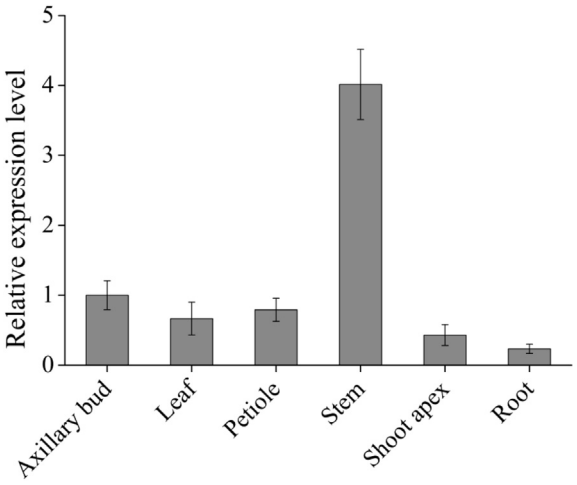


**Fig. 2.** qRT-PCR analysis of *DgD14* expression in chrysanthemum. All samples were taken from pools of 10–12 plantlets, and the experiment was repeated three times. Expression values are relative to the *18S rRNA* reference gene and normalized to axillary bud = 1. Error bars show SDs.

was supplied as $KH_2PO_4$ and N was supplied as $NH_4NO_3$. The pH of all solutions was adjusted to 6.0 with 3 M KOH. The final concentrations in the different solutions were 1.0 mM P and 17.0 mM N (normal solution; that is, the P/N recovery solution), 0 mM P and 17.0 mM N (P absence), and 1.0 mM P and 0.17 mM N (LN). The plants were grown in a growth chamber with a 16/8 h photoperiod at a light intensity of 100–120 μmol $m^{-2}$ $s^{-1}$ at 25/20 °C. The absent P or LN treatments were initiated 12 days after the transfer, then plants were removed to the P/N recovery solution after 2 weeks. The upper one-third section of the plants were regarded as "bud up" compared with the lower one-third of plants, which were regarded as "bud base." Stem has the same status as bud. Leaf, stem up/base, bud up/base, and root tissue was harvested at 0, 1, 10, 24, 48 h after treatments start, snap-frozen in liquid nitrogen, and stored at −80 °C. Three replicates were used for each sample, with 10–12 plants used per replicate.

### 2.11. Plant growth determination

Plants were harvested and separated into shoots and roots after 2 weeks of absent P, LN, or control treatments. We determined the fresh weight (FW) of plants, and counted the number of leaves and shoot branches (>0.2 cm), and primary root (PR) number. Data presented show average values of 10–12 plants.

### 2.12. Elemental analysis

Samples (500 mg) of the shoots and roots used in this study were oven-dried at 65 °C for 72 h, then the dry materials were ground in a mill, and measured as for the plant samples. For each sample, 10 plants were collected. P concentration was determined using an Autoanalyzer (Seal Analytical, Germany), while N concentration was determined by the Kjeldahl method on a Kjeldahl apparatus (KDY-9810; China) and Endpoint titrator DL15 (Mettler Toledo, China). Different letters are used in the table to indicate statistically significant differences between means determined under all examined P conditions by ANOVA with Duncan's test ($p < 0.05$).

## 3. Results

### 3.1. Identification of the D14 gene homolog of chrysanthemum

To address the regulatory role of the *D14* gene during shoot branching of chrysanthemum, a fragment of a putative *D14* ortholog, *DgD14*, was isolated, based on conserved sequences of known *D14* genes from *Arabidopsis*, petunia and rice. Full length cDNA of *DgD14* was isolated by 5′ and 3′ RACE PCR. The full transcript of *DgD14* (accession number KM503100) is 1016 bp in length and contains a 801 bp ORF encoding a predicted protein of 266 amino acids, a 40 bp 5′ untranslated region (UTR) and a 175 bp 3′ UTR. The genomic region corresponding to the *DgD14* coding region is 1716 bp and contains a 915 bp intron (Fig. 1a).

To explore the evolutionary relationship among *DgD14* gene from various plant species, we performed phylogenetic analysis using MEGA5.0. Our analysis showed that DgD14 is more closely related to DAD2, MtD14 and AtD14, which belong to "core D14"of the DWARF14 clade (Waters et al., 2012b) (Fig. 1c). Amino acid sequence comparisons between DgD14 and its orthologs from *Arabidopsis*, pea, rice, and sorghum showed that the predicted DgD14 has 74.91% sequence identity to *DAD2*, 72.28% to *AtD14*, 57.55% to *OsD14*, and 58.60% to *SbD14* (Fig. 1b), and also shares the putative hydrolase catalytic triad of Ser96, Asp219, and His247 residues.

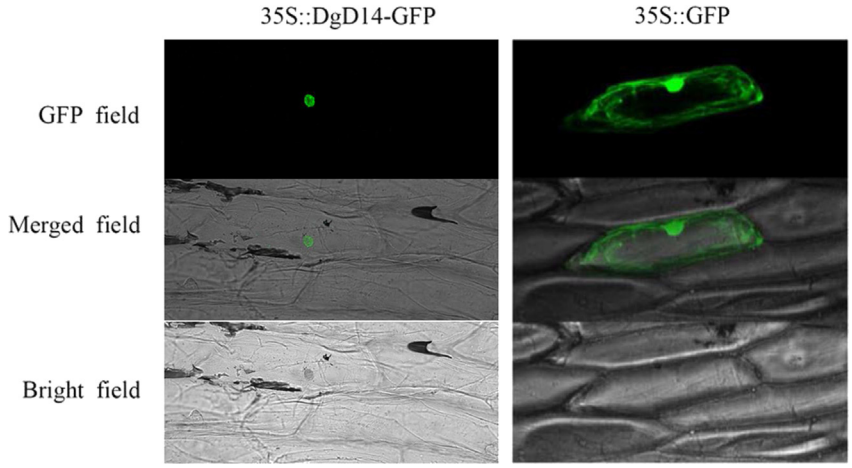


**Fig. 3.** Subcellular localization of DgD14. The photographs were taken in GFP field, merged field, and bright-light field for the morphology of the cell. Left panels show p*35S::DgD14-GFP* plasmid, right panels show p*35S::GFP* control plasmid.

### 3.2. Tissue specificity of DgD14 expression

We used qRT-PCR to determine the expression pattern of *DgD14* in axillary bud, leaf (petiole removed), petiole, stem, shoot apex and root during the vegetative phase of chrysanthemum. As shown in Fig. 2, the highest expression of *DgD14* was in stem, followed by in axillary bud, leaf, and petiole, and it was only weakly expressed in root. Expression levels were higher in shoot than root, and these results were similar to *D14* expression in rice (Arite et al., 2009), suggesting that *DgD14* may have a similar function in control of shoot branching as *D14* in rice.

### 3.3. DgD14 is localized to the nucleus

To further characterize the function of *DgD14*, we investigated the subcellular localization of DgD14. The ORF of *DgD14* was fused to green fluorescent protein (GFP) (in web version) in the pEZS-NL vector, and the construct *35S::DgD14-GFP* was bombarded into onion epidermal cells. The empty *35S::GFP* vectors were used as negative controls. We found that the control *35S::GFP* vector displayed fluorescence throughout the cells (Fig. 3 right), but the fluorescence in the onion cells transformed with *35S::DgD14-GFP* was restricted exclusively to the nucleus, demonstrating that DgD14 is a nuclear-localized protein (Fig. 3 left).

### 3.4. DgD14 complementation of Arabidopsis d14

To confirm the biological function of *DgD14*, a *35S::DgD14* transgene was introduced into the *Arabidopsis d14-1* mutant using a floral dip (Clough and Bent, 1998). Three independent transgenic lines were selected for phenotypic appraisal, and the *DgD14* expression level was checked by RT-PCR analysis. As shown in Fig. 4, all three transgenic lines had elevated levels of *DgD14* mRNA expression. Expression of *DgD14* in *d14-1* reduced the mean number of branches, and their branching phenotypes were close from those of wild-type plants. The expression of *DgD14* in *Atd14-1* plants almost completely restored the phenotype back to that of the WT. These results confirmed *DgD14* as the functional ortholog of *D14*.

### 3.5. Induction of DgD14 expression by auxin

To investigate auxin regulation of *DgD14*, qRT-PCR analysis was performed on two-bud segments cultured in a split-plate system, with or without apical application of auxin (Fig. 5b). Our results showed a 6-fold reduction in *DgD14* expression level at 6 h after decapitation, but this reduction was successfully reversed by apical NAA application. These results indicated that auxins could induce *DgD14* expression.

### 3.6. Feedback control of DgD14 expression

We investigated the effects of SL on *DgD14* expression, using two-bud segments cultured in a split-plate system. As shown in Fig. 5a, *DgD14* expression was reduced in all samples at 6 h after decapitation. In the bottom bud, *DgD14* expression was down-regulated by GR24 in intact plants compared with untreated controls, but this down-regulation was less than that observed upon decapitation. However, in the top bud, GR24 had no effect on *DgD14* expression in intact plants compared with untreated controls, however, *DgD14* was down-regulated upon decapitation. As shown in Fig. 5b, there was a significant difference between control and decapitated plants treated with NAA in all samples. *DgD14* expression was up-regulated by apical NAA or basal GR24 in the bottom bud of decapitated plants compared with untreated controls. Furthermore, there was no significant increase in decapitated plants treated with both apical NAA and basal GR24 compared with the decapitated plants treated individually with apical NAA or basal GR24. However in the top bud, basal GR24 had no effect on *DgD14* expression, while there was a significant increase in decapitated plants treated with both apical NAA and basal GR24 compared with the decapitated plants treated individually with apical NAA or basal GR24. These results indicated the presence of different feedback controls for *DgD14* expression between the top and bottom buds, suggesting that there was relative competitiveness between these two buds in chrysanthemum.

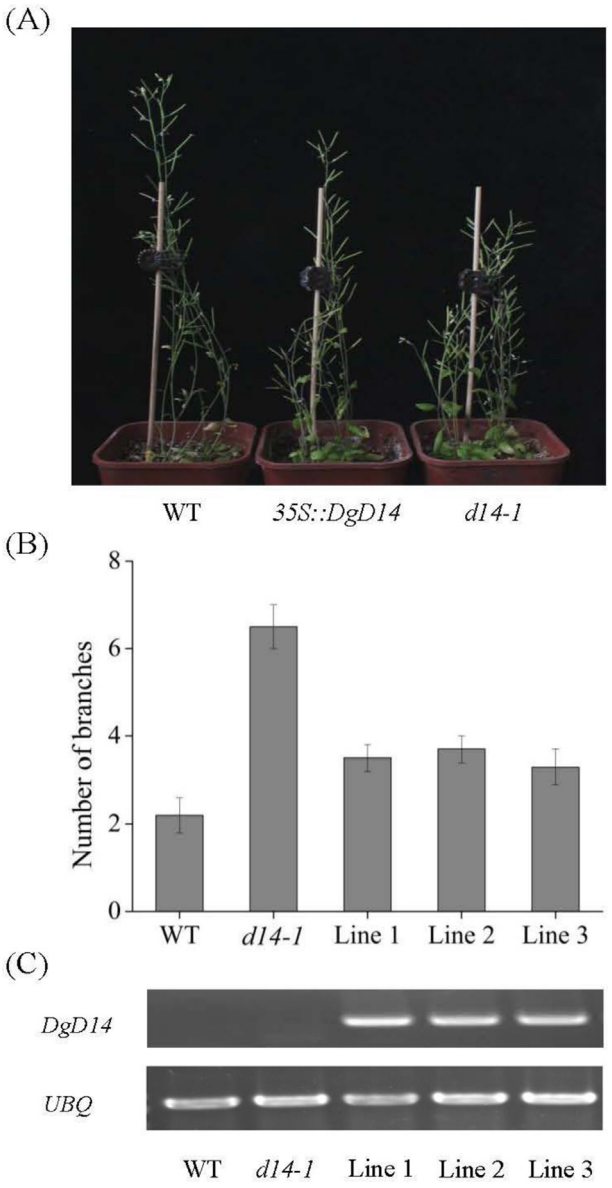


**Fig. 4.** Complementation of *Arabidopsis d14-1* mutant phenotype with *DgD14*. **a** Comparison of phenotypes of wild-type (WT), *d14-1* and *d14-1* transformed with the *35S::DgD14* constructs. **b** The number of secondary rosette branches produced by WT, *d14-1* and three independent homozygous lines transformed carrying *35S::DgD14*. The mean number of rosette branches with a length of at least 5 mm is shown. Data are means ± SE; n = 12. **c** Transcript levels of the overexpressed *DgD14* for the experiment presented in **b** were determined by RT-PCR. Detection of *UBQ* transcript was used as a cDNA normalization control.

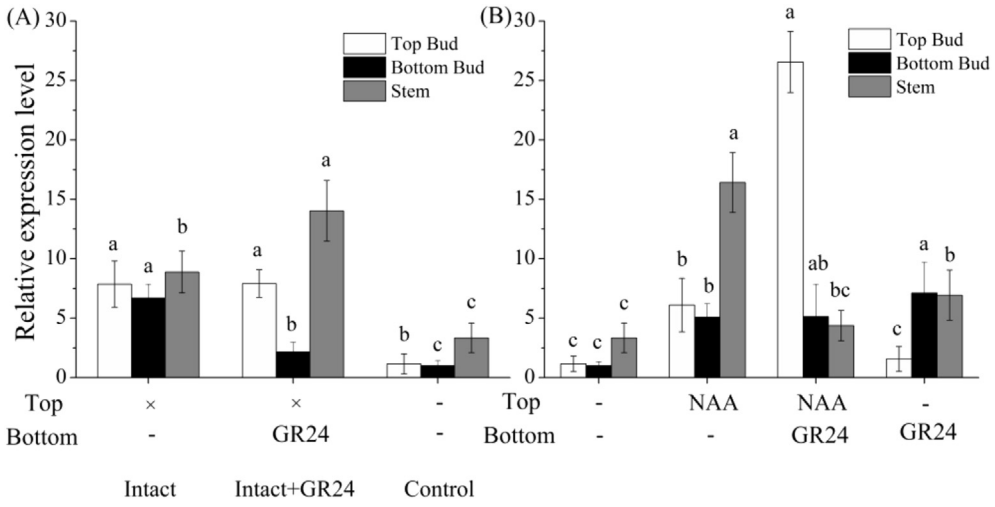


**Fig. 5.** *DgD14* expression response to decapitation, auxin, and GR24. **a** Plantlets were treated with 0 μM or 5 μM GR24 to the intact basal side, and an equal volume of acetone as a control. **b** Decapitated plants were treated with 0 μM or 5 μM NAA to the upper side (with an equal volume of ethanol as a control), and with 0 μM or 5 μM GR24 to the basal side (with an equal volume of acetone as a control). -, vertical control; ×, none treated. Top buds, bottom buds and stems were collected 6 h after treatment. Detection of *18S rRNA* was used as a reference gene and normalized to bottom bud in control samples = 1. Results are means of three biological replicates analyzed by qRT-PCR, with 10–12 plants for each replicate; letters indicate significant differences ($p < 0.05$) between different treatments. ANOVA followed by a Duncan's test. Error bars show SDs.

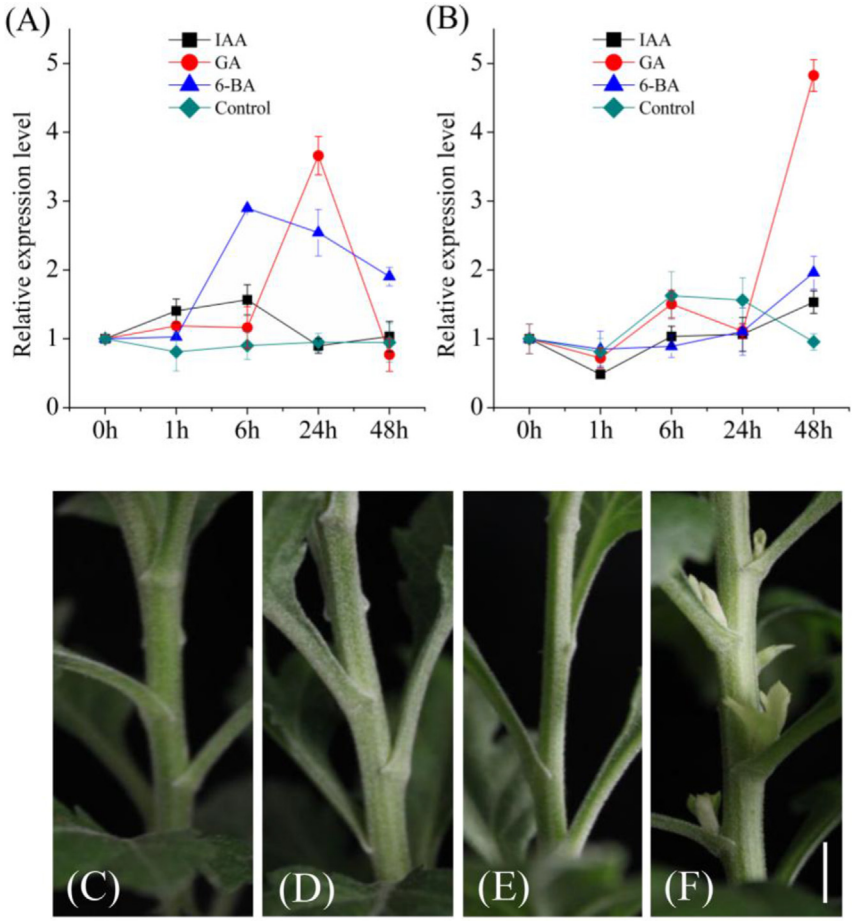


**Fig. 6.** Expression patterns of *DgD14* in response of buds and stems to hormone treatments in chrysanthemum. **a** *DgD14* expression was induced by treatment with 50 mg/L IAA, GA, 6-BA in buds respectively. **b** *DgD14* expression was induced by treatment with 50 mg/L IAA, GA, or 6-BA in stems respectively. **c**–**f** Phenotype of plant treatments: control, and 50 mg/L IAA, GA, or 6-BA after 10 days. Results are means of three biological replicates with 10–12 plants for each replicate. Bar = 1.2 cm.

**Table 2**
Effects of hormone treatments conditions on plant growth.

| Condition | Bud burst rate (%) | Stem diameter (mm) | Internode length (cm) |
|---|---|---|---|
| Control | 5.45 ± 1.87[b] | 4.15 ± 0.44[a] | 1.23 ± 0.12[b] |
| IAA | 4.78 ± 1.68[b] | 4.01 ± 0.51[a] | 1.08 ± 0.11[b] |
| GA | 4.08 ± 2.26[b] | 3.14 ± 0.37[b] | 1.62 ± 0.15[a] |
| 6-BA | 63.41 ± 10.47[a] | 3.99 ± 0.42[a] | 1.19 ± 0.17[b] |

Phenotype observation was performed after 10 days, and measured bud burst rate, stem diameter and internode length. Values are the mean ± SD (n = 10–12). Different superscript letters indicate significant differences ($p < 0.05$) calculated by Duncan's test.

### 3.7. Expression of DgD14 with hormone treatments

We used qRT-PCR analysis to determine expression patterns of *DgD14* in intact plants given hormone treatment. As shown in Fig. 6a, b, the results demonstrated that the expression patterns differed for each type of hormone treatment. The expression level of *DgD14* was induced significantly by 6-BA and GA in buds but was induced only weakly by IAA, while it increased significantly in stems at 48 h with GA treatment. After 10 days, bud burst rates were higher with 6-BA treatment, while stems were narrower and internodes longer, but there was no difference in bud burst rates with GA treatment. There was no obvious influence on any structure with IAA (Fig. 6c–f, and Table 2). Taken together, these data suggested that *DgD14* responded to GA both in buds and stems, and CKs only in buds.

### 3.8. Effects of apical dominance on DgD14 expression

Chrysanthemum has strong apical dominance; the upper part of the bud can rapidly grow out after decapitation. To determine the effects of apical dominance on *DgD14* expression levels, a classical decapitation assay was conducted. The transcript levels of *DgD14* dramatically decreased in all buds but only in apical stem 1 at 1 h after decapitation (Fig. 7a, b), by 48 h after decapitation, levels had recovered almost to pre-decapitation levels in buds, while they were still at a low level in stem 1 and they were increased in basal stems. Substitution of the decapitated apex with exogenous auxin resulted in 0.7–3-fold increase in expression of *DgD14* in all buds but only a single stem – namely, stem 1 (Fig. 7d, e), consistent with the known positive effect of auxins on gene transcription in two-bud segments cultured experimentally. Interestingly, all bud lengths were significantly decreased in decapitated plants treated with exogenous auxin, whereas basal bud burst rate was increased (Fig. 7g, h). Our results suggested that *DgD14* transcription was down-regulated rapidly by decapitation, and increased by exogenous auxin that played the role in the apical dominance.

### 3.9. Response of DgD14 in shoot and root under conditions of phosphate absence

To examine whether *DgD14* can respond to lack of phosphate, we assessed the expression levels of *DgD14* in a hydroponic solution with no phosphate and after 2 weeks in a normal phosphate solution. As shown in Fig. 8, *DgD14* expression was significantly up-

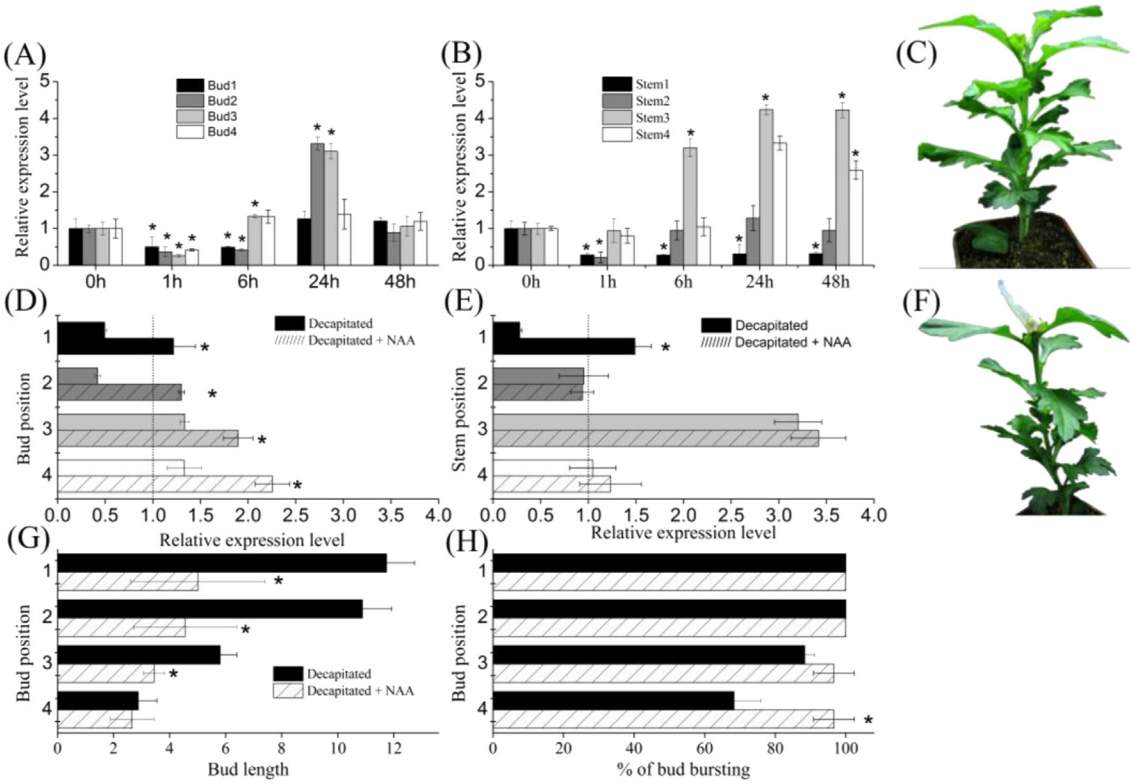


**Fig. 7.** Transcript levels of *DgD14* after decapitation and decapitation + NAA. **a** *DgD14* transcript levels in buds 1–4 were analyzed 0, 1, 6, 24 and 48 h after decapitation. **b** *DgD14* transcript levels in stems 1–4 were analyzed 0, 1, 6, 24 and 48 h after decapitation. **c** Phenotype of decapitation plant after 10 days. **d** *DgD14* transcript levels in buds 1–4 were analyzed 6 h after decapitation and after decapitation + NAA treatment. Black = 6 h post decapitation, striped = 6 h post decapitation + NAA. **e** *DgD14* transcript levels in stems 1–4 were analyzed 6 h after decapitation and decapitation + NAA. Black = 6 h post decapitation, striped = 6 h post decapitation + NAA. **f** Phenotype of decapitation + NAA plant after 10 days. **g** Bud length of decapitation/decapitation + NAA plants after 10 days. **h** Bud burst rates of decapitation/decapitation + NAA plants after 10 days. Bud position was recorded basipetally. Results are means of three biological replicates with 12–15 plants for each replicate. Asterisks indicate statistically significant differences ($p < 0.05$) calculated by Duncan's test between the intact and decapitated plants. Error bars show SDs (n = 12).

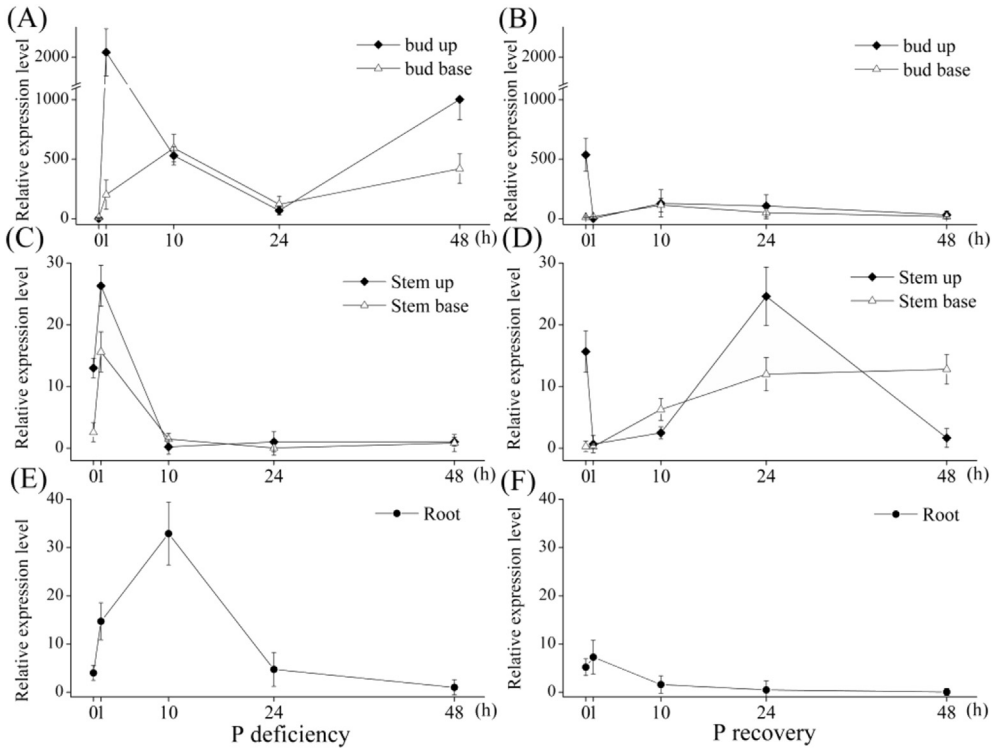


**Fig. 8.** *DgD14* gene expression under phosphate deficiency/recovery conditions in shoot and root. **a** *DgD14* transcript levels in bud up and base were analyzed 0, 1, 10, 24 and 48 h after phosphate deficiency. **b** *DgD14* transcript levels in bud up and base were analyzed 0, 1, 10, 24 and 48 h after phosphate recovery. **c** *DgD14* transcript levels in stem up and base were analyzed 0, 1, 10, 24 and 48 h after phosphate deficiency. **d** *DgD14* transcript levels in stem up and base were analyzed 0, 1, 10, 24 and 48 h after phosphate recovery. **e** *DgD14* transcript levels in root were analyzed 0, 1, 10, 24 and 48 h after phosphate deficiency. **f** *DgD14* transcript levels in root were analyzed 0, 1, 10, 24 and 48 h after phosphate recovery. Detection of *18S rRNA* was used as a reference gene, and normalized to bud up 0 h, stem up 24 h, root 48 h after phosphate deficiency = 1 respectively. The data presented are typical of three independent biological replicates with 10–12 plants for each replicate. Error bars show SDs.

regulated by phosphate absence both in shoot and root; in particular, it was extremely increased in bud at 1 h and attained higher levels after phosphate was removed. There was a more rapid response to phosphate absence in shoot than in root, and in addition, the upper buds increased *DgD14* expression more quickly than the basal buds. When plantlets were treated with normal phosphate solution after a 2-week absence of phosphate, *DgD14* expression then showed a downward trend both in shoot and root, except in basal stem. These results indicated that the response of chrysanthemum to absence of phosphate correlated with *DgD14* transcript levels.

### 3.10. Response of DgD14 in shoot and root under low N conditions

We investigated whether the effects of N treatment on *DgD14* expression were the same as for phosphate by growing plants in a hydroponic solution with low N, and after 2 weeks changing them to a normal N recovery solution. We found that *DgD14* expression level was highest at 1 h in leaf, but there was a mild variation up in bud and stem with low N treatment. In addition, after 48 h of LN conditions, there was a slight increase in *DgD14* expression in both shoot and root, but not in leaves (Fig. 9). After transfer to the normal nitrogen solution treatment, the expression level decreased only in root. Thus, the expression of *DgD14* under low N conditions was not the same as that seen under conditions of absent phosphate. Our results suggested that, unlike phosphate, N did not directly regulate *DgD14* expression in buds and stems of chrysanthemum.

### 3.11. Effects of phosphate deficiency and low N on phosphate levels in plant tissues

Previous reports indicated that the expression levels of SL biosynthesis genes depend on P level, and that N deficiency alters SL production and expression mainly by altering P levels in shoots (Yoneyama et al., 2012; Czarnecki et al., 2013). To determine the relationship by which P level induces different responses in *DgD14* gene expression, we measured effects on plant growth and the soluble P content in shoots and roots of chrysanthemum plants cultivated for 2 weeks in conditions of absent phosphate and low N (Fig. 10). Phosphate absence and low N negatively affected plant growth, decreasing FW and the number of shoot branches, but it did not affect the number of leaves (Table 3). The number of shoot branches was decreased both by low N and by absence of P. Low N promoted PR elongation more significantly than did P absence, whereas P absence significantly decreased P levels in the tissues of both shoot and root of plants (Table 4). By contrast, low N did not significantly decrease P levels in shoots but did decrease them in roots. These results showed that P levels in shoots had a close relationship with the fact that N did not directly regulate *DgD14* expression in buds and stems.

## 4. Discussion

SLs have been recognized as a new class of hormones that can directly regulate shoot branching. Recently, many direct or indirect genes of the SL biosynthesis and signaling pathways have been identified by isolation of mutants (Brewer et al., 2013). In chrysanthemum, only three SL pathway genes have been identified: *DgCCD8*, an SL biosynthesis gene; *DgMAX2*, an SL signaling gene; and *DgBRC1*, a downstream target of SL signaling (Liang et al., 2010; Chen et al., 2013; Dong et al., 2013). Identification of more genes and their functions in bud outgrowth would help to clarify the mechanisms involved in regulating branching in chrysanthemum.

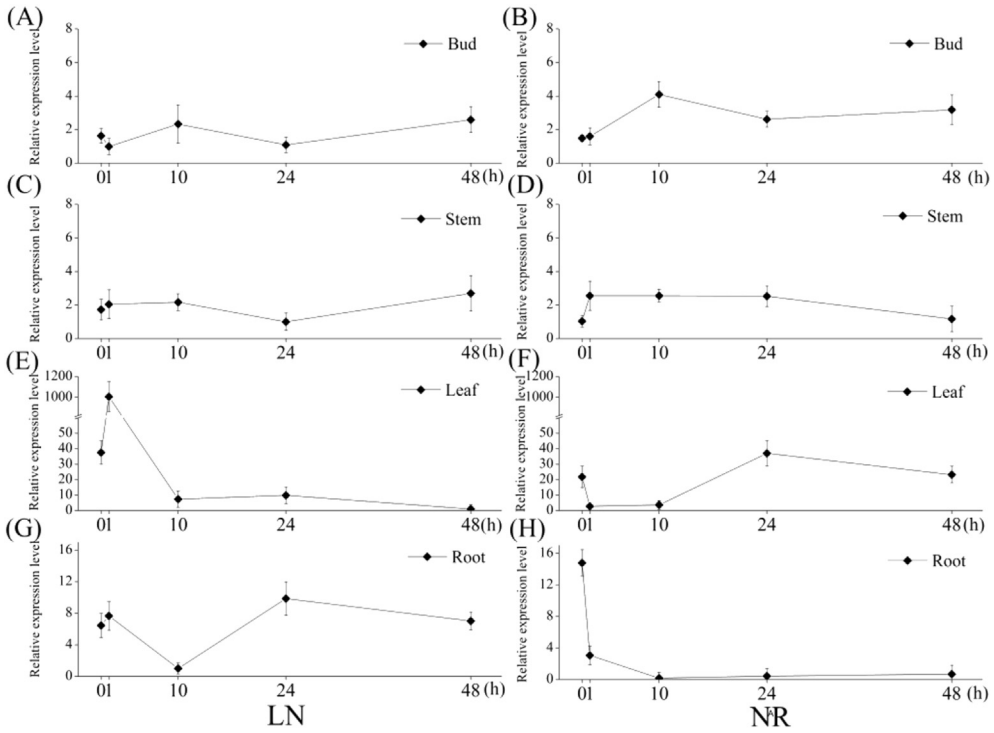


**Fig. 9.** *DgD14* gene expression under low nitrogen/nitrogen recovery conditions in shoot and root. LN: low nitrogen; NR: nitrogen recovery. **a** *DgD14* transcript levels in bud were analyzed 0, 1, 10, 24 and 48 h after low nitrogen. **b** *DgD14* transcript levels in bud were analyzed 0, 1, 10, 24 and 48 h after nitrogen recovery. **c** *DgD14* transcript levels in stem were analyzed 0, 1, 10, 24 and 48 h after low nitrogen. **d** *DgD14* transcript levels in stem were analyzed 0, 1, 10, 24 and 48 h after nitrogen recovery. **e** *DgD14* transcript levels in leaf were analyzed 0, 1, 10, 24 and 48 h after low nitrogen. **f** *DgD14* transcript levels in leaf were analyzed 0, 1, 10, 24 and 48 h after nitrogen recovery. **g** *DgD14* transcript levels in root were analyzed 0, 1, 10, 24 and 48 h after low nitrogen. **h** *DgD14* transcript levels in root were analyzed 0, 1, 10, 24 and 48 h after nitrogen recovery. Detection of *18S rRNA* was used as a reference gene, and normalized to bud 1 h, stem 24 h, leaf 48 h, root 10 h after phosphate deficiency = 1 respectively. The data presented are typical of three independent biological replicates with 10–12 plants for each replicate. Error bars show SDs.

D14 encodes a protein classified as a member of the α/β-fold hydrolase superfamily. There are two D14 homologs found in rice and *Arabidopsis*, but the D14 subfamily consists of genes from angiosperm species only, while the D14-like subfamily consists of genes from gymnosperm, fern, and bryophyte species (Arite et al., 2009; Waters et al., 2012b). In the present study, we isolated a gene of the α/β-fold hydrolase superfamily named *DgD14*, and found that the deduced amino acid sequence of *DgD14* showed high similarity with *P. hybrida* (74.91%), *A. thaliana* (72.28%), *O. sativa* (57.55%), and *S. bicolor* (58.60%). As with other homologs, the *DgD14* gene had only one intron. Phylogenetic analysis further showed that DgD14, along with DAD2, MtD14, AtD14, and OsD14 was placed in "core D14"of the DWARF14 clade (Waters et al., 2012b), indicating that *DgD14* belonged to the D14 subfamily.

In rice, transcription of *D14* has been detected in various tissues, and accumulations of high levels of *D14* were detected in leaves and the first leaf buds, but not in root tip (Arite et al., 2009). In petunia, high expression levels of *DAD2* were evident in axillary bud and leaf, but not in root (Hamiaux et al., 2012). In the current study, we found that *DgD14* had its highest expression in stem, followed by node, and was only weakly expressed in root.

In rice, an approximately 2.7 kb upstream region of *D14*, which was used in the complementation test, and used as a promoter to drive *GUS* (β-glucuronidase) to examine the spatial distribution of *D14* gene expression (Arite et al., 2009). In the current study, we only used 35S promoter of vector in subcellular localization and the complementation test. In future studies, identifying the promoter region of *DgD14* would help to characterize the function of *DgD14*.

The interactions between auxins and SLs in controlling shoot branching have also been studied previously. Auxins were found to be transported basipetally down the main stem in the polar auxin transport stream (PATS), and act indirectly to inhibit bud growth without entering the bud itself (Booker, 2003). By applying the synthetic SL analogue GR24 directly to the axillary buds, SLs can directly inhibit shoot branching (Gomez-Roldan et al., 2008). Auxins can regulate the expression of SL biosynthesis genes (Foo et al., 2005; Johnson et al., 2006), and the SL pathway was shown to regulate auxin transport canalization into the main stem by modulating *PIN* expression (Bennett et al., 2006; Marhavy et al., 2011; Shinohara et al., 2013). In *Arabidopsis*, chrysanthemum, and biomass willow, GR24 was shown to be effective at inhibiting buds in the presence of an auxin source in one-node stem segment assays. However, in two-node assays, the apical bud was always favored over the basal bud in chrysanthemum and biomass willow, while the basal bud dominated in *Arabidopsis* (Crawford et al., 2010; Liang et al., 2010; Ward et al., 2013). In the previous two-node assays studies in chrysanthemum, GR24 was effective at inhibiting bud growth only in the presence of at least one competing auxin source. In the current study, we found that *DgD14* expression could be down-regulated by decapitation and up-regulated by exogenous application of auxin. It was induced by GR24 alone to a similar degree as with apical NAA treatment alone in the bottom bud, but was significantly increased only by apical NAA treatment alone in the top bud. Apical NAA treatment together with basal GR24 was more effective at increasing *DgD14* expression than NAA and GR24 alone in the top bud, but *DgD14* expression was significantly increased only in the bottom bud. Auxins was transported basipetally down the main stem in PATS, SLs was transported upward, differences in apical and basal bud responses may be caused by transport over the stem segment in the 6 h time window. These

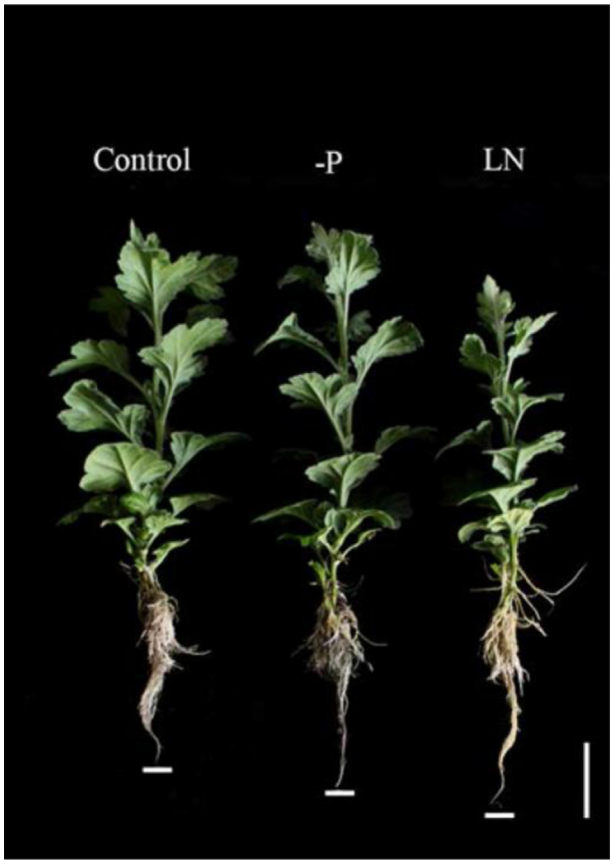


**Fig. 10.** Phenotype of plants treatment with −P and LN conditions after 2 weeks. Bar = 3 cm. Control: normal solution, −P: phosphate deficiency; LN: low nitrogen.

results were consistent with phenotypes resulting from SL being able to inhibit growth of the basal bud in two-node assays, while the apical bud was unaffected (Liang et al., 2010), and suggests that there was relative competitiveness between different buds in chrysanthemum.

Auxin is proposed to be the first hormone that acts to maintain shoot apical dominance in higher plants (Phillips, 1975). Auxins can up-regulate the transcription of SL pathway genes, and the inhibition of *RMS1* and *RMS5* genes expression in the stem can be restored by decapitation (Foo et al., 2005; Ferguson and Beveridge, 2009; Hayward et al., 2009; Zhang et al., 2010), SLs can regulate auxin transport in the stem, and function downstream of auxins to inhibit bud outgrowth (Bennett et al., 2006; Brewer et al., 2009). In previous studies on chrysanthemum, release of the top three buds were released predominantly correlated with down-regulation of *DgBRC1* after decapitation, and the expression of *DgBRC1* could be up-regulated by auxins. In the present study, we determined the effects of apical dominance on *DgD14* expression levels by classic

**Table 4**
P/N levels (mg/g DW) in shoot and root tissues.

| Condition | P level | | N level | |
|---|---|---|---|---|
| | Shoot | Root | Shoot | Root |
| Control | 4.90 ± 0.56$^{a}$ | 13.49 ± 1.3$^{a}$ | 6.62 ± 0.46$^{a}$ | 7.26 ± 0.22$^{a}$ |
| −P | 2.48 ± 0.11$^{b}$ | 3.99 ± 0.19$^{c}$ | 5.82 ± 0.22$^{b}$ | 7.19 ± 0.42$^{a}$ |
| LN | 4.66 ± 0.76$^{a}$ | 6.60 ± 0.69$^{b}$ | 3.58 ± 0.08$^{c}$ | 4.73 ± 0.48$^{b}$ |

Shoot and root tissues of plants subjected to each nutrient condition were collected 14 days after nutrient treatments. Control: normal solution; −P: phosphate deficiency; LN: low nitrogen. Values are means ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$) calculated by Duncan's test.

decapitation assay. Our data showed that *DgD14* expression responded rapidly to the release of apical dominance resulting from decapitation; the expression levels of *DgD14* in buds decreased dramatically at 1 h following decapitation, and then recovered almost to pre-decapitation levels by 48 h after decapitation. Additionally, *DgD14* expression levels in buds were up-regulated after apically application of NAA, but the distal two buds were obviously smaller than the decapitated ones, further demonstrating that *DgD14* as a response regulator is related to auxin regulation of bud outgrowth.

SLs can respond to nutrient supply conditions, and play important roles in regulation plant architecture, including branch numbers, plant height, PR length, LR (the lateral root) number, and LR density (Mayzlish-Gati et al., 2012; Czarnecki et al., 2013). Expression levels of SL biosynthesis genes have been shown to depend on the P level. Under conditions of P and/or N deficiency, the expression levels of the SL pathway genes were up-regulated and SL expression was enhanced, but SL expression was promoted only under P deficiency in modulation of leguminous plants, whereas in other mycotrophic plants it occurred under both P-deficient and N-deficient conditions (Yoneyama et al., 2007; Umehara et al., 2010; Jamil et al., 2011; Bonneau et al., 2013; Foo, 2013). In our preliminary studies, three different types of SLs were found to be expressed in chrysanthemums grown in hydroponic solution without P (data not shown), implying that absence of P also stimulates SL biosynthesis in chrysanthemum. In this study, the results revealed different responses in *DgD14* expression to absent P and low N conditions. Expression of *DgD14* was rapidly increased at the highest level after 1 h in shoot and after 10 h in root under conditions of absent P, but only had the highest level value after 1 h treatment in leaf under low N conditions. The expression levels of *DgD14* in buds were significantly increased after 48 h in a solution lacking P; however, there was almost no difference in a solution with low N. Levels of *DgD14* expression were rapidly induced upon P deficiency, but there was the presence of feedback controls for *DgD14* expression locally in axillary buds; this may be the reason why gene expression dropped off steeply after several hours. In addition, leaves were an important nitrogen source sink, nitrogen would be transferred upon low nitrogen treatment and this may be the reason why the expression levels of *DgD14* changed in the leaves. P levels were significantly decreased in the tissues of

**Table 3**
Effects of −P and LN conditions on plant growth.

| Condition | FW (g) | | Leaves, n | Shoot branches, n | Length of PR (cm) |
|---|---|---|---|---|---|
| | Shoot | Root | | | |
| Control | 13.4 ± 2.4$^{a}$ | 2.5 ± 0.6$^{a}$ | 19.0 ± 1.6$^{a}$ | 10.6 ± 2.1$^{a}$ | 7.2 ± 0.7$^{b}$ |
| −P | 12.1 ± 2.6$^{ab}$ | 2.1 ± 0.4$^{ab}$ | 18.8 ± 1.6$^{a}$ | 8.3 ± 2.3$^{b}$ | 9.3 ± 1.7$^{ab}$ |
| LN | 9.5 ± 1.8$^{b}$ | 1.9 ± 0.3$^{b}$ | 17.9 ± 2.3$^{a}$ | 7.2 ± 1.7$^{b}$ | 11.3 ± 2.4$^{a}$ |

Plants were harvested 14 days after hydroponics to each nutrient condition. Control: normal solution; LN: low nitrogen; −P: phosphate deficiency. Values are the mean ± SD (n = 10–12). Different superscript letters indicate significant differences ($p < 0.05$) calculated by Duncan's test.

both shoots and roots of plants under absent P conditions, but were decreased in root only, not in shoot, under low N conditions. This may be the reason why the expression levels of *DgD14* dramatically changed in conditions with absent P and only steadily changed in low N conditions.

Additionally, there were three reasons that may have caused the difference between the bud (stem) base and upper parts upon P deficiency. i) From Sections 3.5 and 3.6, we know that there was relative competitiveness between the top and bottom buds in chrysanthemum. ii) From Section 2.10, the upper one-third section of the plants was regarded as "bud up" compared with the lower one-third section of the plants, which was regarded as "bud base." The stem had the same status as the bud. There may be some difference in development degree between bud (stem) up and bud (stem) base. iii) The lower one-third section of the plants had more lignification than the upper sections, which hindered the transverse transportation of hormones.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Contributions

Chao Wen and Liangjun Zhao designed the experiments; Chao Wen and Lin Xi carried out split-plate and two-bud section system, hydroponic culture, RNA extraction, and qRT-PCR analysis; Chao Wen and Bin Gao applied hormone treatments; Chao Wen and Keyong Wang performed the nutrient analysis;

Chao Wen and Suhui Lv carried out the subcellular localization experiments; Chao Wen and Yaping Kou did the plant decapitation and exogenous auxin assay; and Chao Wen, Nan Ma, and Liangjun Zhao prepared the manuscript.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.07.030.

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