

RESEARCH PAPER

Strigolactone regulation of shoot branching in chrysanthemum (*Dendranthema grandiflorum*)

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Abstract

Previous studies of highly branched mutants in pea (*rms1–rms5*), *Arabidopsis thaliana* (*max1–max4*), petunia (*dad1–dad3*), and rice (*d3*, *d10*, *htd1/d17*, *d14*, *d27*) identified strigolactones or their derivatives (SLs), as shoot branching inhibitors. This recent discovery offers the possibility of using SLs to regulate branching commercially, for example, in chrysanthemum, an important cut flower crop. To investigate this option, SL physiology and molecular biology were studied in chrysanthemum (*Dendranthema grandiflorum*), focusing on the *CCD8/MAX4/DAD1/RMS1/D10* gene. Our results suggest that, as has been proposed for *Arabidopsis*, the ability of SLs to inhibit bud activity depends on the presence of a competing auxin source. The chrysanthemum SL biosynthesis gene, *CCD8* was cloned, and found to be regulated in a similar, but not identical way to known *CCD8*s. Expression analyses revealed that *DgCCD8* is predominantly expressed in roots and stems, and is up-regulated by exogenous auxin. Exogenous SL can down-regulate *DgCCD8* expression, but this effect can be overridden by apical auxin application. This study provides evidence that SLs are promising candidates to alter the shoot branching habit of chrysanthemum.

Key words: Auxin, *CCD8*, chrysanthemum, shoot branching, strigolactone.

Introduction

Chrysanthemum (*Dendranthema grandiflorum*) accounts for 30% of world cut flower production. Conventionally, the lateral shoots are controlled by manual decapitation or removing the axillary buds, which is labour-intensive and accounts for about one-third of total cultivation costs. Therefore, chrysanthemum producers are in need of novel approaches to control shoot branching.

Shoot branching depends on both the formation of axillary buds in the axils of leaves and their subsequent outgrowth. Outgrowth is a more interesting target for breeders, since it allows greater flexibility in branching. Axillary shoot apical meristems are established in the axils of the leaves produced by the primary shoot apical meristem and after forming only a few leaves, they can enter a dormant state. These dormant buds may be reactivated by endogenous or environmental signals, contributing to the enormous diversity of plant architectures observed in nature (Dun *et al.*, 2006; Leyser, 2009).

Hormones play a central role in shoot branching control. Auxin inhibits bud outgrowth (Thimann and Skoog, 1933; Skoog and Thimann, 1934), whereas cytokinin promotes it (Sachs and Thimann, 1967). These hormones can efficiently regulate shoot branching, but their application also affects flowering, leaf development, and height, reducing their utility for the horticultural industry.

A series of branching mutants with fewer pleiotropic phenotypes has been identified. These include the *more axillary branching* (*max*) mutants of *Arabidopsis*, the *ramosus* (*rms*) mutants of pea (*Pisum sativum*), the *decreased apical dominance* (*dad*) mutants of petunia (*Petunia hybrida*), and the *dwarf* (*d*) or *high tillering dwarf* (*htd*) mutants of rice (*Oryza sativa*) (reviewed in Dun *et al.*, 2006; Leyser, 2009). Reciprocal grafting and double mutant studies demonstrate that these genes are involved in the production and signalling of a graft-transmissible, upwardly moving branch inhibitor, which was recently shown to be

strigolactone or a derivative (SL) (Beveridge *et al.*, 1996, 2000; Napoli, 1996; Foo *et al.*, 2001; Morris *et al.*, 2001; Turnbull *et al.*, 2002; Sorefan *et al.*, 2003; Booker *et al.*, 2004, 2005; Snowden *et al.*, 2005; Simons *et al.*, 2007; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

To date, four loci involved in SL biosynthesis and one locus involved in SL signalling have been molecularly identified (Stirnberg *et al.*, 2002; Sorefan *et al.*, 2003; Booker *et al.*, 2004, 2005; Lin *et al.*, 2009). A sixth gene in the pathway has been described, represented by rice *D14*, which acts either in a biosynthetic step downstream of SL or in SL signalling (Arite *et al.*, 2009). Of the biosynthetic genes, two encode divergent plastidic carotenoid cleavage dioxygenases. *MAX4/RMS1/DAD1/D10* encodes CAROTENOID CLEAVAGE DIOXYGENASE (CCD8) (Sorefan *et al.*, 2003; Snowden *et al.*, 2005; Arite *et al.*, 2007), whereas *MAX3/RMS5/HTD1/D17/SICCD7* encodes CCD7 (Booker *et al.*, 2004; Johnson *et al.*, 2006; Zou *et al.*, 2006; Vogel *et al.*, 2009). CCD8 and CCD7 sequentially cleave a variety of carotenoid substrates (Booker *et al.*, 2004; Schwartz *et al.*, 2004; Aldridge *et al.*, 2006; Alder *et al.*, 2008). *D27* encodes a plastidic iron-containing protein, which possibly acts at an early point in the pathway (Lin *et al.*, 2009). *MAX1* encodes a cytochrome P450 predicted to act downstream of CCD7 and CCD8 (Booker *et al.*, 2005). *MAX2/RMS4/D3* encodes a nuclear-localized F-box protein (Stirnberg *et al.*, 2002; Ishikawa *et al.*, 2005; Johnson *et al.*, 2006), involved in SL signalling (Beveridge *et al.*, 1996; Booker *et al.*, 2005; Stirnberg *et al.*, 2007).

The mode of action of SLs and their relationship with auxin is a matter of debate. One hypothesis is that SLs inhibit branching by reducing auxin transport canalization, preventing the establishment of polar auxin transport out of axillary buds into the main stem, and thus preventing their activation. According to this model, SLs act systemically to modulate auxin transport, and their ability to inhibit buds depends on the presence of competing auxin sources, such as the primary shoot apex or other active buds, for common auxin transport pathways in the main stem (Prusinkiewicz *et al.*, 2009). However, it has also been proposed that SLs act directly in axillary buds to inhibit them by some unknown, but auxin-transport-independent mechanism (Brewer *et al.*, 2009).

Another point of debate is the extent of negative feedback in the SL pathway and the role of auxin in the process. In most species, auxin positively regulates the expression of both *CCD8* and *CCD7* (Sorefan *et al.*, 2003; Bainbridge *et al.*, 2005; Foo *et al.*, 2005; Johnson *et al.*, 2006; Arite *et al.*, 2007; Hayward *et al.*, 2009). However in rice, *CCD7/HTD1* is not stably up-regulated by auxin (Zou *et al.*, 2006; Arite *et al.*, 2007). There is also good evidence for widespread feedback regulation in the SL pathway (Foo *et al.*, 2005; Snowden *et al.*, 2005; Arite *et al.*, 2007; Hayward *et al.*, 2009). *CCD7* and *CCD8* transcripts generally accumulate above wild-type levels in SL mutants. This effect is much stronger in pea than in the other species. In rice, significant up-regulation was observed for *CCD8/D10* but not for *CCD7/HTD1* in the *d3* mutant background

(Arite *et al.*, 2007). The relationship between auxin and feedback regulation of SL biosynthesis is unclear. SL mutants transport increased amounts of applied auxin compared to wild-type (Beveridge *et al.*, 2000; Bennett *et al.*, 2006; Lazar and Goodman, 2006; Lin *et al.*, 2009), and in *Arabidopsis* this correlates with the over-accumulation of auxin in the polar transport streams of their stems (Prusinkiewicz *et al.*, 2009). Furthermore, double mutants defective in both auxin signalling and SL signalling, feedback up-regulation of *CCD7/MAX3* or *CCD8/MAX4* is mostly abolished. This suggests that feedback signalling is substantially mediated by auxin (Hayward *et al.*, 2009). Consistent with this idea, as described above, in rice there is a correlation between auxin-inducibility and feedback regulation (Arite *et al.*, 2007). However, the situation in pea is different since auxin treatments cannot achieve the particularly high level of *CCD8/RMS1* and *CCD7/RMS5* up-regulation observed in *rms* mutants, suggesting an auxin-independent feedback (Foo *et al.*, 2005; Johnson *et al.*, 2006). It is therefore clear that comparative approaches in diverse species have contributed to our understanding of SL function, but also raised some interesting questions about the degree of conservation of the system between species.

Thus the analysis of the SL pathway in additional species has the potential to contribute to both the development of applications for SLs in agriculture and horticulture, and to resolving questions about the mechanism of action of SLs. To these ends, work aimed at the evaluation of SL physiology in chrysanthemum, as well as the identification of chrysanthemum genes required for SL synthesis, is described in this paper.

Materials and methods

Plant materials and growth condition

Chrysanthemum (*Dendranthema grandiflorum*) cv. Jinba plantlets were propagated under sterile conditions in jars containing MS agar medium (Murashige and Skoog, 1962) and grown in a tissue culture room at 21 °C, with 16/8 h light/dark photoperiods and a light intensity of 100–120 $\mu\text{mol m}^{-2} \text{s}^{-2}$. *Arabidopsis thaliana* Columbia (WT) and *max4-1* were grown in 4 cm square compartments containing F2 compost (Levington Horticulture, Ipswich, UK) and transferred to a greenhouse at 21 °C, with 16/8 h light/dark photoperiods.

Hormone stocks

Naphthalene acetic acid (NAA) stock solution was dissolved in 70% ethanol, and GR24 (LeagGen Labs, Orange CT USA) was dissolved in acetone were injected into the apical or basal blocks, as required, to give a final concentration of 5 μM . Solvent treatments were used as controls.

Split plate assay

The hormone responses of buds on isolated stem segments were assessed using the method described by Chatfield *et al.* (2000).

Isolation of the DgCCD8 genes

Total RNA was extracted from the stem and roots of chrysanthemum plantlets using Trizol (Mylab, China). cDNA was

synthesized using Superscript II reverse transcriptase (Invitrogen). Degenerate primers were designed from the amino acid sequence of the Arabidopsis *MAX4*, petunia *DAD1*, and pea *RMS1* gene and used to amplify a fragment of the *DgCCD8* gene from cDNA, with forward primer (5'-GTSGTGAGRATGGAASCHGG-3') and reverse primer (5'-CCATCATCYTCWTSGGTTGC-3'). The amplification products were used to design the primers for Rapid Amplification of cDNA Ends (RACE), which was used to obtain full-length *DgCCD8* transcripts. For 3' RACE, cDNA was synthesized with the adaptor oligo (dT) primer (5'-CTGATCTA-GAAGGTACCGGATCCT(15)-3') and amplified by PCR using the adaptor primer (5'-CTGATCTAGAAGGTACCGGATCCT-3') and a specific primer 3P (5'-GGGTTGGGCGGTTAGGA-TACCATTTCG). For 5' RACE, dC-tailed cDNA was synthesized using a specific primer 5P (5'-AGTCTATCTTAGTCAGAGTGT-3'). The amplification was performed with primer AAP (5'-GGCCACGCGTCGACTAGTACGGGIIIGGGIIGGGIIG-3') and the specific primer 5P. This PCR product was used as the template in an additional nested PCR using specific primer 5P1 (5'-TGGCATCTCGGGCACAATCACA-3') and primer AUAP (5'-GGCCACGCGTCGACTAGTAC-3'). Amplified fragments were cloned into the pMD18-T vector and sequenced. According to the sequences, a forward primer from the 5'-UTR region (5'-TAG-CAAACCTCTTTATTACCGATGG-3') and a reverse primer from 3'-UTR (5'-CTTTATTTCCGACATTTGCCCTTTC-3') region of *DgCCD8* were designed to isolate the complete *DgCCD8* from both cDNA and genomic DNA. Genomic DNA was isolated from young leaves using the DNeasy Plant Mini Kit (Qiagen). The amplification products were used to determine the sequence of the chrysanthemum cDNAs and genomic clones, and the positions of introns in the genes.

Phylogenetic analysis

Orthologues of the known *CCD8* genes were identified using InParanoid (Remm *et al.*, 2001) to perform pairwise comparisons between each of the published or draft complete genome sequences: *Arabidopsis thaliana* (Rhee *et al.*, 2003), *Brachypodium distachyon* (<http://www.brachypodium.org/>), *Lotus japonicus* (Sato *et al.*, 2008), *Medicago truncatula* (<http://www.medicago.org/>), *Oryza sativa* (Ouyang *et al.*, 2007), *Physcomitrella patens* (Rensing *et al.*, 2008), *Populus trichocarpa* (Tuskan *et al.*, 2006), *Selaginella moellendorffii* (<http://genome.jgi-psf.org/Selmo1/Selmo1.home.html>), *Sorghum bicolor* (Paterson *et al.*, 2009), *Vitis vinifera* (Jaillon *et al.*, 2007), and *Zea mays* (Schnable *et al.*, 2009). The orthology of candidate genes was confirmed during phylogenetic reconstruction. Sequences were aligned using MUSCLE v3.6 (Edgar, 2004) using default settings. The maximum likelihood phylogeny was reconstructed under the Dayhoff substitution matrix using RA×ML (Stamatakis, 2006). Support for the maximum likelihood phylogenies was estimated from 100 rapid bootstrap resamplings (Stamatakis *et al.*, 2008).

Generation of transgenic plants

For complementation experiments, the ORFs of *DgCCD8a* and *DgCCD8b* were cloned into pART7 fusing them with the 35S promoter, and then these fusions were transferred into the binary vector pART27 (Gleave, 1992). The constructs were transformed into *Arabidopsis thaliana* mutant *max4-1* plants via *Agrobacterium tumefaciens* strain GV3101 using the floral dip method (Clough and Bent, 1998). The independent transformants were screened on *Arabidopsis thaliana* salts (ATS) agar medium containing 50 mg l⁻¹ kanamycin. Independent, single insertion site, homozygous T₃ lines were used to analyse the branching phenotype.

Decapitation assay

To quantify branching, a decapitation assay was used (Bainbridge *et al.*, 2005). Seeds of *max4-1*, WT, [*35S::DgCCD8a*] *max4-1* and

[*35S::DgCCD8b*] *max4-1* lines were sown in F2 compost and grown in a growth cabinet at 21 °C, 8/16 h light/dark photoperiods. After 28 d, plants were shifted to 16/8 h light/dark photoperiods to induce flowering. Primary bolts were decapitated once they reached 10–15 cm in height. Rosette branches were counted 10 d after decapitation.

Hormone treatments

For analysis of *DgCCD8* expression after decapitation, auxin, or GR24 treatment, *in vitro*-grown chrysanthemum plantlet cuttings, 15 cm in height, were transferred into jars containing MS-agar medium with 5 μM GR24 or an equal volume of acetone. They were left intact or were decapitated, and Eppendorf tubes containing MS-agar medium with 5 μM NAA or an equal volume of ethanol were placed over the stumps. After 6 h treatment, the stem spanning the basal three nodes of six plantlets per treatment was excised and the tissue pooled to extract total mRNA, which was used to examine the accumulation of total *DgCCD8* transcripts.

Semi-quantitative RT-PCR

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using Superscript II (Invitrogen) according to the manufacturer's manual. To investigate the total expression of *DgCCD8* in chrysanthemum, the common primers RT1 (5'-GTTTGAATGGCATCCTGAGTCG-3') and RT2 (5'-GTTGAGCACCACAAGCGTAAGC-3') were used. Specific primers (5'-TTATTACCGATGGCTTCTCCCTT-3') and (5'-CCGGCACAATCACATAATGTTTCG-3') were used to analyse the expression of *DgCCD8b*. Amplification of ribosomal RNA (Yang *et al.*, 2005) was performed using 25 cycles as a normalization control. To analyse the transcription from the transgenes in [*35S::DgCCD8b*] and [*35S::DgCCD8a*] *max4* lines, RT1 (5'-GTTTGAATGGCATCCTGAGTCG-3') and RT3 (5'-TGTTCACTGGGGTTAAGAGCGTC-3') were used. UBQ was used as a normalization control with primers UF (5'-AACCCCTGAGGTTGAATCATC-3') and UR (5'-GTCCTTCTTCTGGTAAACGT-3').

Results

The inhibition of chrysanthemum buds by SL and auxin

To assess whether SLs can inhibit chrysanthemum bud outgrowth, the hormone responses of buds on one-node stem segments held between two agar blocks in Petri dishes were tested (Chatfield *et al.*, 2000). The segments were treated with basally supplied GR24, a synthetic strigolactone, apically supplied auxin (NAA) or both. Although NAA was sufficient to inhibit bud outgrowth, GR24 alone was surprisingly ineffective (Fig. 1). However, the combined application of GR24 and NAA completely inhibited bud outgrowth and was more effective than NAA applied alone.

These results suggest that GR24 is only effective at inhibiting buds in the presence of an auxin source. To test this further, the activity of SLs supplied to two-node stem segments was determined, with the rationale that the second bud would provide a natural auxin source. In this configuration, the two buds respond in different ways to the treatments. The apical bud behaves similarly to buds in the one-node segment experiment (compare Fig. 1 with Fig. 2A). There is no effect of basally supplied GR24, but apical auxin has a strong inhibitory effect. By contrast, the

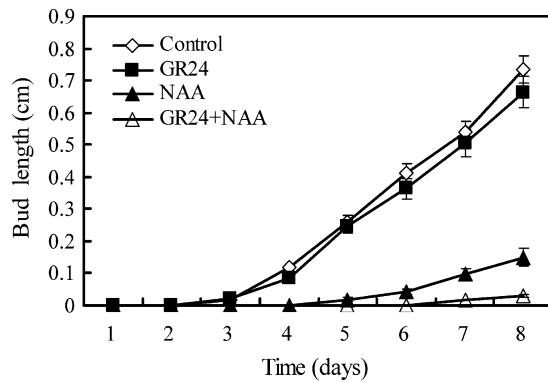


Fig. 1. Effect of GR24 and NAA on bud outgrowth on single-node isolated stem segments in chrysanthemum. One-node stem segments were excised from chrysanthemum plantlets and inserted between two agar blocks. The apical agar blocks contained either 5 μ M NAA or 0 μ M NAA (with an equal volume of ethanol as a control). The basal agar blocks contained either 5 μ M GR24, or 0 μ M GR24 (with an equal volume of acetone as a control). Bud lengths were measured every 24 h and the mean lengths are presented. Error bars represent the standard error of the means, $n=19-20$. The data presented are typical of three independent experiments.

basal bud was inhibited by GR24 alone to a similar degree as for apical NAA treatment alone (Fig. 2B). GR24 together with NAA was more effective at inhibiting both the apical and the basal buds than NAA and GR24 alone (Fig. 2A, B). NAA alone was less effective at inhibiting the basal bud compared with the apical bud (Fig. 2A, B). These results are consistent with the idea that GR24 is only effective at inhibiting a bud in the presence of at least one competing auxin source.

Isolation of DgCCD8

To study SL biosynthesis in chrysanthemum, its putative *MAX4* orthologue, *DgCCD8* was isolated. Amino acid sequence comparison of *CCD8* from *Arabidopsis thaliana* (*MAX4*), pea (*Pisum sativum*, *RMS1*), petunia (*Petunia hybrida*, *DAD1*), and rice (*Oryza sativa*, *D10*) enabled the design of degenerate primers in the most conserved domains of these genes. A 750 bp fragment with strong sequence similarity to the *CCD8* family was successfully amplified. A full-length cDNA of *DgCCD8* was recovered by 5'- and 3' Rapid Amplification of cDNA Ends (RACE) PCR. Subcloning and sequencing of 21 fragments resulted in the identification of three distinct *DgCCD8* cDNAs, designated as *DgCCD8a*, *DgCCD8b*, and *DgCCD8c*. *DgCCD8a* cDNA comprises 1740 bp containing an open reading frame of 1671 bp encoding a predicted protein of 556 amino acids. *DgCCD8b* and *DgCCD8c* coding sequences are both 1668 bp in length and are predicted to encode proteins of 555 amino acids. Southern blot analysis showed that at least three copies of the *DgCCD8* gene are present in the chrysanthemum genome (data not shown), consistent with its known hexaploidy (Nazeer and Khoshoo, 1983), so the

three related sequences identified as *DgCCD8a*, *DgCCD8b*, and *DgCCD8c* may represent transcripts from these three loci, which are 98–99% identical. Alternatively, they could be alleles at two of those loci since, while the chrysanthemum line used is clonally propagated and thus of a single genotype, it is not inbred. Amino acid sequence comparisons between these three genes and their orthologues from *Arabidopsis*, pea, petunia, and rice show that the predicted *DgCCD8s* are 60–61% identical to *AtMAX4*, 72–73% identical to *PsRMS1*, 78% identical to *PhDAD1*, and 58% identical to *OsD10* (Fig. 3A).

To investigate further the relationship between the predicted *CCD8* proteins, a phylogenetic tree of *CCD8* proteins was constructed from a taxonomically diverse set of species (Fig. 3B). In line with taxonomy, *DgCCD8s* are placed in a well-supported clade with petunia.

Genomic fragments corresponding to *DgCCD8* genes were isolated by PCR using a forward primer from the 5'-UTR regions and a reverse primer from the 3'-UTR regions of *DgCCD8*. Sequencing of clones led to the identification of five different *CCD8*-related genomic sequences. Comparison of the *DgCCD8* cDNAs to the genomic sequence revealed that two genomic fragments correspond to the *DgCCD8a* and *DgCCD8c* cDNA, respectively. However, none corresponds to *DgCCD8b*. Thus, in total, six different *DgCCD8* sequences, that are likely to represent two alleles at each of three *DgCCD8* loci have been identified.

Alignment of all cDNAs with their corresponding genomic DNA revealed that all the *DgCCD8* genes have the same intron–exon structure (Fig. 3A). Both the number of introns (five) and their positions in the genes are conserved with the previously reported gene structures.

Functional conservation of the DgCCD8s

To determine whether the *DgCCD8* genes are functionally conserved, the *DgCCD8a* and *DgCCD8b* ORFs from the Cauliflower Mosaic Virus 35S promoter (*35S::DgCCD8a*, *35S::DgCCD8b*) were expressed in the *Arabidopsis max4-1* mutant background. For each construct, multiple independent transgenic lines were generated, and those showing segregation patterns in the T_2 consistent with a single insertion site (data not shown) were taken to homozygosity for detailed analysis. Branching was assessed using a decapitation assay previously shown to provide sensitive discrimination between wild-type and *max4* mutant plants (Bainbridge et al., 2005). The mean number of rosette branches with a length of at least 2 cm was scored 10 d after decapitation to quantify the degree of rescue of the *max4* mutant phenotype by the *DgCCD8* genes. Figure 4B represents the three most strongly rescuing lines for each construct out of 11 *35S::DgCCD8b* and 15 *35S::DgCCD8a* *max4* characterized lines. Constitutive expression of *DgCCD8b* reduced the mean number of branches from 10.8 in *max4* to 5–6, resulting in plants indistinguishable from wild-type (WT), which had a mean of 5.25 branches. However, constitutive expression of *DgCCD8a* was less effective, reducing the number of branches to 7.05–7.35

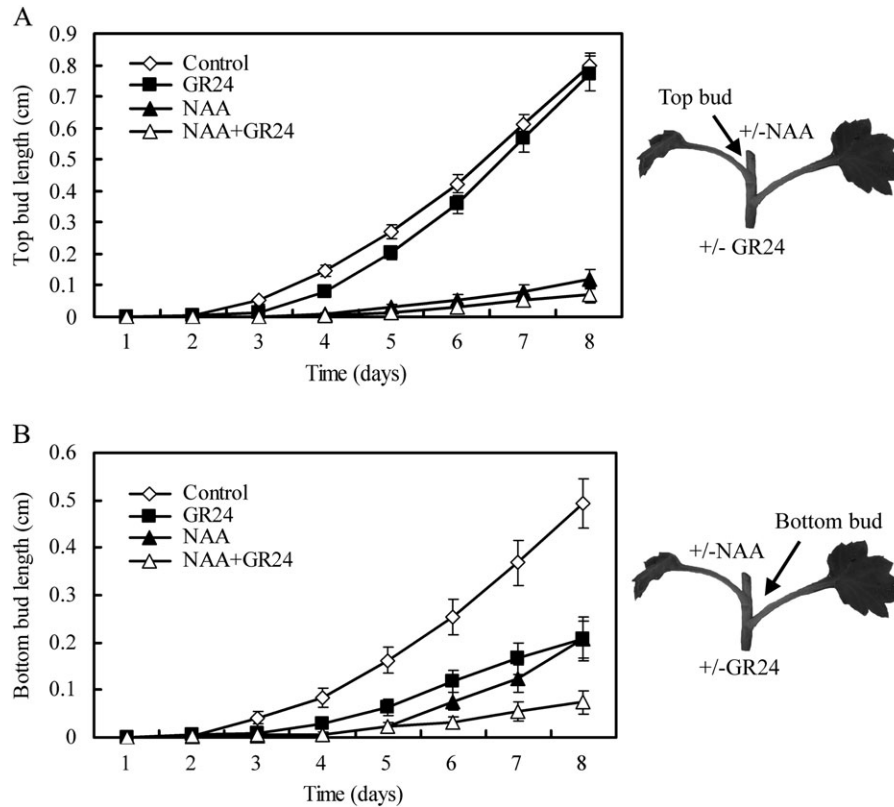


Fig. 2. Effect of GR24 and NAA on bud outgrowth on two-node isolated stem segments in chrysanthemum. Two-node stem segments were excised from chrysanthemum plantlets and inserted between two agar blocks. The apical agar blocks contained either 5 μ M NAA or 0 μ M NAA (with an equal volume of ethanol as a control). The basal agar blocks contained either 5 μ M GR24, or 0 μ M GR24 (with an equal volume of acetone as a control). The length of the top bud (A) or bottom bud (B) was measured every 24 h and the mean lengths are presented. The arrows indicate the axil for which bud length was measured. Error bars represent the standard error of the mean, $n=23-24$. The data presented are typical of three independent experiments.

(Fig. 4A, B). This difference was not due to different levels of expression of the transgenes, because when RT-PCR was used to determine transcript abundance, the *DgCCD8* transgenes were found to accumulate to similar levels between the *35S::DgCCD8b* and *35S::DgCCD8a* lines (Fig. 4C). Therefore, the increased effectiveness of *DgCCD8b* in rescuing the *max4* phenotype compared to *DgCCD8a* is probably a post-transcriptional effect.

Tissue specificity of *DgCCD8* expression

To gain an overall picture of the tissue specificity of *DgCCD8* expression in chrysanthemum, the combined levels of all *DgCCD8* transcripts in mRNA extracted from different tissues were examined by RT-PCR, using primers that do not discriminate between the different copies/alleles. *DgCCD8* mRNA was detected in all tissues examined, except for the leaf. Expression in stems was higher than in roots and shoot apices (Fig. 5).

To gain insight into whether there is any sub-functionalization within the family, the expression pattern of *DgCCD8b* was analysed further using specific primers. *DgCCD8b* was not detected in shoot apices, but only in stem and root tissues, with strongest expression in the stem (Fig. 5).

Induction of *DgCCD8* expression by auxin

To investigate auxin regulation of *DgCCD8*, RT-PCR analysis was performed on basal stem segments following decapitation, with or without apical application of auxin (Fig. 6). Decapitation resulted in a drastic reduction in total *DgCCD8* transcript abundance after 6 h but this reduction was successfully compensated by apical NAA application. These data indicate that auxin regulates *DgCCD8* gene expression in a similar manner to that described in other species.

Feedback control of *DgCCD8* expression

To investigate feedback control by SL on *DgCCD8* expression, total *DgCCD8* transcript abundance after 6 h treatment was compared with basal GR24 on intact and decapitated plants (Fig. 6). It was observed that, in intact plants, *DgCCD8* expression is down-regulated by GR24 compared with untreated controls. However, this down-regulation is modest and is less than that observed upon decapitation. Furthermore, down-regulation by GR24 was not observed in decapitated plants, and *DgCCD8* expression in decapitated plants treated with NAA was indistinguishable from the decapitated plants treated with both apical NAA and basal GR24.

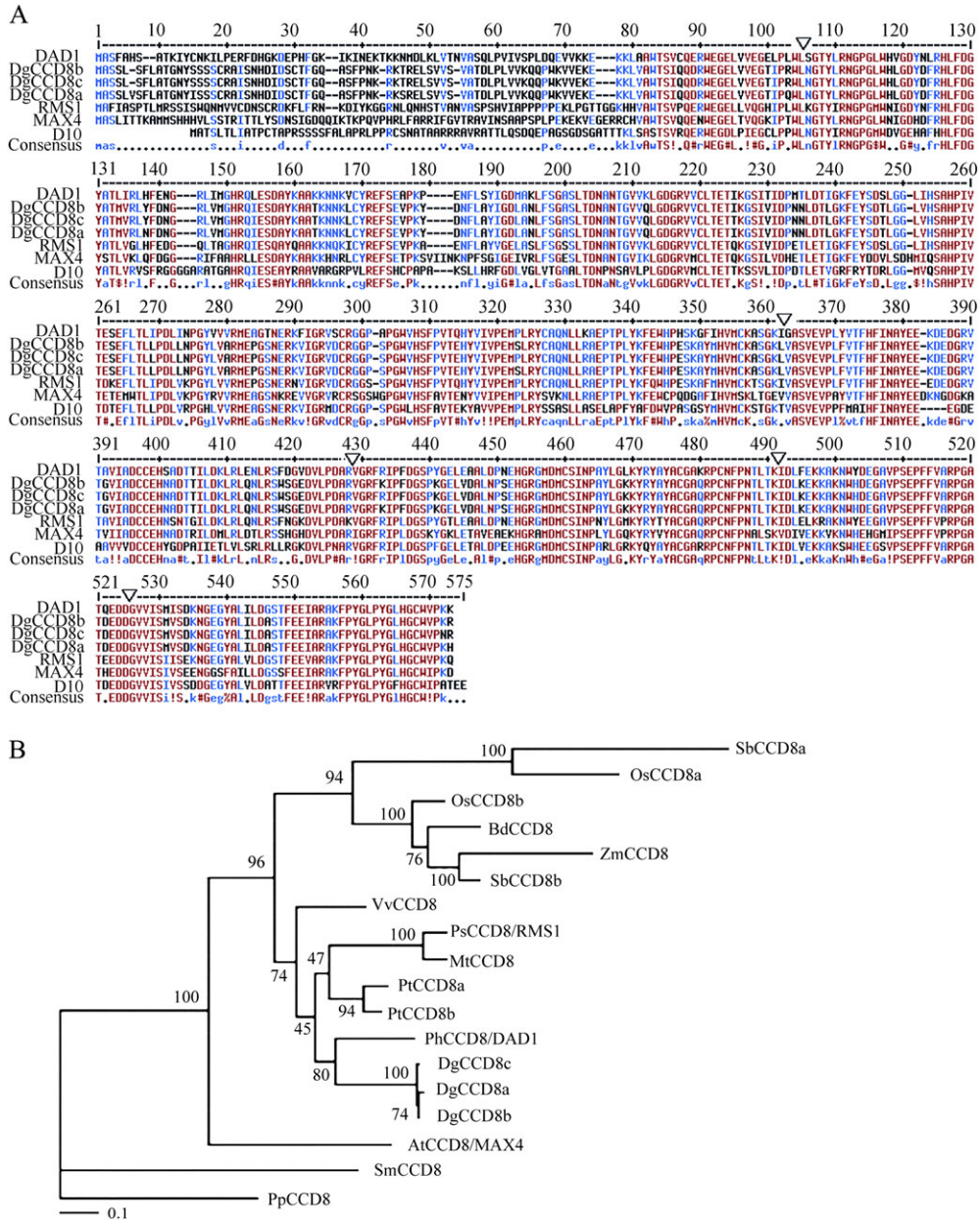


Fig. 3. Isolation of *DgCCD8*. (A) Alignment of the predicted amino acid sequences of *DgCCD8* compared with *Arabidopsis* (*MAX4*), pea (*RMS1*), petunia (*DAD1*), and rice (*D10*). Intron positions corresponding to the genomic DNA sequence are denoted by triangles. (B) Phylogenetic analysis. Maximum likelihood phylogeny of *CCD8* orthologues reconstructed using RAxML (Stamatakis, 2006) under the Dayhoff substitution matrix. Node support values were estimated from 100 rapid bootstrap resamplings (Stamatakis et al., 2008). Proteins are labelled with a prefix that represents the species origin of the sequence: *Arabidopsis thaliana AtCCD8* (At4g32810.1); *Brachypodium distachyon BdCCD8* (Bd2g49670.1); *Dendranthema grandiflorum DgCCD8a*, *DgCCD8b*, and *DgCCD8c*; *Medicago truncatula MtCCD8* (CR9563923.4); *Oryza sativa OsCCD8a* (Os01g38580.1), *OsCCD8b* (Os01g54270.1); *Pisum sativum PsCCD8/RMS1* (AAS66906.1); *Petunia hybrida PhCCD8/DAD1* (AAW33596.1); *Populus trichocarpa PtCCD8a* (eugene 300061708), *PtCCD8b* (gw1.XV111.1171.1); *Physcomitrella patens PpCCD8* (e gw 1.14.69.1); *Sorghum bicolor SbCCD8a* (Sb05g00950), *SbCCD8b* (Sb03g034400); *Selaginella moellendorffii SmCCD8* (egw1.86.30.1); *Vitis vinifera VvCCD8* (GSVIVT0003 2423001); *Zea mays ZmCCD8* (Zm2g147254).

Discussion

The mechanisms of bud inhibition by SL and auxin

Inhibition of bud outgrowth by an actively growing shoot apex is mediated by apically-derived auxin transported down the main stem (Thimain and Skoog, 1933), which

acts indirectly without entering the bud (Morris, 1977; Booker et al., 2003). SLs have been proposed to act as second messengers for auxin, relaying the inhibitory signal from the main stem into the buds (Brewer et al., 2009). An alternative model is that apically-derived auxin moving in the main stem inhibits bud activity by reducing the sink

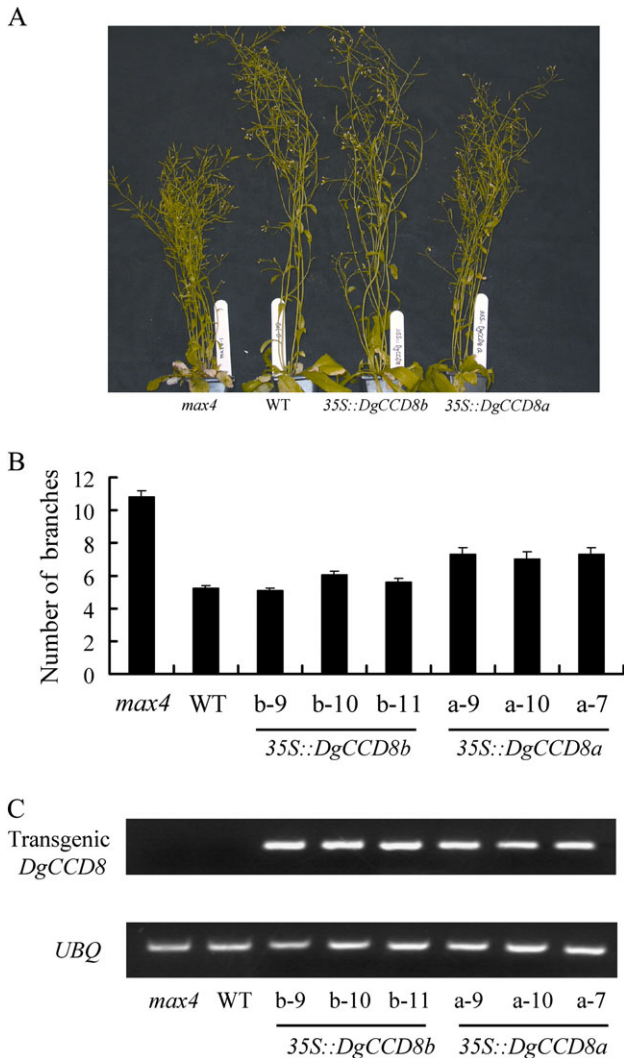


Fig. 4. Complementation of *Arabidopsis max4-1* mutant phenotype with *DgCCD8*. (A) Comparison of phenotypes of wild type, *max4-1*, and *max4-1* transformed with the *35S::DgCCD8a* and *35S::DgCCD8b* constructs. (B) The number of secondary rosette branches produced by WT, *max4-1*, and the three most-strongly rescued independent homozygous lines transformed carrying either *35S::DgCCD8a* or *35S::DgCCD8b*. Branching was assessed using a decapitation assay. The mean number of rosette branches with a length of at least 2 cm 10 d after decapitation is shown (error bars =SEM, $n=20$). The data presented are typical of two independent experiments. (C) Analysis of *DgCCD8* expression for the experiment presented in (B). Transcripts were assayed by reverse transcriptase PCR from total RNA from rosette leaves. One leaf from each of the 20 plants in each sample was collected and pooled after branching had been assessed. Detection of the *UBQ* transcript was used as a cDNA normalization control. The data presented are typical of two independent experiments.

strength of the stem for auxin, thus preventing canalization of auxin transport out of the bud (Prusinkiewicz *et al.*, 2009). In this model, SLs inhibit bud activation by systemically dampening auxin transport canalization (Prusinkiewicz *et al.*, 2009).

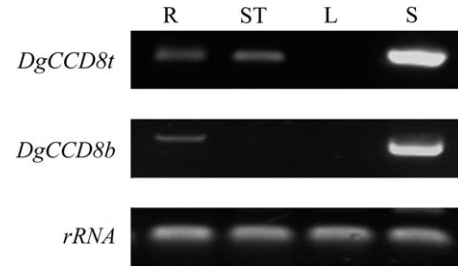


Fig. 5. RT-PCR analysis of total *DgCCD8* (*DgCCD8t*) and *DgCCD8b* expression in chrysanthemum. Total RNA was extracted from R, root; ST, shoot tips; L, leaf; and S, stem, with tissue samples taken from pools of six *in vitro*-grown plantlets. Detection of *rRNA* was used as a normalization control. The data presented are typical of three independent experiments.

The effect of SL on the activity of solitary buds, in the absence of a competing auxin source has not previously been described, and has the potential to distinguish between these alternative models. When a solitary bud was treated with SL, it was activated with similar kinetics to an untreated bud. This argues against the idea that SLs act in a straightforward way by entering buds and inhibiting their activity. However, the result is entirely consistent with the idea that SLs act by dampening auxin transport canalization. Such a mechanism would only be effective in inhibiting bud activation in the presence of a competing auxin source, reducing the sink strength of the main stem for auxin. Accordingly, SL was effective at inhibiting solitary buds when apical auxin was simultaneously applied. Furthermore, SL was able to inhibit growth of the basal bud on an explant with two buds, while the apical bud was unaffected. The active apical bud presumably exports auxin into the stem, as has been demonstrated for *Arabidopsis* buds, providing a competing auxin source similar to apical auxin application (Prusinkiewicz *et al.*, 2009).

Isolation of the chrysanthemum *CCD8* genes

To investigate further the operation of the SL pathway in chrysanthemum, the chrysanthemum orthologues of *CCD8* were isolated, and their orthology was confirmed by phylogenetic analysis and functional complementation of the *Arabidopsis ccd8/max4-1* mutant. Our results suggest that there are at least three loci encoding *CCD8* in chrysanthemum, consistent with its known hexaploidy. Furthermore, some evidence was found for sub-functionalization within the family. The two cDNAs tested were apparently not equally effective in rescuing *max4-1*, despite differing by only three non-conserved amino acids, and the more effective rescuing cDNA is expressed in only a sub-set of the tissues where the *CCD8* transcripts were detected.

Despite the high functional conservation of the *CCD8* genes between species, there are interesting differences in expression pattern. In *Arabidopsis* (Auldridge *et al.*, 2006), petunia (Snowden *et al.*, 2005), and pea (Foo *et al.*, 2005), root expression of *CCD8* is at least 10 times higher than shoot expression. By contrast, in rice (Arite *et al.*, 2007) and

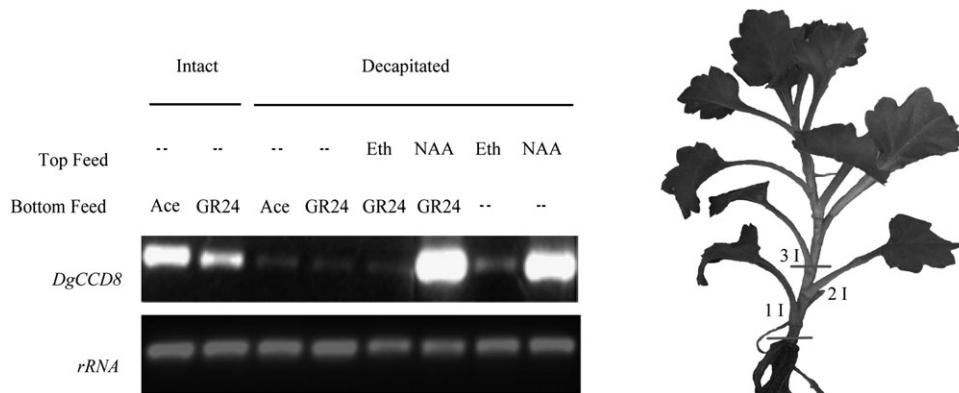


Fig. 6. *DgCCD8* transcript response to decapitation, auxin, and GR24. Top panel, *DgCCD8* gene expression in the three basal internodes (1I–3I as represented by the lines in the scheme on the right). Pools of six plantlets were sampled either intact (left two lanes) or 6 h after decapitation (right six lanes). Plantlets were treated with 5 μ M NAA (NAA) or 0 μ M NAA [with an equal volume of ethanol as a control (Eth)] supplied to the decapitated stump, and/or with 5 μ M GR24 (GR24) or 0 μ M GR24 [with an equal volume of acetone as a control (Ace)] to the basal node. Bottom panel, *rRNA* gene expression was used as a cDNA normalization control. The data presented are typical of three independent experiments.

chrysanthemum, shoot expression exceeds root expression. These differences may reflect different contributions of the root and shoot in the SL-regulation of shoot branching in the different species.

Feedback regulation of *DgCCD8* expression

Previously negative feedback regulation has been observed for *CCD8*s in all species examined (Foo *et al.*, 2005; Snowden *et al.*, 2005; Arite *et al.*, 2007; Hayward *et al.*, 2009). However, the molecular basis for this feedback is unresolved. Three non-exclusive mechanisms have been proposed (Hayward *et al.*, 2009). SL biosynthesis genes may be directly negatively regulated by SLs; feedback could be mediated indirectly by auxin since SL signalling results in a decreased auxin export from buds, and auxin is required for optimal *CCD8* gene expression; or indirect feedback may be mediated by an unknown signal, which in pea is suggested to depend on the *RMS2* gene (Foo *et al.*, 2005; Dun *et al.*, 2006).

In our studies, decapitation and exogenous application of auxin result in down- and up-regulation of *DgCCD8* expression, respectively, suggesting positive regulation by auxin similar to other species. Furthermore, GR24 can down-regulate the expression of the *DgCCD8* gene, demonstrating feedback control. However, application of GR24 to decapitated plants did not further repress *DgCCD8* expression. Moreover, GR24 could not down-regulate *DgCCD8* expression in the presence of exogenous apically applied auxin. In both these situations, GR24 would be expected to have only a modest effect on the amount of auxin in the stem, and thus these results are consistent with the hypothesis that GR24 acts mainly via auxin to mediate feedback regulation on *DgCCD8*.

Conclusion

It has been demonstrated that chrysanthemum buds can be inhibited by GR24 and thus there is potential to use SLs for

the regulation of branching in chrysanthemum horticultural production. However, large-scale exogenous treatment techniques need to be developed and the cost of GR24 or analogues needs to be reduced for this to be practicable. In addition, chrysanthemum *CCD8* genes have been isolated, providing the opportunity to use genetic modification to improve the chrysanthemum growth habit. Our results also contribute data from an additional species to ongoing debates about the mode of action of SLs in the inhibition of shoot branching, and its relationship with auxin. The effects of GR24 on bud activity and *DgCCD8* expression support the idea that SLs inhibit bud activation by modulating auxin transport canalization, and that auxin is a major mediator of feedback by SL on SL synthesis.

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