Control of chrysanthemum flowering through integration with an aging pathway

Qian Wei¹, Chao Ma¹, Yanjie Xu¹, Tianle Wang¹, Yiyu Chen¹, Jing Lü¹, Lili Zhang¹, Cai-Zhong Jiang²,³, Bo Hong¹ & Junping Gao¹

Age, as a threshold of floral competence acquisition, prevents precocious flowering when there is insufficient biomass, and ensures flowering independent of environmental conditions; however, the underlying regulatory mechanisms are largely unknown. In this study, silencing the expression of a nuclear factor gene, CmNF-YB8, from the short day plant chrysanthemum (Chrysanthemum morifolium), results in precocious transition from juvenile to adult, as well as early flowering, regardless of day length conditions. The expression of SQUAMOSA PROMOTER BINDING-LIKE (SPL) family members, SPL3, SPL5, and SPL9, is upregulated in CmNF-YB8-RNAi plants, while expression of the microRNA, cmo-MIR156, is downregulated. In addition, CmNF-YB8 is shown to bind to the promoter of the cmo-MIR156 gene. Ectopic expression of cmo-miR156, using a virus-based microRNA expression system, restores the early flowering phenotype caused by CmNF-YB8 silencing. These results show that CmNF-YB8 influences flowering time through directly regulating the expression of cmo-MIR156 in the aging pathway.
Flowering at the appropriate time is critical for optimal sexual reproductive success, and is determined by a complex interplay of environmental cues and internal signals. Angiosperms have evolved several mechanisms to coordinately regulate floral transition, including signaling controlled by photoperiod and vernalization, as well as by the gibberellin and aging pathways. These flowering pathways converge on a common set of downstream flowering time integrators, such as FLOWERING LOCUS T (FT), SUPPRESSOR OF OVER-EXPRESSION OF CO1 (SOC1), and APETALA1 (API), and a plant-specific transcription factor, LEAFY (LFY). Among these flowering pathways, the aging pathway provides an endogenous developmental cue that prevents flowering during the juvenile phase, and ensures flowering during the adult phase, even in the absence of exogenous inductive factors. A microRNA (miRNA), miR156, and its targets, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE, have been identified as key components of the aging pathway that underlies the transition from the juvenile-to-adult phases, and that promotes flowering. In Arabidopsis thaliana, overexpression of miR156 prolongs the juvenile phase, as characterized by leaf morphology, whereas a reduction in miR156 levels accelerates the expression of adult traits. The expression of miR156 is high in seedlings, decreases with time, and is downregulated by the accumulation of photosynthetic activity during vegetative growth. The decline in miR156 levels results in an increase in the expression of SPL genes, which in turn induce the expression of the floral pathway integrators SOC1, API, FUL, and LFY in the shoot apical meristem. A key role for miR156 in flowering has been shown to be conserved among angiosperms and ectopic expression of miR156 alters flowering time in many plant species, including rice (Oryza sativa), potato (Solanum tuberosum), Arabis alpina, and Cardamine flexuosa. However, while some components of the complex aging pathway regulatory system have been elucidated, the upstream transcription effectors that modulate miR156 levels are still largely unknown.

Previous studies have demonstrated that flowering time, abiotic stress tolerance, and various aspects of plant development are regulated by nuclear factor Y (NF-Y) proteins. NF-Y proteins comprise NF-YA, NF-YB, and NF-YC subunits that form conserved heterotrimeric transcription factor complexes in all higher eukaryotes. NF-Y complexes bind to the CCAAT motif in the promoters of many genes. In plants, each NF-Y subunit is encoded by 8–39 genes, so there are potentially thousands of unique heterotrimeric NF-Y complexes that may enable the specific regulation of a variety of transcriptional profiles. As an example, A. thaliana, NF-YB2 and NF-YB3 bind to NF-YC3, NF-YC4, or NF-YC9 in a photoperiod-dependent flowering pathway. The heterodimers then physically interact with CONSTANS to induce the expression of FT. In addition, the NF-Y complexes can function as epigenetic regulators in both the photoperiod and GA pathway. However, less is known about the involvement of NF-Y proteins in the aging pathway.

Chrysanthemum (Chrysanthemum morifolium) is a typical obligate short day (SD) herbaceous perennial species, and has provided a useful model to study the role of day length in the transition from vegetative to inflorescence meristem identity. Studies of chrysanthemum flowering to date have mainly focused on the interplay of environmental cues and internal signals.
Results

CmNF-YB8 expression is associated with aging. To investigate the function of NF-YB family genes in the aging pathway of chrysanthemum, unigenes encoding eight putative NF-YB family proteins were identified in an in-house chrysanthemum transcriptome database (Supplementary Fig. 1). Of these, UN69086, which exhibited a trend of decreasing expression in apical buds as they develop (Supplementary Fig. 2), is predicted to encode a protein (Supplementary Fig. 3) with conserved NF-YB domains, including a DNA-binding domain, and the NF-YA and NF-YC subunit interaction domains (Fig. 1a)32, 33. The secondary structure of the deduced UN69086 polypeptide is composed of four alpha-helices separated by three strand-loops domains, as is characteristic of the conserved domains of NF-YB family members (Fig. 1a)34, 35. Phylogenetic analysis revealed that the closest relationship between UN69086 and 13 A. thaliana NF-YB proteins was with AtNF-YB8 and AtNF-YB10 (Fig. 1b), with the greatest similarity to AtNF-YB8, so UN69086 was re-named CmNF-YB8.

The expression of CmNF-YB8 was evaluated in different organs of young chrysanthemum plants 5 days after propagation, and transcript abundance was found to be relatively high in apical buds and leaves (Fig. 2a). In both cases, transcript levels were highest in young plants and subsequently declined during development (Fig. 2b). We also examined the expression of CmNF-YB8 in plants grown under different day lengths, in low-temperature conditions, and following treatment with gibberellic acid (GA). No difference was observed in the expression of CmNF-YB8 between long day (LD) and SD conditions, between plants exposed to room temperature and a low temperature, or between GA-treated or non-treated control plants (Fig. 2c). These results suggested that the expression of CmNF-YB8 is regulated by age, but not by other known flowering signals, such as photoperiod, vernalization, or GA.

We performed a transactivation assay of CmNF-YB8 in yeast and saw no evidence that CmNF-YB8 had transcriptional activity (Supplementary Fig. 4). This is consistent with previous reports that transcriptional activation of the NF-Y heterotrimer is mediated by the NF-YA and NF-YC subunits, but not by the NF-YB subunit21, 36.

CmNF-YB8 silencing promotes the juvenile-to-adult transition. To investigate whether CmNF-YB8 influences growth and flowering time, we used the 3′ region of CmNF-YB8 to specifically silence CmNF-YB8 expression in chrysanthemum, generating a population of 23 CmNF-YB8-RNAi-expressing lines. A reduction in CmNF-YB8 expression in the transgenic lines compared with wild-type (WT) plants was confirmed by qRT-PCR analysis (Fig. 3a). We also evaluated the expression of other NF-YB family members in the CmNF-YB8-RNAi lines and determined that only CmNF-YB8 was silenced (Supplementary Fig. 5). Three representative CmNF-YB8-RNAi lines (4, 13, and 22) were selected for functional analysis (Fig. 3a).

We compared the morphology of leaves from the WT and CmNF-YB8-RNAi lines, as the number of juvenile leaves can be used as an indicator of the juvenile-to-adult transition12, 37. All the two first-emerging leaves and 89% of the third leaves from the WT plants were typically juvenile, in that they were small and had no, or minimal, marginal serration (Fig. 3b). In contrast, more than 20, 65, and 93% of the first- second- and third-emerged leaves, respectively, of the CmNF-YB8-RNAi lines had adult critical regulator of age-dependent flowering time by directly regulating cmo-MIR156 expression in chrysanthemum.

on the significance and molecular regulation of the photoperiod pathways28-30. It has been shown that the juvenile-to-adult transition during vegetative growth, which is required for chrysanthemum flowering, must occur before any response to SD period can take place31. However, little is known about the mechanism that controls the juvenile-to-adult transition in chrysanthemum. Here we report that the chrysanthemum nuclear factor CmNF-YB8 modulates flowering time by mediating the juvenile-to-adult transition. Further transcriptome analysis reveals that CmNF-YB8 regulates the expression of genes related to aging pathway. Notably, CmNF-YB8 is shown to bind to the promoter of the cmo-MIR156 gene. Ectopic expression of cmo-miR156 restores the early flowering phenotype caused by CmNF-YB8 silencing. Our findings uncover that CmNF-YB8 is a
CmNF-YB8 in the transgenic lines compared to WT was prior to the WT plants (Fig. 4d). In addition, we overexpressed CmNF-YB8 (OX) 42 days in the floral buds emerged at 67 days in WT plants, but after only 28, 29 days in the WT plants (Fig. 4b). Under SD conditions, CmNF-YB8-OX plants had a prolonged juvenile phase since the appearance of abaxial trichomes was delayed by more than 1.3 plastochrons (Supplementary Fig. 7c, d). Furthermore, CmNF-YB8-OX A. thaliana lines flowered later than WT, as evidenced by the fact that, at the bolting stage, the transgenic plants had more rosette leaves than did the WT plants (Supplementary Fig. 7b, c).

Taken together, these results suggested that the influence of CmNF-YB8 on flowering time might be exerted through the aging pathway.

CmNF-YB8 regulates the expression of aging pathway genes. We performed a large-scale screen of genes that were differentially expressed between the aerial organs of CmNF-YB8-RNAi and WT chrysanthemum plants using an RNA-sequencing (RNA-seq) approach. In total, we identified 683 upregulated and 74 downregulated differentially transcribed genes (DTGs) in CmNF-YB8-RNAi line 4 (Supplementary Data 1). In the context of the regulation of flowering time by CmNF-YB8, we focused on DTGs that were annotated as components of flowering pathways. These included three putative aging pathway genes, encoding SPL proteins, which were expressed at higher levels in the CmNF-YB8-RNAi lines than in WT plants (Table 1). Based on an alignment with, and sequence homology to, A. thaliana SPL proteins (Supplementary Fig. 8), we designated these three genes as CmSPL3, CmSPL5, and CmSPL9 (Table 1).

We also performed a dual-luciferase-based miRNA sensor assay to quantitatively evaluate the cleavage of the CmSPL genes by cmo-miR156 in a Nicotiana benthamiana heterologous expression system. The cmo-MIR156 gene has two local morphology, as they were larger and had serrated margins (Fig. 3c).

We also observed a significant difference in flowering time between CmNF-YB8-RNAi expressing and WT plants (Fig. 4), but no difference in flower diameter or the number of flowers. Under LD conditions, at 62 days, flower bud emergence was observed in CmNF-YB8-RNAi line 4, and at 66 and 68 days, lines 13 and 22 plants had flowering buds, while flower buds only emerged from the WT plants at 89 days (Fig. 4a, b). Subsequently, flower buds bloomed in CmNF-YB8-RNAi-expressing plants ~20 days before the WT plants (Fig. 4b). Under SD conditions, flower buds emerged at 67 days in WT plants, but after only 42 days in the CmNF-YB8-RNAi lines (Fig. 4c, d). Flower buds then bloomed in CmNF-YB8-RNAi-expressing plants ~25 days prior to the WT plants (Fig. 4d). In addition, we overexpressed (OX) CmNF-YB8-GFP (green fluorescent protein) in chrysanthemum, obtaining six CmNF-YB8-OX lines. Increased expression of CmNF-YB8 in the transgenic lines compared to WT was confirmed by qRT-PCR analysis (Supplementary Fig. 6a). The flowering time of CmNF-YB8-OX plants was slightly delayed compared with WT plants (Supplementary Fig. 6b, c).

We also tested whether CmNF-YB8 has the capacity to regulate juvenile-to-adult leaf transition and flowering time in A. thaliana. CmNF-YB8 was overexpressed in A. thaliana, and three of the resulting 15 CmNF-YB8-OX lines (OX7, OX8, and OX11) were chosen for functional analysis (Supplementary Fig. 7a). In A. thaliana, a marker for juvenile leaves is no, or few, trichomes on the abaxial side. Compared to WT plants, the CmNF-YB8-OX plants had a prolonged juvenile phase since the appearance of abaxial trichomes was delayed by more than 1.3 plastochrons (Supplementary Fig. 7c, d). Furthermore, CmNF-YB8-OX A. thaliana lines flowered later than WT, as evidenced by the fact that, at the bolting stage, the transgenic plants had more rosette leaves than did the WT plants (Supplementary Fig. 7b, c).

Taken together, these results suggested that the influence of CmNF-YB8 on flowering time might be exerted through the aging pathway.
CmSPL9 cleavage sites fused to the ORF of the CmSPL9 UTR of the CmNF-YB8 was co-expressed with the LUC gene (Fig. 6c). The potential cmo-miR156 cleavage sites are located at the 3′UTR of the miR156a, 101 bp) and pre-cmo-miR156b (122 bp) (Figs. 6b and 7a). The potential cmo-miR156 cleavage sites were located at the 3′UTR of CmSPL3 and 5, and in the open-reading frame (ORF) of CmSPL9. Co-expression in N. benthamiana of pre-cmo-miR156a (3SS-pre-cmo-miR156a) with either of the CmSPL3 cleavage sites fused to the 3′UTR of the Firefly luciferase (LUC) gene or the CmSPL5 cleavage sites fused to the ORF of the LUC gene led to reduced LUC activity. However, this was not observed when pre-cmo-miR156a was co-expressed with the CmSPL5 cleavage site fused to the 3′UTR of the LUC gene (Fig. 6c).

CmNF-YB8 is an upstream regulator of cmo-MIR156. To determine whether CmNF-YB8 is associated with specific regulatory regions of the cmo-MIR156 promoter, we performed a chromatin immunoprecipitation (ChIP) assay using CmNF-YB8-GFP overexpressing chrysanthemum. We observed that the association of CmNF-YB8 with the cmo-MIR156 promoter had an enrichment peak in the P5 fragment, −1121 ∼ −1318 bp relative to a major transcription start site (TSS), but not with other fragments of the cmo-MIR156 promoter, or with a pre-cmo-miR156a (P0) control fragment (Fig. 7a, b).

It has been reported that the DNA-binding property of NF-YB can be evaluated in yeast, since the yeast HAP complex members, HAP2 and HAP5, can act as surrogate NF-YA and NF-YC partners, and combine with NF-YB, thereby forming a functional NF-Y complex24. Using a yeast one-hybrid assay, we observed that CmNF-YB8 bound to the P9 fragment (−1083 ∼ −1436 bp relative to the TSS) of the cmo-MIR156 promoter (Fig. 7d). A putative CCAAT box cis-element (−1217 ∼ −1221 bp), which is a known binding site for NF-Y proteins21, was localized at the overlapping region of P5 and P9 (Fig. 7a). The binding activity was completely abolished by mutation of the CCAAT box (Fig. 7c, d). In addition, we performed a dual-luciferase reporter assay to analyze the regulation by CmNF-YB8 of the promoter activity of cmo-MIR156 in vivo (Fig. 7e). N. benthamiana leaf cells co-transformed with 3SS:CmNF-YB8 (CmNF-YB8) and promoter cmo-MIR156:LUC (Pro-miR156) had significantly higher LUC activity than cells transformed with CmNF-YB8 or Pro-miR156 alone, or with Pro-miR156 carrying a mutated CAAT cis-element (Fig. 7e).

Finally, we evaluated whether CmNF-YB8 can bind to the MIR156 promoters in A. thaliana, as was suggested by the observation that the expression of SPL3, 5, 9 was lower, and miR156 abundance was higher in the CmNF-YB8-OX A. thaliana lines (Supplementary Fig. 9). A ChIP assay using the CmNF-YB8-GFP-OX A. thaliana lines demonstrated that CmNF-YB8 associated with MIR156A in a promoter region ∼824 ∼ −351 bp relative to the TSS of MIR156A, and in MIR156C in a region ∼759 ∼ −446 bp relative to the major TSS of MIR156C (Supplementary Fig. 10).

### Table 1 Genes related to flowering time with upregulated expression in CmNF-YB8-RNAi-expressing chrysanthemum

| Gene    | Annotation | WT fold change | RNAi fold change |
|---------|------------|----------------|-----------------|
| Aging related |            |                |                 |
| UN052587 | CmSPL3     | 14.18          | 28.61           |
| UN016109 | CmSPL5     | 0.60           | 1.50            |
| UN033721 | CmSPL9     | 21.77          | 42.36           |
| Flowering integrators |          |                |                 |
| UN043322 | CmTFL1-like protein | 0 4.85 CNC      |                 |
| UN062942 | CmTFL3     | 5.87           | 10.97           |
| UN074378 | SOCI       | 7.28           | 11.26           |
| UN055907 | SOCI-like protein 1 | 9.09 12.79 1.90 |                 |
| UN026081 | CsAPETALA1/FRUITFULL | 5.64 25.50 4.52 |                 |
| UN045503 | APETALA2-like protein | 2.29 5.59 2.45 |                 |

CNC indicates cannot calculate. WT, wild type

Data in the columns are RPKM values

*a* Reported as flowering integrators in chrysanthemum28, 29.
cmo-miR156 restores early flowering by CmNF-YB8 silencing. To gain additional evidence that CmNF-YB8 influences flowering time through a cmo-miR156-SPL module, we overexpressed cmo-miR156 in WT and CmNF-YB8-RNAi chrysanthemum lines, using a modified cabbage leaf-curl geminivirus vector (CaLCuV) containing pre-cmo-miR156a (CaLCuV + miR156)39. As expected, in both WT and RNAi plants, expression of cmo-miR156 was increased in CaLCuV + miR156-infected plants compared to the Mock and CaLCuV-only infected control plants (Fig. 8a). Under SD conditions, at 50 days, scanning electron microscopic observation showed that in both WT and RNAi plants, inflorescence development was slower in CaLCuV + miR156-infected plants than in control plants (Fig. 8b). In RNAi plants, CaLCuV + miR156 infection restored inflorescence development to the stage of involucre formation; the same stage as the WT control plants (Fig. 8b). Subsequently, following CaLCuV + miR156 infection, flower bud emergence occurred on day 98 and 77 in WT and RNAi plants, respectively, which represented a significant delay compared to the control plants (Fig. 8c, d). In addition, while we noted a 10 days difference in flowering time between the RNAi and the WT control plants as a consequence of CaLCuV + miR156 infection, in the context of this experiment this was not statistically significant (Fig. 8d).

Discussion
Plants undergo a transition from a vegetative to a reproductive developmental phase after germination, the former of which can be further divided into juvenile and adult stages40. These two stages can be distinguished morphologically by leaf shape, size, and patterns of epidermal differentiation41. For example, the juvenile leaves of A. thaliana are smaller and rounder than the adult leaves, and they lack abaxial trichomes14. In the present study, we noted that juvenile leaves of chrysanthemum have similar morphological characteristics to those of A. thaliana (Fig. 3b), although no equivalent differences were observed with respect to abaxial trichome distribution or the structures of the juvenile and adult leaves.

Plants acquire the ability to flower and undergo the transition to reproductive development during the transition from juvenile-to-adult42, and plants in the juvenile phase are thought to have no flowering competence, and will remain vegetative even when exposed to flowering cues such as photoperiod or vernalization. The propensity of plants to flower and to initiate reproduction increases with age43, so the control of age-related competence to flower should be distinct from competence mechanisms regulated by environmental responses such as photoperiod, or by general endogenous pathways, such as the GA pathway43. The expression of genes in the aging-related pathway should be affected by age, rather than by other flowering cues8. In several plant species, such as A. thaliana44, 45, A. alpina10, Acacia confusa12, Eucalyptus globulus12, Hedera helix12, Z. mays44, O. sativa45, and Glycine max46, the miR156/SPL module shows a typical temporally regulated expression pattern, regardless of other flowering cues. In young A. thaliana seedlings, the expression levels of miR156 are high, and decline with growth, a trend that appears to be independent of known floral regulators, including vernalization, GA, and photoperiod48. Here we found that the expression of CmNF-YB8 in chrysanthemum...
silencing (Fig. 5b), implying that other miR156 variants, such as other family members or iso-miR156s, might contribute to the cleavage.

In this study, we found that CmNF-YB8 regulates the transcription of the cmo-MIR156 gene by associating with its promoter (Fig. 7). ChiP-PCR and yeast one-hybrid assays indicated that CmNF-YB8 binds to the promoter of cmo-MIR156 (Fig. 7a–d). Interestingly, dual-luciferase reporter assay showed that in N. benthamiana leaf cells, the promoter activity of cmo-MIR156 could be induced by overexpression of CmNF-YB8 (Fig. 7e), although CmNF-YB8 has no transcriptional activity (Supplementary Fig. 4). It is well known that the regulation of downstream gene by NF-YB subunit need interact with NF-YA and NF-YC subunits to form heterotrimERIC complex39, 40. Therefore, we speculate that CmNF-YB8 can induce the promoter activity of cmo-MIR156 through recruiting NF-YA and NF-YC of N. benthamiana to form a functional NF-Y complex. In A. thaliana, the AGAMOUS-like (AGL) transcription factors, AGL15 and AGL18, act as upstream regulators of MIR156 expression by forming a heterodimer, and binding to the CArG motifs of the MIR156A and MIR156C promoters40. Transgenic A. thaliana plants overexpressing AGL15 or AGL18 had shortened petioles and wavy/curled leaves, rather than abnormal juvenile leaf numbers51. AGL15 or AGL18 can therefore be considered to function in a complex, along with other components to regulate miR156 expression. Further studies are required to investigate whether NF-YB8 associates with AGL15/18 in the aging pathway.

In A. thaliana, genes of NF-YB subunit have been reported as regulators of flowering time in photoperiod and GA pathways. NF-YB2 was discovered and it can physically associate with the CCAAT box of the FT promoter in photoperiod-dependent flowering pathway23, 24, 26. Subsequently, it was found that NF-YB2 directly regulates SOC1 expression in response to photoperiod and GA flowering pathways by interacting with NF-YA2 and NF-YC42. Here we found a new function of NF-YB subunit members in flowering time. CmNF-YB8 regulates the transition of juvenile-to-adult through directly targeting cmo-MIR156 in chrysanthemum. It suggests broad-spectrum functions of NF-YB subunit members in regulation of flowering time in response to environmental cues and internal signals.

In addition, the NF-Y complex has been demonstrated to be an epigenetic regulator that associates with SOC1 promoter to mediate H3K27me3 demethylation, partly through a H3K27 epigenetic regulator that associates with N. benthamiana to form a functional NF-Y complex. In A. thaliana, silencing of miR156, or overexpression of SPL genes, shortens the juvenile phase and causes a decrease in the number of juvenile leaves6, 14, 47, 48. In this current study, silencing of a NF-YB subunit member, CmNF-YB8, in chrysanthemum resulted in a significant reduction in the number of juvenile leaves, indicating a precocious transition from juvenile to adult (Fig. 3b, c), with a concomitant acceleration in the transition to flowering under both LD and SD conditions (Fig. 4). These observations suggest that CmNF-YB8 functions in regulating flowering time predominantly through the aging pathway.

It has been reported that miR156 plays a critical role in the aging pathway by directly regulating SPL transcription factors at a posttranscriptional level18, 49. Here we showed that the major cmo-miR156 cleavage sites in CmSPL genes were different (Fig. 6a). Although the cleavage site in CmSPL9 was located between the canonical tenth and eleventh nucleotides, the site in CmSPL3 was between the ninth and tenth nucleotide (Fig. 6a). In addition, although no site was identified in CmSPL5 (Fig. 6a, c), the expression of CmSPL5 was increased by CmNF-YB8

**Methods**

**Plant materials and treatments.** A ground-cover chrysanthemum cultivar (Chrysanthemum morifolium var. Fall Color) used in this study was propagated by vitro culturing. Chrysanthemum shoots, including 2 nodes, were cultured on Murashige and Skoog (MS) medium comprising 1/2 Murashige and Skoog (MS) for 10 days, then transplanted into 9 cm diameter pots containing a mixture of 1:1 (v/v) peat and vermiculite and watered once a week. The plants were grown in a culture room at 23 ± 1 °C, 40% relative humidity, and 100 μmol m−2 s−1 illumination with fluorescent lamps under a LD cycle (16 h light/8 h dark).

For SD treatments, plants grown for 5 days after transplanting were transferred to a culture room and maintained under an SD cycle (8 h light/16 h dark). For low-temperature treatments, plants were transferred to a 4 °C chamber under a LD cycle.

![Image](56x511 to 99x634)

**Fig. 6** cmo-miR156 targets CmSPL3 and CmSPL9. a cmo-miR156-directed cleavage sites in the CmSPL genes were determined by 5′ RLM-RACE, and are highlighted by arrows. The number indicates the frequency of clones when validating the cleavage sites of the target miRNAs. b The precursor structures of cmo-miR156 were predicted by RNA structure software. miRNA sequences are highlighted in red. c A dual-luciferase reporter assay was used to quantify cmo-miR156-mediated repression of target gene CmSPLs. 3SS-cmo-miR156 precursor a (pre-cmo-miR156a) was co-transformed with CmSPL3 or 5 cleavage sites fused to the 3′ UTR of the LUC gene, CmSPL9 cleavage site fused to the ORF of the LUC gene. LUC vectors carry a REN gene under the control of the 35S promoter as a positive control. Synonymous negative control (SNC) is a negative control with disruption of CmSPL9 cleavage site while maintaining identical amino acid sequence. Spacer is a 20 nt random sequence as negative control for CmSPL3 and CmSPL5. Nicotiana benthamiana leaves were infiltrated with the samples, and LUC and REN activities were assayed 3 days after infiltration. Three independent experiments were performed and error bars indicate standard deviation.
cycle (16 h light/8 h dark) for 30 days. For GA treatments, plants at 5 days after transplanting were sprayed with 100 μM GA₃ (Sigma) and maintained under a LD cycle (16 h light/8 h dark). The GA₃ treatment was performed twice every week for 30 days.

Gene isolation. A partial *CmNF-YB8* sequence was obtained from a chrysanthemum RNA-seq data set, and the full-length sequence was obtained using a SMARTTM RACE cDNA amplification kit (Clontech), according to the manufacturer’s instructions.

Alignments of the deduced *CmNF-YB8* full-length amino acid sequence with *A. thaliana* NF-YB sequences, and of the deduced CmSPLs amino acid sequence with *A. thaliana* SPL sequences were performed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Phylogenetic analyses were performed using MEGA version 5 and the neighbor-joining method with 1000 bootstrap replicates.

The full-length *cmo-MIR156* primary transcript was captured using the FirstChoice® RLM-RACE kit (Ambion) for 5’ RLM-RACE, 1 μg total RNA was directly ligated to the 5’ adapter using the T4 RNA ligase treated with calf intestine alkaline phosphatase and tobacco acid pyrophosphatase. The ligated RNA sample was used to synthesize cDNA using M-MLV reverse transcriptase. *cmo-MIR156* gene-specific primers were designed based on sequence immediately upstream of the predicted foldback structure. 3’ RLM-RACE was performed according to the manufacturer’s instructions (Ambion). PCR primers are listed in Supplementary Table 1.

The genome sequence of *cmo-MIR156* was obtained from in-house unpublished *chrysanthemum* genome sequencing data. *A. thaliana* NF-YB family sequences were obtained from the TAIR database (www.arabidopsis.org) and OsNF-YB8 (AB095440); BrNF-YB8 (AH949926) and MtNF-YB8 (AFK96568) sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/); PtNF-YB8 (Potri.008G044800.1) sequences were obtained from PopGenIE (http://popgenie.org/). *A. thaliana* NF-YB family sequences were obtained from the TAIR database: AtNF-YB1 (AT2G37060), AtNF-YB2 (AT3G47640), AtNF-YB3 (AT4G14540), AtNF-YB4 (AT1G90350), AtNF-YB5 (AT2G7810), AtNF-YB6 (AT3G47670), AtNF-YB7 (AT2G13570), AtNF-YB8 (AT2G37060), AtNF-YB9 (AT1G21970), AtNF-YB10 (AT3G33340), AtNF-YB11 (AT2G27470), AtNF-YB12 (AT3G581890), AtNF-YB13 (AT5G23090). A partial sequence of *CmNF-YB8* has been deposited as NYF87 (KT253142) in the NCBI database; however, based on our phylogenetic analysis, we re-named it as *CmNF-YB8*.

Quantitative RT-PCR analysis. Total RNA samples were extracted from chrysanthemum apical buds, leaves, stem, and roots using TRIzol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). First-strand cDNAs were synthesized from 1 μg total RNA using an oligo d(T) primer and the SuperScript III RT-PCR system, according to the manufacturer’s instructions.
Chrysanthemum transformation. To construct the RNAi vector, 312 bp sense and antisense CmNF-YB8 fragments were amplified by two pairs of primers containing either XhoI/KpnI or XbaI/BamHI sites. The PCR products were digested with the corresponding restriction enzymes, and directionally inserted into the two sides of the PdK Intron in the pHANNIBAL vector to form an intron-containing “hairpin” RNA (ihpRNA) construct. The ihpRNA construct, containing a constitutive 35S promoter and a Nos terminator, was then subcloned into the pART27 binary vector for transformation56.

To construct the 35S:CmNF-YB8-GFP overexpressing vector, the CmNF-YB8 ORF fused to GFP was digested with XbaI and Smal from the Super:CmNF-YB8-GFP vector, and then cloned into the pBI121 vector. PCR primers used for vector construction are listed in Supplementary Table 1.

The resulting plasmids were introduced into Agrobacterium tumefaciens strain EHA105 and transformed into chrysanthemum by Agrobacterium-mediated transformation57. The kanamycin-resistant primary transformants were screened on MS basal medium containing 50 mg l\(^{-1}\) kanamycin. T3 plants were used in this study.

A. thaliana transformation. To construct the CmNF-YB8 overexpression vector, the ORF of CmNF-YB8 was amplified by a pair of primers containing Psil and Solf restriction sites. The PCR products were digested with the corresponding restriction enzymes, and inserted into the Super1300 vector at the 5′ end of the sequence encoding GFP. The PCR primers are listed in Supplementary Table 1. The Super:CmNF-YB8-GFP plasmid was introduced into A. tumefaciens strain GV3101, which was then used to transform A. thaliana using the floral dip method58. Independent transformants were screened on MS basal medium containing 50 mg l\(^{-1}\) kanamycin. T3 plants were used in this study.

Trichome staining. Whole rosettes were washed in acetic acid:ethanol (1:3) for 3 h, and then washed three times in deionized water. The samples were incubated overnight in aniline blue solution (150 mM KH\(_2\)PO\(_4\) and 0.01% (w/v) aniline blue, pH 9.5). The autofluorescence of callose in the trichomes was observed using a 405-nm laser with an emission window of 490–510 nm59.

RNA-seq analysis. Total RNA samples were extracted from the aerial parts of CmNF-YB8-RNAi line 4 and WT plants (three independent plants of each genotype) 15 days after transplanting, using the RNeasy Plant Mini Kit (QIAGEN). Six RNA-Seq libraries were prepared60 and sequenced using a HiSeq 2000 (Illumina) at the RIBO Biotechnology Co. Ltd. (www.ribobo.com). RNA-seq data were processed, assembled, and annotated as previously described61. Briefly, RNA-seq reads were examined by a custom R script based on the ShortRead package to remove low-quality (Q value <20) reads, adapters, and barcode
sequences. High-quality clean reads were assembled de novo into contigs by Trinity with strand-specific option “-SS_lib_type” set to “F” and “min_kmer_cov” set to 2. The resulting contigs were blasted against GenBank nonredundant, UniProt, and Arabidopsis protein databases with a cutoff E value of 1e−5.

5' RLM-RACE. To map the cmo-miRNA cleavage site of the CmSP3 transcripts, a modified 5' RLM-RACE procedure was used with the FirstChoice® RLM-RACE kit (Ambion). One μg total RNA was directly ligated to the 5' adapter using the T4 RNA ligase without treatment with calf intestine alkaline phosphatase and soybean agglutinin lectin. The ligated RNA was used to synthesize the cDNA using M-MLV reverse transcriptase according to the manufacturer’s instructions (Ambion). PCR products were gel-purified and cloned into the pGem T-Easy vector (Promega), and 10 clones were randomly selected and sequenced. The primers used in this assay are listed in Supplemental Table 1.

ChIP assay. For chrysanthemum, expanded leaves of 3SS:CmNF-YB-GFP chrysanthemum plants 7 days after transplanting were used as materials. For A. thaliana, two-week-old Super:CmNF-YB-GFP A. thaliana leaves were used. The plant tissues were fixed in formaldehyde (final concentration, 1%) for 40 min. Fixed tissues were homogenized and the chromatin complexes were isolated and recovered under a vacuum at 0.7 MPa for 3 min, and then washed in deionized water. After washing, the plants were transplanted into pots containing a mixture of 1:1 (v/v) peat and vermiculite and grown in a culture room at 23 ± 1 °C, 40% relative humidity, and 100 μmol m−2 s−1 illumination with fluorescent lamps, under a SD cycle (8 h light/16 h dark). Three independent experiments were performed with 40 plantlets in each experiment. Prior to the flowering time test, the plants were screened by PCR to determine the expression of cmo-miR156. cmo-miR156 overexpressing plants were then used for the flowering time test.

Data availability. RNA-seq data associated with this study has been deposited in NCBI SRA database under accession code PRJNA393366. The authors declare that all data supporting the finding of this study are available within the article and its Supplementary files or are available from the corresponding author on request.

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Author contributions
B.H., J.G., and C.M. conceived and designed the experiments; Q.W. performed most of the experiments; C.M. and C.-Z.I. provided technical support and conceptual advice and analyzed the data; Y.X., T.W., and L.Z. contributed to the ChiP assay and 5′ RLM-RACE; Y.X. and J.L. contributed to chrysanthemum transformation; T.W. and Y.C. contributed to miR156 cloning and functional analysis. C.M., B.H., and J.G. wrote the manuscript.

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