Two Cyc2CL transcripts (Cyc2CL-1 and Cyc2CL-2) may play key roles in the petal and stamen development of ray florets in chrysanthemum

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Abstract

**Background:** *Chrysanthemum morifolium* is one of the most popular ornamental crops. The capitulum, which is the main ornamental part of chrysanthemum plants, consists of ligulate marginal ray florets, an attractive corolla (petals), and radially hermaphroditic disc florets, but no stamens. In Asteraceae species, the zygomorphic ray florets evolved from the actinomorphic disc florets. During this process, the zygomorphic ligulate corolla arose and the stamens were aborted. Although molecular genetic research has clarified ray floret development to some extent, the precise molecular mechanism underlying ray floret development in chrysanthemum remained unclear.

**Results:** A CYC2-like gene, Cyc2CL, was cloned from *C. morifolium* ‘Fenditan’. Subsequent analyses revealed that the alternative splicing of Cyc2CL, which occurred in the flower differentiation stage, resulted in the production of Cyc2CL-1 and Cyc2CL-2 in the apical buds. Prior to this stage, only Cyc2CL-1 was produced in the apical buds. A fluorescence in situ hybridization analysis of labeled Cyc2CL-1 and Cyc2CL-2 RNA indicated that Cyc2CL-2 was first expressed in the involucre tissue during the final involucre differentiation stage, but was subsequently expressed in the receptacle and floret primordia as the floral bud differentiation stage progressed. Moreover, Cyc2CL-2 was highly expressed in the inflorescence tissue during the corolla formation stage, and the expression remained high until the end of the floral bud differentiation stage. Furthermore, the overexpression of Cyc2CL-1 and Cyc2CL-2 in transgenic Arabidopsis inhibited stamen and petal development. Therefore, both Cyc2CL-1 and Cyc2CL-2 encode candidate regulators of petal development and stamen abortion and are important for the ray floret development in chrysanthemum.

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**Background**

Asteraceae is the largest family of flowering plants, and it belongs to the euasterid II clade of the core eudicots. The unique head-like inflorescence of Asteraceae species, known as a capitulum, often consists of the following two morphologically and functionally differentiated florets: bilateral (zygomorphic) ray florets and radial (actinomorphic) disc florets [18]. The marginal ray florets are ligulate and female, with an attractive corolla (petals), but no stamens. The inner disc florets with fertile pollen grains are radially pentamerous and hermaphroditic. In Asteraceae species, zygomorphic ray florets evolved from the actinomorphic disc florets [41]. In angiosperms, the transition to bilateral floral symmetry is considered to represent one of three critical evolutionary events, and is related to the evolution of specialized flower–pollinator interactions that contributed to the diversification of flowering plant lineages [1, 12, 20].

The development of the zygomorphic female ray floret was an important event associated with the evolutionary success of the capitulum in Asteraceae species because the ray floret is highly attractive to pollinators and can significantly improve the outcrossing rate [44]. The following two fundamental changes occurred during the development of the zygomorphic female ray floret: the zygomorphic ligulate corolla arose and the stamens were aborted. Many recent phylogenetic and biological studies have focused on this trait.

In *Antirrhinum majus* (Lamiales), the TCP transcription factors CYCLOIDEA (CYC) and DICHTOMA (DIC) were isolated and characterized as key regulators of floral zygomorphy [33]. Several CYC-like TCP-encoding genes have been identified in Asteraceae species, and the CYCLOIDEA/TEOSINTE BRANCHED1 (CYC/TB1)-like subfamily was classified into the following three CYC clades: *CYC1*, *CYC2*, and *CYC3*, of which *CYC2* clade genes underwent multiple duplication events, resulting in the functional diversification of these genes within the Asteraceae lineage [43]. The 10 CYC/TB1-like genes identified in sunflower belong to three distinct clades (*CYC1*, *CYC2*, and *CYC3*), which is consistent with what has been determined for other eudicot species. Previous studies proposed that gene duplication and functional divergence have greatly facilitated the diversification of the sunflower CYC gene family [3, 5]. Additionally, a phylogenetic analysis of CYC-like genes revealed that different paralogs of these genes may have been independently recruited to mediate the zygomorphy in different Asteraceae species [7].

In *Senecio vulgaris*, the CYC-like homolog *GhCYC2* is specifically expressed in the marginal zygomorphic ray florets, but not in the center-most actinomorphic disc flowers. The overexpression of *GhCYC2* results in the production of disc flowers that are morphologically similar to ray flowers. Moreover, GhCYC2 is reportedly important for the differentiation of flower types in *G. hybrida* [2]. In *Gerbera hybrida*, RAY1 is important for regulating floret identity (i.e., ray or disc florets), whereas RAY2 promotes the ventral identity of ray florets. The RAY1 and RAY2 genes belong to a subfamily of TCP genes, and RAY2 may be an ortholog of GhCYC2 in *G. hybrida* [34]. In a previous study of sunflower mutants, a CYC-like gene, *HaCYC2*, was expressed throughout the inflorescence, and disc florets developed bilateral symmetry [7]. Therefore, in Asteraceae species, some CYC2 clade genes that are specifically expressed in ray florets determine floret identity (i.e., ray or disc florets). Examples include GhCYC2 in *G. hybrida*, HaCYC2d and HaCYC2c in sunflower, and RAY1 and RAY2 in *Senecio vulgaris*.

In an earlier investigation, tissue slices were analyzed to study early stamen development and the subsequent abortion in *Gerbera* species. The results indicated that in the early stages, the stamen primordia in ray and disc florets develop similarly, but the development of the stamen primordium in ray florets subsequently starts to lag behind the corresponding development in disc florets. Additionally, the stamens of ray florets are gradually aborted [29]. However, little is known about the molecular mechanisms responsible for stamen abortion in ray florets.

The alternative splicing (AS) of precursor mRNAs (pre-mRNAs) enables the same gene to generate multiple transcripts that may encode various protein isoforms. Alternative splicing has profound functional consequences because of the resulting changes to protein production. Moreover, AS is broadly useful for enhancing molecular versatility. Specifically, it is a key gene regulatory process that influences almost all analyzed biological functions. Additionally, AS increases the coding potential of genomes, and represents an important post-transcriptional regulatory mechanism for increasing...
the proteomic diversity and functional complexity of higher eukaryotes. Furthermore, AS is common in plants [4, 39]. However, whether CYC2-like genes are affected by AS remains unknown.

*Chrysanthemum morifolium*, which is one of the most popular ornamental crops, is cultivated worldwide [19, 40]. The main ornamental part of *C. morifolium* plants is the capitulum, and its typical structure contains morphologically distinct ray and disc florets (Supplemental Figure S1). Ray florets, which are ligulate and zygomorphic, have a showy corolla (petals) and lack stamens. Their primary function is to attract pollinators. The central disc florets, which are radially symmetrical and hermaphroditic, have fertile pollen grains and are mainly required for reproduction [31]. In this study, a CYC2-like gene, *Cyc2CL*, was cloned from *C. morifolium* 'Fenditan'. The AS of *Cyc2CL* resulted in two distinct transcripts (*Cyc2CL-1* and *Cyc2CL-2*), which were produced in the apical buds after the flower differentiation stage was initiated. Prior to this stage, only *Cyc2CL-1* was produced in apical buds. Additionally, *Cyc2CL-2* was first expressed in the involucre tissue during the final involucre differentiation stage. As the floral bud differentiation stage proceeded, *Cyc2CL-2* was expressed in the receptacle and floret primordia. Studies involving transgenic Arabidopsis plants revealed that the overexpression of *Cyc2CL-1* and *Cyc2CL-2* can inhibit the development of stamens and petals. Therefore, *Cyc2CL* may play a key role in controlling the stamen abortion and petal development of ray florets and is likely a crucial regulator of chrysanthemum ray floret development. The results of this study are important for clarifying the molecular mechanisms underlying ray floret development in chrysanthemum and may be useful for identifying important candidate genes for the breeding of chrysanthemum and related species.

**Results**

**Isolation of chrysanthemum CYC homologs**

We isolated the pre-mRNA sequences (1146 bp) and the two alternatively spliced transcripts (*Cyc2CL-1* and *Cyc2CL-2*) of the CYC-like gene *Cyc2CL* (Fig. 1). The full-length *Cyc2CL-1* and *Cyc2CL-2* genomic sequences were also cloned (Supplemental Figure S2). As shown in Fig. 1b, the splice variants are derived from a pre-mRNA transcript and alternative splicing produces two types of mRNA. The *Cyc2CL-1* exon sequences contained 819 bp. Regarding *Cyc2CL-2*, the first exon comprised 795 bp and was similar to the *Cyc2CL-1* exon, whereas the second exon consisted of 105 bp, which matched part of the *Cyc2CL-1* intron. The *Cyc2CL-2* exons were separated by an intron sequence (246 bp). Thus, *Cyc2CL-1* and *Cyc2CL-2* shared partly similar exon sequences, but had diverse intron sequences. The splice sites of the *Cyc2CL-2* exons and intron are consistent with the canonical GT-AG splice sites, whereas the splice sites of the *Cyc2CL-1* exon and intron are non-canonical sequences [38]. The *Cyc2CL-1* and *Cyc2CL-2* coding sequences were 819 and 900 bp, respectively. The encoded *Cyc2CL-1* and *Cyc2CL-2* amino acid sequences were 89% similar (Fig. 1c). The deduced *Cyc2CL-1* and *Cyc2CL-2* amino acid sequences included the conserved TCP and R domains typical of CYC/TB1 subfamily members (Figs. 2 and 3). During phylogenetic analyses, *Cyc2CL-1* and *Cyc2CL-2* were clustered with the other CYC2 members in *C. morifolium* as well as with the CYC2-like genes of *Helianthus annuus*, *G. hybrida*, and *S. vulgaris*, implying there may have been several CYC2
Fig. 2 Coding sequences and deduced amino acid sequences of Cyc2CL-1 and Cyc2CL-2. The blue single and double underlines indicate the TCP and R domains, respectively. The differences in the alternatively spliced transcripts are underlined in red.
subclade gene duplication events in Asteraceae species (Fig. 4).

Expression analyses of chrysanthemum CYC homologs
A quantitative real-time polymerase chain reaction (qRT-PCR) assay was conducted to analyze the expression levels of CYC-like genes in chrysanthemum floral parts. Relatively low Cyc2CL-1 expression levels were detected in the leaves and vegetative buds, whereas Cyc2CL-2 expression was undetectable in the vegetative buds (Fig. 5). During chrysanthemum bud development, the Cyc2CL-1 and Cyc2CL-2 expression levels tended to increase. Additionally, Cyc2CL-1 and Cyc2CL-2 were highly expressed in ray florets, but were expressed at low levels in disc florets. These results implied that Cyc2CL-2 is not expressed in the vegetative buds, but is expressed when floral bud differentiation is initiated. An analysis of gene expression patterns in various floral tissues revealed that Cyc2CL-1 was highly expressed in the ray floret corolla, involucral bract, and receptacle, and relatively highly expressed in the pistil (stigma, style, and ovary) (Fig. 6). In contrast, Cyc2CL-2 was mainly expressed in the ray floret corolla. Both Cyc2CL-1 and Cyc2CL-2 were expressed at extremely low levels in the disc floret tissues, including the corolla, stamen, and pistil (stigma, style, and ovary) (Fig. 6). Thus, both Cyc2CL-1 and Cyc2CL-2 were mainly expressed in floral reproductive organs and weakly expressed in vegetative organs. This is consistent with the results of an earlier investigation on Gerbera species and sunflower [2].

A fluorescence in situ hybridization (FISH) analysis of labeled Cyc2CL-1 and Cyc2CL-2 RNA indicated that Cyc2CL-2 was expressed at low levels in the involucre tissue during the final involucre differentiation stage (Fig. 7b, d). During the floret primordia differentiation stage, Cyc2CL-2 was expressed in multiple tissues, including the involucre, receptacle, and floret primordia. During the corolla formation stage, which follows the
Fig. 4 (See legend on next page.)
floral bud differentiation stage, *Cyc2CL-2* was highly expressed in the inflorescence tissue. Fluorescence was undetectable in the FISH assay negative controls, in which the sense probes for *Cyc2CL-1* and *Cyc2CL-2* RNA were used (Supplemental Figure 3). Thus, *Cyc2CL-2* was initially expressed in the involucre tissue, but was also expressed in other tissues during the floral bud differentiation stage. The qRT-PCR data confirmed that before the floral bud differentiation stage, *Cyc2CL-2* was not expressed. However, unlike *Cyc2CL-2*, *Cyc2CL-1* was expressed in all inflorescence tissues at relatively high levels.

Morphological effects of chrysanthemum CYC homologs in transgenic Arabidopsis

Two constructs respectively expressing *Cyc2CL-1* and *Cyc2CL-2* under the control of the 35S promoter (35S::*Cyc2CL-1* and 35S::*Cyc2CL-2*) were inserted into Arabidopsis according to the floral-dip method, resulting in 42 and 48 independent lines of 35S::*Cyc2CL-1* and 35S::*Cyc2CL-2* plants, respectively. Most of the transgenic Arabidopsis lines grew weakly compared with the wild-type Arabidopsis plants, and many seedlings died (Supplemental Figure 4). The transgene expression levels of six randomly selected transgenic *Cyc2CL-1*-overexpressing (OE) and *Cyc2CL-2*-OE Arabidopsis lines were analyzed in a qRT-PCR assay (Fig. 8). Of the six *Cyc2CL-1*-OE lines, the highest and lowest *Cyc2CL-1* expression levels were detected in lines #35 and #8, respectively. Among the six *Cyc2CL-2*-OE lines, the highest and lowest *Cyc2CL-2* expression levels were detected in lines #37 and #15, respectively (Fig. 8). The gene expression data confirmed that *Cyc2CL-1* and *Cyc2CL-2* were overexpressed in the transgenic Arabidopsis plants (Figs. 9 and 10). On the basis of *Cyc2CL-1* and *Cyc2CL-2* expression levels, *Cyc2CL-1*-OE Arabidopsis lines #35 and #8 as well as *Cyc2CL-2*-OE Arabidopsis lines #37 and #15 were selected for a subsequent phenotype analysis.
In the analyzed Cyc2CL-1-OE and Cyc2CL-2-OE Arabidopsis lines, the stamens were aborted and the petals were very short or absent, whereas the pistils and sepals were normal. Additionally, wild-type plants had six stamens, but the Cyc2CL-1-OE Arabidopsis plants produced only two stamens that were aborted and lacked pollen grains (Fig. 9e). The wild-type Arabidopsis plants had four petals, which was in contrast to the suppressed petal development in the Cyc2CL-1-OE Arabidopsis plants. In the examined Cyc2CL-2-OE Arabidopsis lines, the petals grew, but were considerably shorter than the wild-type petals, suggesting that petal development was severely inhibited in the Cyc2CL-2-OE Arabidopsis lines (Fig. 10e). Similar to the Cyc2CL-2-OE Arabidopsis lines, the Cyc2CL-2-OE Arabidopsis lines produced two stamens that were aborted and lacked pollen grains. Additionally, normal pistils and sepals were detected in the Cyc2CL-2-OE Arabidopsis plants. Therefore, the Cyc2CL-1-OE and Cyc2CL-2-OE Arabidopsis lines were phenotypically similar. In both lines, the petals and stamens developed abnormally, and the stamens were aborted, resulting in a lack of pollen production. However, the pistils and sepals in the Cyc2CL-1-OE and Cyc2CL-2-OE Arabidopsis lines developed normally. To functionally characterize Cyc2CL-1 and Cyc2CL-2 regarding their inhibitory effects on native TCP genes, a qRT-PCR assay was completed to compare the TCP2, TCP3, TCP4, TCP10, and TCP24 expression levels between the wild-type Arabidopsis plants and the transgenic plants that were selected for the subsequent analysis of phenotypes [27]. The results indicated these genes were similarly expressed in the wild-type and transgenic Arabidopsis plants (Supplemental Figure 5). These results implied that Cyc2CL-1 and Cyc2CL-2 are important for stamen and petal development, but have no effect on pistil and sepal development in transgenic Arabidopsis plants.

**Discussion**

**Discovery of the alternative splicing of Cyc2CL represents an important step toward revealing the subtle molecular mechanism regulating ray floret development in chrysanthemum**

**Genetic control of ray floret development in Asteraceae**

Previous studies involving *G. hybrida*, *S. vulgaris*, and *H. annuus* indicated that some CYC2 clade genes specifically expressed in ray florets determine the production of ray or disc florets. The regulatory mechanism underlying floral symmetry was originally characterized in *A. majus*. Two partially redundant CYC2 paralogs, CYC and DICH, determine the dorsal identity of flowers and control the establishment of the zygomorphy (monosymmetry) in petal and stamen whorls [32, 33]. There has recently been considerable research focused on the regulatory functions of CYC-like genes affecting the zygomorphic flowers in Asteraceae, Leguminosae, Gesneriaceae, and other plant families.

In Asteraceae, the CYC-like genes are included in the CYC1, CYC2, and CYC3 subclades. Functional studies have confirmed that CYC-like genes are important for controlling organ growth, both as positive and negative regulators involved in cell proliferation and/or expansion.
Some studies have confirmed that CYC orthologs have a conserved role in controlling petal growth, and changes in their expressed domains determine whether zygomorphic or actinomorphic flowers are produced [2, 7, 22, 33, 43, 45, 48]. In *S. vulgaris*, *H. annuus*, and *G. hybrida*, CYC functions as a floral symmetry regulator, with a key role in determining floret identity (i.e., disc or ray florets) within the capitulum [2, 5, 43]. Duplication events considerably increased the diversity of the CYC2 clade genes within the Asteraceae lineage. Additionally, six, six, and five CYC2 clade genes have been identified in *G. hybrida*, *S. vulgaris*, and *H. annuus*, respectively. Some CYC2-like genes expressed exclusively in ray florets are essential for the formation of ray and disc florets [26]. Therefore, previous studies in *G. hybrida*, *S. vulgaris*, and *H. annuus* indicated that some CYC2 clade genes that are specifically expressed in ray florets determine the production of ray or disc florets. These genes include GhCYC2 in *G. hybrida*, HaCYC2d and HaCYC2c in *H. annuus*, and RAY1 and RAY2 in *S. vulgaris*.

**Identification of the CYC2 clade gene (Cyc2CL) and its two transcripts (Cyc2CL-1 and Cyc2CL-2) expressed in ray and disc florets**

In this study, one chrysanthemum CYC2-like gene (Cyc2CL) and its two alternatively spliced transcripts (Cyc2CL-1 and Cyc2CL-2) were revealed. The AS of Cyc2CL was initiated during the floral bud differentiation stage in the apical buds. Gene expression analyses in a qRT-PCR assay proved that during the vegetative growth stage, Cyc2CL-1 is expressed at low levels, whereas Cyc2CL-2 is not expressed. Thus, Cyc2CL is not alternatively spliced during the vegetative growth stage.
Fig. 8 Cyc2CL-1 and Cyc2CL-2 expression patterns in transgenic Arabidopsis. 

a Confirmation of gene expression by PCR amplification. 

b Relative gene expression levels as determined in a qRT-PCR assay.

Fig. 9 Phenotypes of Arabidopsis plants overexpressing Cyc2CL-1. 

a Wild-type flower.

b Flower of an Arabidopsis plant overexpressing Cyc2CL-1. The pistil and sepal grew normally.

c Inflorescence of an Arabidopsis plant overexpressing Cyc2CL-1. The inflorescence had normal pistils and sepals, but the petals and stamens did not grow normally.

d Flower of an Arabidopsis plant overexpressing Cyc2CL-1. Petal and stamen development was suppressed.

e Flower of an Arabidopsis plant overexpressing Cyc2CL-1 after sepals were removed. Petals did not develop in this flower. The stigma was normal, but the stamens were aborted.

f Arabidopsis plants overexpressing Cyc2CL-1.
However, the initiation of the floral bud differentiation stage resulted in up-regulated *Cyc2CL-1* expression levels as well as induced *Cyc2CL-2* expression, but at low levels. During the subsequent bud development stage, *Cyc2CL-1* expression was initially down-regulated, but then gradually increased as the buds developed (Fig. 5). Similarly, the *Cyc2CL-2* expression levels also increased as the buds developed. When the flowers bloomed, *Cyc2CL-1* and *Cyc2CL-2* were most highly expressed in the ray florets, whereas they were weakly expressed in the disc florets. Therefore, at some point during the floral bud differentiation stage, *Cyc2CL* was alternatively spliced to generate *Cyc2CL-1* and *Cyc2CL-2*.

Chrysanthemum floral bud differentiation is believed to comprise the shoot-tip doming stage, the initial and final involucre differentiation stages, the initial and final floret primordia differentiation stages, and the corolla formation stage [30]. In this study, a FISH analysis of labeled *Cyc2CL-1* and *Cyc2CL-2* RNA was conducted to identify the particular developmental stage and tissue in which the AS of *Cyc2CL* was initiated to produce *Cyc2CL-2*. We detected low *Cyc2CL-2* expression levels in the involucre tissue during the final involucre differentiation stage (Fig. 7b, d). In the subsequent floret primordia differentiation stage, *Cyc2CL-2* was expressed in the receptacle and floret primordia in addition to the involucre tissue (Fig. 7f, h). During the corolla formation stage, *Cyc2CL-2* was highly expressed in the inflorescence tissue (Fig. 7j, l). Therefore, we determined that *Cyc2CL-2* is first produced in involucre tissue and then in other tissues during the floral bud differentiation stage.

Our qRT-PCR analysis confirmed that the AS of *Cyc2CL* was initiated in the floral bud differentiation stage, resulting in the production of *Cyc2CL-2*. As the buds developed, the *Cyc2CL-2* expression levels increased. These results were consistent with those of the FISH analysis. Additionally, *Cyc2CL-1* was expressed in all inflorescence tissues and at higher levels than *Cyc2CL-2*. In sunflower, *HaCYC2c* is expressed in the corolla region of ray flowers and in the tubular flower reproductive organs, including the stamen, stigma, style, and ovary [43]. However, unlike the CYC2 clade genes specifically expressed in *G. hybrida*, *H. annuus*, and *S. vulgaris* ray florets, both *Cyc2CL-1* and *Cyc2CL-2* were weakly expressed in disc floret tissues.

Both *Cyc2CL-1* and *Cyc2CL-2* may be important for regulating stamen and petal growth

In chrysanthemum, the outer-layer ray florets vary regarding petal types and the inner-layer disc florets have diverse orientations. Additionally, the arrangement and combination of both floret types determine the daedal...
capitula types. Moreover, flower head types and petal types develop independently. Therefore, diverse petal types exist in specific types of flower heads. The complexity in the chrysanthemum petal and flower head types is indicative of a characteristic molecular mechanism involving CYC clade regulators. Huang et al. [22] cloned six CYC2 genes from two chrysanthemum cultivars and their F1 progenies. They observed that in one CYC2 gene-overexpressing Chrysanthemum lavandulifolium line, some trans-like florets had short petals and abnormal stamens and were morphologically similar to ray florets [22]. Chen et al. [8] analyzed six CYC2-like genes in some Asteraceae species and evaluated the effects of CICYC2d overexpression in C. lavandulifolium. They proved that the constitutive expression of CICYC2d suppresses corolla growth during ray floret development [8]. Therefore, the CYC genes may be functionally diverse and the encoded proteins may interact to regulate the development of chrysanthemum ray florets.

Phenotypic analyses of the Cyc2CL-1-OE and Cyc2CL-2-OE Arabidopsis lines verified that the stamens were aborted, and the petals were very short or absent, in contrast to the normal pistils and sepalas. Alternative splicing can result in the production of multiple mRNAs from a single gene, thereby increasing the proteomic diversity of higher eukaryotes. It is also a vital mechanism for regulating gene expression during the growth and development of higher plants [25, 42]. The data presented herein imply that Cyc2CL-1 and Cyc2CL-2 likely play key regulatory roles related to stamen and petal growth.

The observed inhibitory effects of Cyc2CL-1 and Cyc2CL-2 expression on stamen and petal growth are consistent with the results of previous studies. In A. majus, both CYC and DICH are expressed in the dorsal domain of the young floral meristem, thereby retarding petal and stamen growth. At a later stage, CYC is expressed throughout the dorsal domain to facilitate petal lobe growth and inhibit stamen development [33]. In Primulina heterotricha, when flowers are dorsoventrally differentiated during floral development, two CYC2 clade genes, CYC1C and CYC1D, are highly expressed in the dorsal petals and the dorsal/lateral stamens. Additionally, the substantial transcription of CYC1C in the lateral staminodes at the late development stage is related to the abortion of the dorsal and lateral stamens. Both CYC1C and CYC1D may regulate the decrease in the dorsal petal size and the abortion of the dorsal/lateral stamens [15, 47]. Fabio et al. confirmed that in H. annuus, TURF and CHRY are crucial for establishing the corolla symmetry of disc and ray flowers, but these genes also influence carpel and stamen development. Additionally, a loss-of-function mutation to the CYC gene reportedly leads to hermaphroditic tubular-like ray florets replacing the normal sterile ray florets [13, 14, 16, 17, 35]. Another study indicated that CYC-like genes help regulate stamen and carpel differentiation, likely via their association with the genes controlling the cell cycle and flower organ identity [15]. In G. hybrida, the ectopic overexpression of GhCYC2 disrupts stamen development and causes disc flowers to acquire enlarged and markedly fused petals [2].

Nag et al. [36] confirmed that the expression of TCP4 (i.e., a key target of miR319a) must be appropriately controlled to ensure normal petal and stamen development, with high TCP4 expression levels disrupting petal and stamen development in Arabidopsis. The ectopic expression of CyeCL-1 and CyeCL-2 similarly inhibits petal and stamen development. Therefore, the CYC and TCP target genes are likely conserved in chrysanthemum and Arabidopsis.

**The molecular mechanism underlying Cyc2CL-1 and Cyc2CL-2 functions remains to be revealed.**

We identified a CYC2 clade gene (Cyc2CL) and its two transcripts (Cyc2CL-1 and Cyc2CL-2) and assessed the effects of the ectopic expression of this gene in Arabidopsis to functionally characterize Cyc2CL-1 and Cyc2CL-2. However, the Cyc2CL-1-OE and Cyc2CL-2-OE Arabidopsis lines were phenotypically similar, and the differences in the functions of the two transcripts were not obvious in the transgenic Arabidopsis plants, likely because of the substantial differences in the flower types and molecular mechanisms between chrysanthemums and Arabidopsis. Chrysanthemums have a capitulum consisting of bilateral (zygomorphic) ray florets and radial (actinomorphic) disc florets, whereas Arabidopsis plants produce only one type of radial flowers. Additionally, the gene regulatory mechanism is more complex in chrysanthemums than in Arabidopsis. The regulatory functions of Cyc2CL-1 and Cyc2CL-2 cannot be thoroughly analyzed based solely on the phenotypic changes in the transgenic Arabidopsis plants. Thus, we were unable to elucidate the precise molecular mechanism underlying Cyc2CL-1 and Cyc2CL-2 functions. Future investigations should examine the consequences of overexpressing and silencing Cyc2CL-1 and Cyc2CL-2 in chrysanthemum to clarify the regulatory functions of Cyc2CL-1 and Cyc2CL-2 related to ray floret development. Additionally, our qRT-PCR analysis revealed the Cyc2CL-1 and Cyc2CL-2 expression levels in the corolla region and reproductive organs of flowers, but the exact floral organ regions in which these genes are expressed should be determined via in situ RNA hybridization in future studies. Analyses of the proteins interacting with Cyc2CL-1 and Cyc2CL-2 as well as the downstream target genes should also be performed to provide new insights into the molecular mechanism underlying their functions.
insights into the molecular mechanism controlling ray floret development in chrysanthemum.

Methods

Plant materials and RNA extraction

The individual plants used in this study were all derived by tissue culture from a hybrid of chrysanthemum varieties (i.e., *C. morifolium* ‘Fenditan’, which is a ground cover chrysanthemum variety). The plants were developed in our laboratory and were cultivated in a greenhouse at Beijing Forestry University (116.3°E, 40.0°N) under long-day conditions (16-h light/8-h dark) for 180 days and then under short-day conditions (8-h light/16-h dark) at 24 ± 1 °C. Under long-day conditions, approximately 100 vegetative buds were harvested between 9:00–12:00 am. When the plants were first exposed to short-day conditions, about 100 apical buds were harvested between 9:00–12:00 am every week until visible floral buds formed, after which 100 buds were harvested between 9:00–12:00 am every week until the ray florets developed an observable color. Some harvested plant tissues were immediately placed in liquid nitrogen and stored at −70 °C for a subsequent RNA extraction step. Other plant tissues were treated and underwent a FISH analysis. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Beijing, China). The quantity and quality of the extracted RNA were determined with the NanoDrop ND2000 spectrophotometer.

Isolation of *Chrysanthemum morifolium* CYC2 genes and construction of phylogenetic trees

Degenerate primers specific for CYC2 genes (Table 1) were designed based on the CYC2 homologs in the NCBI database [2, 5]. Full-length cDNA sequences were obtained with the 3′ and 5′ RACE cDNA amplification kits (Takara, Japan). Total DNA was extracted from harvested tissues with the DNAsecure Plant Kit (TianGen, Beijing, China). Total RNA was extracted from chrysanthemum capitula with the RNAprep Pure Kit (for plants) (TianGen, China). The target sequences were amplified by PCR, which was completed in a final reaction volume of 50 μl comprising 2 μl cDNA (40 ng), 0.4 μl Taq DNA polymerase (Promega), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 5 μl 10× PCR buffer (Promega), 1 μl dNTP (10 mM), 3 μl MgCl₂ (25 mM), and 36.6 μl ddH₂O. The PCR conditions were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; 72 °C for 10 min. The amplified gene sequences were ligated to the pMD18-T vector and inserted into *Escherichia coli* DH5α cells, after which the accuracy of the inserted fragments was verified by sequencing. The Cyc2CL-1 and Cyc2CL-2 coding sequences were deposited in the GenBank database (accession numbers: KP696775.1 and KP696776.1). We obtained CYC-like sequences from the NCBI database. The MEGA program (version 10) [28] was used to construct phylogenetic trees based on the maximum likelihood method involving the JTT matrix-based model [23].

Gene expression analysis in a qRT-PCR assay

The abundance of two transcripts (Cyc2CL-1 and Cyc2CL-2) in chrysanthemum flowers at different developmental stages was investigated in a qRT-PCR assay, which was completed with the PikoReal Real-Time PCR system (Thermo Fisher Scientific, Germany). To analyze the Cyc2CL-1 and Cyc2CL-2 expression patterns in the shoot apices and buds at different developmental stages, we sampled the vegetative buds (i.e., apical buds during the vegetative growth stage), flower buds (transsection diameter of about 2 mm), and buds at three flower developmental stages (first stage: bud transsection diameter of about 3 mm; second stage: bud transsection diameter of about 5 mm; and third stage: bud transsection diameter of about 6–7 mm and colors were detectable in the outer ray florets; Fig. 5). To analyze the Cyc2CL-1 and Cyc2CL-2 expression patterns in ray and disc florets at different flower developmental stages, we collected ray and disc florets at the early and full flowering stages. In the early stage, colors were detectable in both ray and disc florets. During the full flowering stage, ray and disc florets had bloomed. Furthermore, to analyze the Cyc2CL-1 and Cyc2CL-2 expression patterns in different floral tissues, we collected the involucral bract, receptacle, corolla, and pistil (stigma, style, and ovary) of ray florets as well as the corolla, stamen, and pistil (stigma, style, and ovary) of disc florets (Fig. 6). The leaves of wild-type and transgenic Arabidopsis plants were collected to analyze the CYC2 and TCP gene expression levels. We collected three biological replicates for each tissue. The qRT-PCR was completed with SYBR Premix Ex Taq (TaKaRa) and the PikoReal Real-Time PCR system (Thermo Fisher Scientific). Each reaction was prepared in a total volume of 20 μl containing 2 μl first-strand cDNA as the template. The qRT-PCR conditions were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, the optimal annealing temperature for 30 s, and 60 °C for 30 s. The *C. morifolium* protein phosphatase 2A gene (*PP2Ac*) was used as the reference gene. Details regarding the qRT-PCR primers are provided in Table 2. The qRT-PCR primers specific for Arabidopsis TCP genes are listed in Supplementary Table 1. The qRT-PCR assay was completed with three biological replicates, with samples analyzed in triplicate in each replicate.

| Table 1 | Degenerate primers used for amplifying *C. morifolium* CYC genes |
|---------|---------------------------------------------------------------|
| Gene    | Forward primer (5′-3′)                                       |
|         | Reverse primer (5′-3′)                                        |
| CmCyc   | ATGTTTTCYTCAAAACCCYTTTC                                       |
|         | GTTTGCCTGTGTTGTCRAAMCCTA                                      |

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Vector construction and Arabidopsis transformation

Full-length Cyc2CL-1 and Cyc2CL-2 cDNA sequences were amplified by a PCR with gene-specific primers (Table 3). The amplicons were inserted into the pGEM-T vector (Promega), after which the accuracy of the inserted fragments was confirmed by sequencing. The Cyc2CL-1 and Cyc2CL-2 sequences were then digested with restriction enzymes and subcloned into pCAMBIA1304 and modified pCAMBIA1304 vectors. The resulting plasmids were inserted into Agrobacterium tumefaciens strain EHA105 cells, which were then used to transform Arabidopsis (Col-0) cells according to a floral-dip method [11]. The putative transgenic lines were screened on Murashige and Skoog medium containing 50 mg/l hygromycin. The hygromycin-resistant seedlings (T0 generation) were transferred to soil after 14 days and grown at 21–23 °C under long-day conditions. The T3 generation plants were subsequently analyzed. Col-0 seeds were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org).

Fluorescence in situ hybridization

The expression of Cyc2CL-1 and Cyc2CL-2 in shoot apices and buds at different developmental stages was analyzed by FISH. We designed LNA-based probes to target the mature Cyc2CL-1 and Cyc2CL-2 mRNA sequences (Table 4). Sense probes were used as the FISH assay negative controls (Supplemental Table 2). Shoot apices and buds collected at different developmental stages were fixed with a 4% paraformaldehyde solution for 30 min, after which frozen tissue sections were prepared. The fixed slides were washed twice with PBS, treated with proteinase K at 37 °C for 10 min, and then dehydrated by FISH. We designed LNA-based probes to target the mature Cyc2CL-1 and Cyc2CL-2 sequences were then digested with restriction enzymes and subcloned into pCAMBIA1304 and modified pCAMBIA1304 vectors. The resulting plasmids were inserted into Agrobacterium tumefaciens strain EHA105 cells, which were then used to transform Arabidopsis (Col-0) cells according to a floral-dip method [11]. The putative transgenic lines were screened on Murashige and Skoog medium containing 50 mg/l hygromycin. The hygromycin-resistant seedlings (T0 generation) were transferred to soil after 14 days and grown at 21–23 °C under long-day conditions. The T3 generation plants were subsequently analyzed. Col-0 seeds were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org).

Fluorescence in situ hybridization

The expression of Cyc2CL-1 and Cyc2CL-2 in shoot apices and buds at different developmental stages was analyzed by FISH. We designed LNA-based probes to target the mature Cyc2CL-1 and Cyc2CL-2 mRNA sequences (Table 4). Sense probes were used as the FISH assay negative controls (Supplemental Table 2). Shoot apices and buds collected at different developmental stages were fixed with a 4% paraformaldehyde solution for 30 min, after which frozen tissue sections were prepared. The fixed slides were washed twice with PBS, treated with proteinase K at 37 °C for 10 min, and then dehydrated in 70, 85, and 100% ethanol for 5 min each. After a denaturing treatment at 78 °C for 5 min, the probes were added to the slides and allowed to hybridize overnight at 42 °C under humid conditions. The slides were washed with 50% formamide/2× SSC at 43 °C and then

Table 3 Gene-specific primers used for the PCR amplification of full-length Cyc2CL-1 and Cyc2CL-2 cDNA sequences

| Gene  | Forward primer (5'-3') | Reverse primer (5'-3') |
|-------|------------------------|------------------------|
| Cyc2CL-1 | ACTAGATGGTTTTTCTCAAACC CTTTCCTAG | CCTTCCAG |
| Cyc2CL-2 | ACTAGATGGTTTTTCTCAAACC CTTTCCTAG | CCTTCCAG |

Table 4 Gene-specific probe sequences for the fluorescence in situ hybridization of Cyc2CL-1 and Cyc2CL-2

| Gene  | Probes sequence (5'-3') |
|-------|------------------------|
| Cyc2CL-1 | GTAT GTAT TCCGTC TATA TCTAG |
| Cyc2CL-2 | CAGTACTGACCTCTTGGGCAGGAAATATGTTGGAGCTGG ACTGATGTGTGAA |

with 2× SSC at room temperature to eliminate the non-specific and repetitive RNA hybridizations. Finally, the slides were counterstained with DAPI (Sigma) for 10 min and then observed with a Zeiss LSM 700 Meta confocal microscope.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-02884-z.

Additional file 1.

Abbreviation

AS: Alternative splicing

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Authors’ contributions

H L and QX Z designed the whole experiments; H L conducted the experiments and wrote the manuscript; H L, M S, HT P, TR C and J W analyzed the data. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and/or analysed during the current study are available in the GenBank repository at NCBI (accession numbers: KP696775.1 and KP696776.1).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Berger BA, Thompson V, Lim A, Ricigliano V, Howarth DG. Elaboration of bilateral symmetry across Knautia macedonica capitula related to changes in ventral petal expression of CYCLOIDEA -like genes. EvoDevo. 2016;7:1–10.
2. Broholm SK, Sari TH, Laitinen RAE, Albert VA, Teeri TH, Paula E. A TCP domain transcription factor controls flower type specification along the
radial axis of the Gerbera (Asteraceae) inflorescence. Proc Natl Acad Sci USA. 2008;105:9117–22.
3. Carlson SE, Howarth DG, Donoghue MJ. Diversification of CYCLOIDEA-like genes in Dipsacaceae (Dipsacales): implications for the evolution of capitulum inflorescences. BMC Evol Biol. 2011;11:1–13.
4. Carvalho PF, Feijão CV, Duque P. On the physiological significance of alternative splicing events in higher plants. Prototplatea. 2013;250:539–50.
5. Chapman MA, Leebeens-Mack JH, Burke JM. Positive selection and divergence following gene duplication in the sunflower CYCLOIDEA gene family. Mol Biol Evol. 2008;25:1260–73.
6. Chapman MA, Shunxue T, Rthe D, Savithri N, Hunter S, Barb JG, Knapp SJ, Burke JM. Genetic analysis of floral symmetry in van Gogh’s sunflowers reveals independent recruitment of CYCLOIDEA genes in the Asteraceae. PLoS Genet. 2012;8:e1002628.
7. Chapman MA, Tang S, Draeger D, Nambereesan S, Shaffer H, Barb JG, Knapp SJ, Burke JM. Genetic analysis of floral symmetry in van Gogh’s sunflowers reveals independent recruitment of CYCLOIDEA genes in the Asteraceae. PLoS Genet. 2012;8:e1002628.
8. Chen, J. Shen C-Z, Guo Y, Yao G-Y. Patternning the Asteraceae Capsulatum: duplications and differential expression of the flower symmetry CYC2-like genes. Front Plant Sci. 2018;9:551 https://doi.org/10.3389/fpls.2018.00551.
9. Citerne HL, Reyes E, Le Guilloux M, Delannoy E, Simonnet F, Sauquet H, Weston PH, Nadot S, Darnelval C. Characterization of CYCLOIDEA-like genes in Proteaceae, a basal eudicot family with multiple shifts in floral symmetry. Ann Bot. 2017;119:367–78.
10. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J Cell Mol Biol. 1998;16:735–43.
11. Ditcher D. Toward a new synthesis: major evolutionary trends in the angiosperm fossil flora. Proc Natl Acad Sci USA. 2000;97:7030–6.
12. Fabio B, Marco F, Maurizio T, Daniele B, Claudio P. Mutations of corolla symmetry affect carpel and stamen development in Helianthus annuus. Can J Bot. 2005;83:1065–72.
13. Fambrini M, Michelotti V, Pugliesi C. The unstable tubular ray flower allele of sunflower: inheritance of the reversion to wild-type. Plant Breed. 2007;126(5):548–50.
14. Fambrini M, Puglicci C. CYCLOIDEA 2 clade genes: key players in the control of floral symmetry, inflorescence architecture, and reproductive organ development. Plant Mol Biol Report. 2017;35(20–36 https://doi.org/10.1007/s11105-016-1005-z.
15. Fambrini M, Salvini M, Puglicci C. A transcription-mediated inactivation of a CYCLOIDEA-like gene generates polysemic and androgynous ray flowers in Helianthus annuus[J]. Genetic. 2011;19(11–12):2152–9.
16. Fambrini M, Salvini M, Basile A, Puglicci C. Transcription-dependent induction of Vincent van Gogh’s sunflowers: exceptions revealed. Genesis. 2014;52(4):315–27 https://doi.org/10.1111/gene.22743.
17. Garces HM, Spencer VM, Kim M. Control of floret symmetry by RAY3, SvDIV1B and SvrAD in the capitulum of Senecio vulgaris. Plant Physiol. 2016;171:2055–69.
18. García-Fernández SA, Leal F, Fernández-Sánchez P, Sánchez-Melgar JA, de la Serna MM. Identification and characterization of CYC-like genes in regulation of ray floret development in Chrysanthemum morifolium. Front Plant Sci. 2016;7:551 https://doi.org/10.3389/fpls.2016.01633.
19. Gu C, Chen S, Liu Z, Shan H, Luo H, Guan Z, Chen F. Reference gene selection for quantitative real-time PCR in Chrysanthemum subjected to abiotic and abiotic stress. Mol Biotechnol. 2011;49:192–7.
20. Hileman LC. Bilateral flower symmetry—how, when and why? Curr Opin Plant Biol. 2014;17:146–52.
21. Howarth DG, Donoghue MJ. Phylogenetic analysis of the “ECE” (CYC/CTB1) clade reveals duplications predating the core eudicots. Mol Biol Evol. 2018;35(6): 1547–9 https://doi.org/10.1093/molbev/msy096.
22. Huang D, Li X, Sun M, Zhang T, Pan H, Cheng T, Wang J, Zhang Q. Whole-transcriptome analysis of differentially expressed genes in the ray florets and disc florets of Chrysanthemum morifolium. BMC Genomics. 2016;17:1–17.
23. Luo D, Carpenter R, Copsey L, Vincent C, Clark J, Coen E. Control of organ asymmetry in flowers of Antirrhinum. Cell. 1999;99:367–76.
24. Luo D, Carpenter R, Vincent C, Copsey L, Coen E. Origin of floral asymmetry in Antirrhinum. Nature. 1996;383:794–9.
25. Mizzotti C, Fambrini M, Caporal E, Maisero S, Pugliesi CA. CYCLOIDEA-like gene mutation in sunflower determines anunnual floret type able to produce filled achenes at the periphery of the euphytan. Botany. 2015;93:71–81.
26. Nag A, King S, Jack T, mIR319a targeting of TCP4 is critical for petal growth and development in Arabidopsis. Proc Natl Acad Sci. 2009;106(52):22534–9 https://doi.org/10.1073/pnas.0908718106.
27. Preston JC, Hileman LC. Developmental genetics of floral symmetry evolution. Trends Plant Sci. 2009;14:147–54.
28. Pucker B, Brockington SF. Genome-wide analyses supported by ma-seq reveal non-canonical splice sites in plant genomes. BMC Genomics. 2018;19(1):980.
29. Raddy ASN, Marquez Y, Kalyna M, Barta A. Complexity of the alternative splicing landscape in plants. Plant Cell. 2013;25:3657.
30. Siwa JAT. Chrysanthemum advances in tissue culture, cyoposynthesis, postharvest technology, genetics and transgenic biotechnology. Biotechnol Adv. 2003;21:715–6.
31. Stuessy TF, Urtubey E. Phylogenetic implications of corolla morphology in subfamily Barnadesioideae (Asteraceae). Flora. 2006;201(5):540–52 https://doi.org/10.1016/j.flora.2005.07.009.
32. Syed NH, Kalyna M, Marquez Y, Barta A. Round. JWS. Alternative splicing in plants – coming of age. Trends Plant Sci. 2012;17:616–23.
33. Tahtharaju S, Rijpkema AS, Vetterli AR, Albert VA, Teeri TH, Elomaa P. Evolution and diversification of the CYC/CTB1 gene family in Asteraceae—a comparative study in Gerbera (Mitsutieae) and sunflower (Helianthus). Mol Biol Evol. 2012;29:1155–66.
34. Tonices R, Méndez M, Gómez JM. Where do monomorphic sexual systems fit in the evolution of dioecy? Insights from the largest family of angiosperms. New Phytol. 2011;190:2344–48.
35. Xia Y, Hong-Bo P, Bo-Ling L, Zh-Ling Q, Qiu G, Lai W, Yang D, Yin-Zheng W. Evolution of double positive autoregulatory feedback loops in CYCLOIDEA2 clade genes is associated with the origin of floral zygomorphy. Plant Cell. 2012;24:834–47 https://doi.org/10.1105/tpc.112.105802.
36. Yafei Z, Kai P, Barbara DA, Oriane B, Paula E. Evolutionary diversification of cyctbl -like tcp homologs and their recruitment for the control of branching and floral morphology in papaveraceae (basal eudicots). New Phytol. 2018;210(3):317–31.
37. Yang X, Pang H-B, Liu B-L, Qiu Z-J, Gao Q, Wei L, Dong Y, Wang Y-Z. Evolution of double positive autoregulatory feedback loops in CYC2 clade genes is associated with the origin of floral zygomorphy. Plant Cell. 2012;24:834–47.
38. Yang X, Zhao X, Li C, Liu J, Qiu Z, Dong Y, Wang Y. Distinct regulatory changes underlying differential expression of TDSERNE BRANCHED1- CYCLOIDEA-PROLIFERATING CELL FACTOR genes associated with petal variations in zygomorphic flowers of Petrosocoea sp. of the family Gesneriaceae. Plant Physiol. 2015;169:2138–51.

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