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Characterization of the sequence and expression pattern of *LFY* homologues from dogwood species (*Cornus*) with divergent inflorescence architectures

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• *Background and Aims* *LFY* homologues encode transcription factors that regulate the transition from vegetative to reproductive growth in flowering plants and have been shown to control inflorescence patterning in model species. This study investigated the expression patterns of *LFY* homologues within the diverse inflorescence types (head-like, umbel-like and inflorescences with elongated internodes) in closely related lineages in the dogwood genus (*Cornus s.l.*). The study sought to determine whether *LFY* homologues in *Cornus* species are expressed during floral and inflorescence development and if the pattern of expression is consistent with a function in regulating floral development and inflorescence architectures in the genus.

• *Methods* Total RNAs were extracted using the CTAB method and the first-strand cDNA was synthesized using the SuperScript III first-strand synthesis system kit (Invitrogen). Expression of *CorLFY* was investigated by RT–PCR and RNA *in situ* hybridization. Phylogenetic analyses were conducted using the maximum likelihood methods implemented in RAxML-HPC v7.2.8.

• *Key Results* cDNA clones of *LFY* homologues (designated *CorLFY*) were isolated from six *Cornus* species bearing different types of inflorescence. *CorLFY* cDNAs were predicted to encode proteins of approximately 375 amino acids. The detection of *CorLFY* expression patterns using *in situ* RNA hybridization demonstrated the expression of *CorLFY* within the inflorescence meristems, inflorescence branch meristems, floral meristems and developing floral organ primordia. PCR analyses for cDNA libraries derived from reverse transcription of total RNAs showed that *CorLFY* was also expressed during the late-stage development of flowers and inflorescences, as well as in bracts and developing leaves. Consistent differences in the *CorLFY* expression patterns were not detected among the distinct inflorescence types.

• *Conclusions* The results suggest a role for *CorLFY* genes during floral and inflorescence development in dogwoods. However, the failure to detect expression differences between the inflorescence types in the *Cornus* species analysed suggests that the evolutionary shift between major inflorescence types in the genus is not controlled by dramatic alterations in the levels of *CorLFY* gene transcript accumulation. However, due to spatial, temporal and quantitative limitations of the expression data, it cannot be ruled out that subtle differences in the level or location of *CorLFY* transcripts may underlie the different inflorescence architectures that are observed across these species. Alternatively, differences in *CorLFY* protein function or the expression or function of other regulators (e.g. *TFL1* and *UFO* homologues) may support the divergent developmental trajectories.

Key words: *Cornus*, dogwood, inflorescence evolution, *LFY* homologues, *CorLFY* expression, RT–PCR, *in situ* hybridization.

INTRODUCTION

FLORICAULA/*LEAFY* (*FLO*/*LFY*) homologues are transcription factors regulating the transition from vegetative growth to reproductive growth in flowering plants. They were first identified in the analysis of two mutants, *floricaula* in *Antirrhinum majus* (Coen *et al.*, 1990) and *lfy* in *Arabidopsis thaliana* (Schultz and Haughn, 1991; Weigel *et al.*, 1992). *FLO*/*LFY* homologues have since been identified and studied in many lineages of land plants (e.g. Mouradov *et al.*, 1998; Ahearn *et al.*, 2001; Chujo *et al.*, 2003; Dornelas and Rodriguez, 2005; Maizel *et al.*, 2005; Sliwinski *et al.*, 2006, summarized in Benlloch *et al.*, 2007; Bosch *et al.*, 2008; Hamès *et al.*, 2008). In land plants, *LFY* is known to encode a protein with highly conserved N-terminal and C-terminal domains that are connected by a more variable interdomain region (Maizel *et al.*, 2005). The C-terminal contains a DNA-binding domain that is structurally related to helix–turn–helix domains (Hamès *et al.*, 2008), while the N-terminal encodes a homodimerization domain (Siriwardana and Lamb, 2012), and the interdomain region is of unknown function. Unlike many plant developmental regulators that exist as multigene families (Riechmann and Ratcliffe, 2000; Martinez-Castilla and Alvarez-Buylla, 2003; Shiu *et al.*, 2005), *LFY* exists as a single-copy gene in most angiosperms that have been investigated (Southerton *et al.*, 1998; Frohlich and Parker, 2000; Yoon and Baum, 2004), although two or more

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copies of *LFY* homologues have been reported in some angiosperm lineages, including *Ionopsidium acaule* (violet cress; Shu *et al.*, 2000), *Nicotiana* (tobacco; Ahearn *et al.*, 2001), *Zea mays* (maize; Bomblies *et al.*, 2003), Rosaceae (Maloideae; Wada *et al.*, 2002; Esumi *et al.*, 2005), Fabaceae (Caesalpinoideae, Archambault and Bruneaua, 2004), Lamiales (Aagaard *et al.*, 2005), Alliaceae (garlic, Rotem *et al.*, 2007) and *Idahoa scapigera* (Brassicaceae, Sliwinski *et al.*, 2007).

The role of the *LFY* gene in controlling floral meristem identity (Weigel *et al.*, 1992; Weigel and Meyerowitz, 1993; Weigel and Nilsson, 1995; Blázquez *et al.*, 1997; Nilsson *et al.*, 1998) and floral organ initiation and patterning (Weigel *et al.*, 1992; Parcy *et al.*, 1998; Chae *et al.*, 2008; Hamès *et al.*, 2008), which was initially reported in *Arabidopsis* and *Antirrhinum*, has been supported by gene expression and/or functional data from a number of other plants, including both monocots [*Z. mays*, Bomblies *et al.*, 2003; *Allium sativum* (garlic), Rotem *et al.*, 2007; *Oryza sativa* (rice), Rao *et al.*, 2008] and dicots [*Pisum sativum* (pea), Hofer *et al.*, 1997; *Petunia*, Souer *et al.*, 1998, 2008; *Solanum lycopersicum* (tomato), Molinero-Rosales *et al.*, 1999; *Ionopsidium acaule* (violet cress), Shu *et al.*, 2000; *Vitis vinifera* (grapevine), Carmona *et al.*, 2002; *Eschscholzia californica* (California poppy), Busch and Gleissberg, 2003, Wreath *et al.*, 2013; *Hevea brasiliensis* (rubber tree), Dornelas and Rodriguez, 2005; *Idahoa*, Sliwinski *et al.*, 2007; and *Populus*, An *et al.*, 2011]. A recent study has demonstrated that LEAFY (LFY) stimulates flower development and the formation of floral primordia via control of auxin response pathways (Li *et al.*, 2013). Furthermore, several studies have supported a role for *LFY* in controlling inflorescence architectures. For instance, constitutive expression of *LFY* resulted in a solitary terminal flower in *Nicotiana* (Ahearn *et al.*, 2001; Koes, 2008); ectopic expression of *LFY* caused internodal compression of inflorescences in *Arabidopsis* (Sliwinski *et al.*, 2007) and *Malus domestica* (apple; Flachowsky *et al.*, 2010); changes in the activity or expression of *LFY* were associated with the origins of rosette flowering in *Idahoa*, *Ionopsidium* and *Leavenworthia* (Brassicaceae) (Shu *et al.*, 2000; Bosch *et al.*, 2008); and *LFY* activity repressed pedicel elongation and orientation in *Arabidopsis*, contributing to the variation of inflorescence architecture in the species (Yamaguchi *et al.*, 2012). In maize, the *LFY* homologue (*ZFL*) promoted spikelet formation (Ikeda-Kawakatsu *et al.*, 2012), while double mutants of *LFY* homologues (*zfl1* and *zfl2*) exhibited reduced tassel branching (Bomblies *et al.*, 2003). In rice, RNAi knockdowns of the *LFY* homologue, *RFL*, severely decreased panicle branching, while overexpression of *RFL* resulted in small panicles (Rao *et al.*, 2008) due to the suppression of spikelet meristem formation (Ikeda-Kawakatsu *et al.*, 2012). In wheat, the expression pattern of the *LFY* orthologue (*WFL*) was associated with spikelet formation (Shitsukawa *et al.*, 2006). These data seem to suggest contrasting effects of LFY activity on inflorescence size between dicots and monocots (reduction in dicots but enlargement in monocots).

In the current study, we investigated the expression of *LFY* homologues during floral and inflorescence development of several dogwood species (*Cornus*, *s.l.*) in an effort to detect differences in *LFY* expression that might explain the evolutionary divergence in this genus of determinate umbel-like and head-like inflorescences from inflorescences with elongated internodes. The dogwood genus consists of four closely related lineages with similar inflorescence branching patterns (Feng *et al.*, 2011), but differing in their aspect (Fig. 1). All inflorescences have cymous lateral branches and possess a terminal flower (the thyrsoids of Endress, 2010; Feng *et al.*, 2011). Species within the blue- or white-fruited lineage (BW) produce determinate, elongated, large compound inflorescences. Within the cornelian cherry (CC) lineage, the inflorescences are determinate and umbel-like. Species within the dwarf dogwood lineage (DW) bear depauperate, condensed inflorescences with up to four lateral dichasia with very short, but evident inflorescence branches and pedicels (the minidichasia of Feng *et al.*, 2011). Those within the big-bracted dogwood lineage (BB) produce flowers in head-like structures (Harris, 1999; Harris and Harris, 2001) (Fig. 1).

Developmental studies using scanning electron microscopy and histology have shown that the structural differences among these inflorescence types are largely determined during early development of inflorescences and are due to variations in the pattern, number and elongation of inflorescence branch meristems. Inflorescence branch meristems (IBMs) are generated in all four types during early development, but in umbel-like and head-like inflorescences the inflorescence branches and the rachis supporting the central inflorescence meristem (IM) do

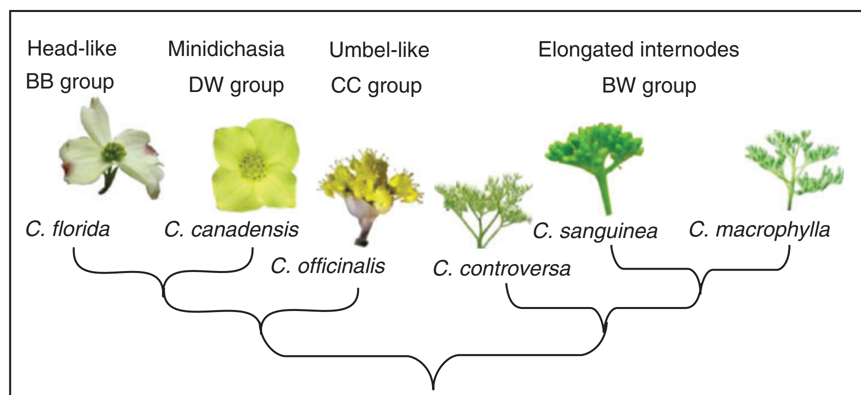

Fig. 1. Four major clades of *Cornus* showing their phylogenetic relationships (from Xiang *et al.*, 2008) and inflorescence types (modified from Xiang *et al.*, 2008 and Feng *et al.*, 2011). BB, big-bracted; DW, dwarf dogwood; CC, cornelian cherry; BW, blue- or white-fruited.

not elongate during development (for detailed differences among the four types, see Feng *et al.*, 2011). The observation that the generation of new inflorescence meristems ceases in all four inflorescence types when the central inflorescence meristem transitions into a floral meristem (as evidenced by floral organ formation) clearly indicates that these four inflorescence types are all determinate (Feng *et al.*, 2011). The inflorescence buds are preformed in the fall and expand in the following spring (Feng *et al.*, 2011). Previous studies have indicated that the determinate umbel-like and head-like inflorescences in *Cornus* evolved in parallel from elongated, branched inflorescences (Xiang and Thomas, 2008; Feng *et al.*, 2011).

The primary goal of this study was to identify orthologues of *Arabidopsis LFY* from the different lineages of dogwood (*Cornus*) species (hereafter *CorLFY*) and examine their expression patterns during floral and inflorescence development in the genus. We also hoped that a comparison of the expression patterns of the *LFY* genes among these species would provide insight into the potential role of these genes in the evolution of umbel-like and head-like inflorescences in *Cornus*.

MATERIALS AND METHODS

Sampling and cDNA preparation

Six species of *Cornus* representing the four major clades of the genus were included in the study. These were *C. officinalis* (CC group), *C. florida* (BB group) and *C. canadensis* (DW group) and *C. macrophylla*, *C. sanguinea* and *C. controversa* (BW group). Inflorescence buds were collected from plants growing on the NCSU campus and JC Raulston Arboretum for all species except *C. canadensis*, which were grown in the NCSU Phytotron and introduced from several wild populations in West Virginia and New Hampshire.

Inflorescence buds in early and late developmental stages were collected from these species for investigation of *CorLFY* expression. The early stages (Stages I–IV in Feng *et al.*, 2011) span from the initiation of the inflorescence meristems to the formation of floral organ primordia. These early developmental events occur in the fall season. The late stage (Stage V in Feng *et al.*, 2011) represents the subsequent development (or maturation) of the inflorescence and flowers in the following spring. Inflorescence buds of the late development stage were further divided into three different developmental phases: (1) bud unopened; (2) inflorescence bud opened, flower buds enlarging and bracts expanding; and (3) flower buds open and bracts fully expanded and white (in *C. canadensis* and *C. florida*). Table 1 provides the dates of inflorescence sample collections. Samples of developing young leaves were also collected from each species for analysis as a comparison with inflorescence buds. All samples of buds and leaves were stored in RNAlater solution (Ambion) immediately after their removal from the living plants to stabilize the RNA. Total RNA was extracted from bracts, flowers and young leaves using the CTAB method (Chang *et al.*, 1993). For buds in early developmental stages it was not possible to separate bracts from the flowers manually. The RNA samples obtained were used as the template for first-strand cDNA synthesis using the SuperScript III First-Strand Synthesis System kit and the supplied oligo(dT) primer (Invitrogen, Carlsbad, CA, USA). The cDNAs were used for

TABLE 1. *Dates (month/day) samples were collected for RT–PCR analysis*

Cornus species	Stage 0	Stage 1	Stage 2	Stage 3
C. florida	7/19	2/16	3/30	4/7
C. canadensis	0·8–1·5 cm	>1·5 cm		
C. officinalis	4/27	6/8	10/20	2/16
C. macrophylla	7/27	4/7	4/20	5/27
C. sanguinea	7/11	3/16	3/30	5/27
C. controversa	7/11	7/27	3/16	4/20

Stage 0, formation of IM, IBM, FM; Stage 1, inflorescence bud unopened; Stage 2, bud open, flower buds and inflorescence bracts (if present) expanding, except in *C. officinalis*, in which buds are expanding but bracts are not fully expanded; Stage 3, flower mature and bracts fully expanded. For *C. canadensis* grown in the phytotron, developmental stages were identified by morphology (size of bud) as plants in a growth chamber do not grow in synchrony with external seasons.

cloning *CorLFY* and the gene expression analyses described below.

CorLFY *gene cloning*

The cDNA from early-stage inflorescences was used to isolate *CorLFY* homologues using PCR with degenerate primers. The cDNA sequences of *CorLFY* were obtained by amplification and sequencing of three overlapping fragments (I, II and III) (see Table 2 for the primer sequences). Degenerate primers were first designed based on *LFY*-like sequences of other flowering plants from GenBank to amplify fragments I and II using PCR (Table 2). The 3′ end of *CorLFY* (fragment III) was amplified in two steps. First, an oligo-dT primer (Table 2) containing a unique adaptor sequence was used for synthesis of the first strand. The products were then used as the templates for PCR with the forward primer lfyF$_{2\text{-}3\text{-}2}$ and the reverse primer lfy3′, which were designed from the sequence of 3′RT. Unless specified, the T_m value for PCR was set at two degrees higher than the average value of the T_m of forward and reverse primers. PCR products were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). For each *Cornus* species, at least five to ten positive clones from PCR products of each fragment were sequenced. Sequencing reactions were performed using the M13 primers and DNA sequencing kit (Applied Biosystems, Warrington, UK). Sequences obtained for the three fragments were assembled manually to build the cDNA sequence based on overlapping regions of approximately 100 base pairs (bp), which showed no polymorphisms among clones in all regions, including the overlapping regions. The complete cDNA sequence for *Cornus canadensis* was also amplified as a single fragment using PCR with primers (P5LFY_COcaN GAGGG ATTGTAATTGTGTTGC and P3LFY_COca CGTACTACAG ATGATATAAGG) to confirm the sequence assembled from fragments. The cDNA sequences of *CorLFY* were aligned and translated into amino acid sequences using the translation tool implemented in Geneious v5·1 (http://www.geneious.com/; Drummond *et al.*, 2010). The N and C domains were identified in the amino acid sequences of *CorLFY* by comparison with that in *Arabidopsis* (see Results and Fig. 2). Identity of *CorLFY* was confirmed by a BLAST search using the amino acid sequence from *Cornus* as the query sequence and the

TABLE 2. *Primers designed for* CorLFY *cloning, RT-PCR and probe synthesis for* in situ *hybridization in Cornus*

PCR		Forward primer (5′–3′)	Reverse primer (5′–3′)
Gene cloning	I	$lfyL_2$ TTCWCGGCSAGYTTRTTCAAGTGGG	$lfyR_{3\text{-}1}$ ACGTAGTGYCKCATYTTS GGCTTGTTKATGT
	II	$lfyF_{2\text{-}3\text{-}2}$ GAGGTKGCRCGYGGRAAGAAGAACGG	Cornus_3R_1_yg CAGAGCTGGCGGAGCTTGGTNGGG
	RT	3′ RT CCGGATCCTCTAGAGCGGCCGC(T)17	
	III	$lfyF_{2\text{-}3\text{-}2}$ GAGGTKGCRCGYGGRAAGAAGAACGG	lfy3′ CCGGATCCTCTAGAGCGGCCGC
RT–PCR	P-I, T_m 60 °C	P_F *C. florida* TCTATGAGCAGTGCCGTGATTTCTTG	P_R *C. florida* CGTAGTGTCGCATT TTGGGCTTGTTA
		P_F *C. canadensis* TCTACGAGCAGTGCCGTGATTTCTTG	P_R *C. canadensis* CGTAGTGCCGC ATCTTGGGCTTGTTA
		P_F *C. officinalis* TCTACGAGCAGTGCCGTGATTTCTTG	P_R *C. officinalis* CGTAGTGCCGC ATTTTGGGCTTGTTA
		P_F *C. macrophylla* TCTACGAGCAGTGCCGTGATTTCTTG	P_R *C. macrophylla* CGTAGTGCCG CATTTTGGGCTTGTTA
		P_F *C. sanguinea* TCTACGAACAGTGCCGTGATTTCTTG	P_R *C. sanguinea* CGTAGTGTCGCA TCTTAGGCTTGTTA
		P_F *C. controversa* TCTACGAGCAGTGCCGTGATTTCTTG	P_R *C. controversa* CGTAGTGTCGC ATTTTGGGCTTGTTA
	26S, T_m 55 °C	12F GTCCTAAGATGAGCTC	2782R GGTAACTTTTCTGACACCTC
In situ probe	T_m 55 °C	$lfyL_2$ TTCWCGGCSAGYTTRTTCAAGTGGG	$lfyR_1$ CAACGCMCTTGATGCACWCT

non-redundant protein database on GenBank as well as by phylogenetic analyses of the *CorLFY* sequences with other *LFY* homologous sequences from Genbank.

Analyses of gene expression using PCR

Expression of *CorLFY* in early and late development of inflorescences and young leaves was investigated by PCR using the cDNA libraries derived from total RNA samples. Species-specific PCR primers (P-I) in *CorLFY* binding to exons II and III, respectively, were designed and used to amplify a fragment of 150 bp spanning exons II and III. The primer sequences are listed in Table 2. The amplicons from each species and each type of organ (i.e. leaf, bract and flower) were obtained by 30 cycles of PCR and sequenced to confirm their identity. The PCR analyses were repeated at least once for the same cDNA samples with each pair of primers for all six species. For the three species with involucral bracts (bracts appearing to subtend the entire inflorescence) (*C. florida*, *C. canadensis* and *C. officinalis*), PCR analyses were further repeated using different cDNA samples from at least one additional biological replicate. For all PCR analyses, we were able to use 26S rDNA as the internal control. Although 26S rDNA is usually not expected to be present in the cDNA libraries prepared using oligo(T) due to the lack of a poly(A) tail in 26S rRNA molecules, polyadenylation of 26S rRNA was found in *Nicotiana* shoots (Lewandowska *et al.*, 2007) and reverse transcription of 18S rRNA with poly(dT)18 and other homopolymers was successful in several diverse flowering plant species representing bryophytes, ferns, monocots and eudicots (Bogdanović *et al.*, 2013). Our sequencing results for the PCR products amplified using 26S rDNA primers confirmed the amplicon as a part of the gene. PCR controls using an RNA sample in an amount similar to that of the cDNA samples with the species-specific primers or 26S rDNA primers did not amplify a band.

Analyses of gene expression using RNA in situ *hybridization*

RNA *in situ* hybridization was used to examine the spatial expression pattern of *CorLFY* during early development of inflorescences and flowers. Young inflorescence buds at Stages I–IV were collected in summer and autumn from the six species. The sampled inflorescence buds were fixed in formaldehyde for at least 8 h at 4 °C and then dehydrated in a series of cold ethanol concentrations, permeated with an analytical grade xylene (Fisher, Fair Lawn, NJ, USA) series and embedded in Paraplast Plus (Fisherbrand, Houston, TX, USA) for sectioning, following Feng *et al.* (2012).

Before *in situ* hybridization, RNA probes were transcribed *in vitro* following the methods described in Franks *et al.* (2002) using the primers listed in Table 2. The probes were approximately 400 bp long and were derived from the first exon of *CorLFY*. The *in situ* hybridization protocol followed that of Feng *et al.* (2012), which was modified from Franks *et al.* (2002), Kim *et al.* (2005) and Souer *et al.* (2008). The tissues were sectioned at a thickness of 8 μm using a microtome and mounted on slides. The slides were treated with proteinase K (0·5 ng μl^{-1}) at 37 °C for 30 min and hybridized with species-specific probes (0·8–1·5 ng μl^{-1}) at 60 °C overnight. Slides were washed twice in 0·2 × SSC at 65 °C with agitation. Signals were detected using

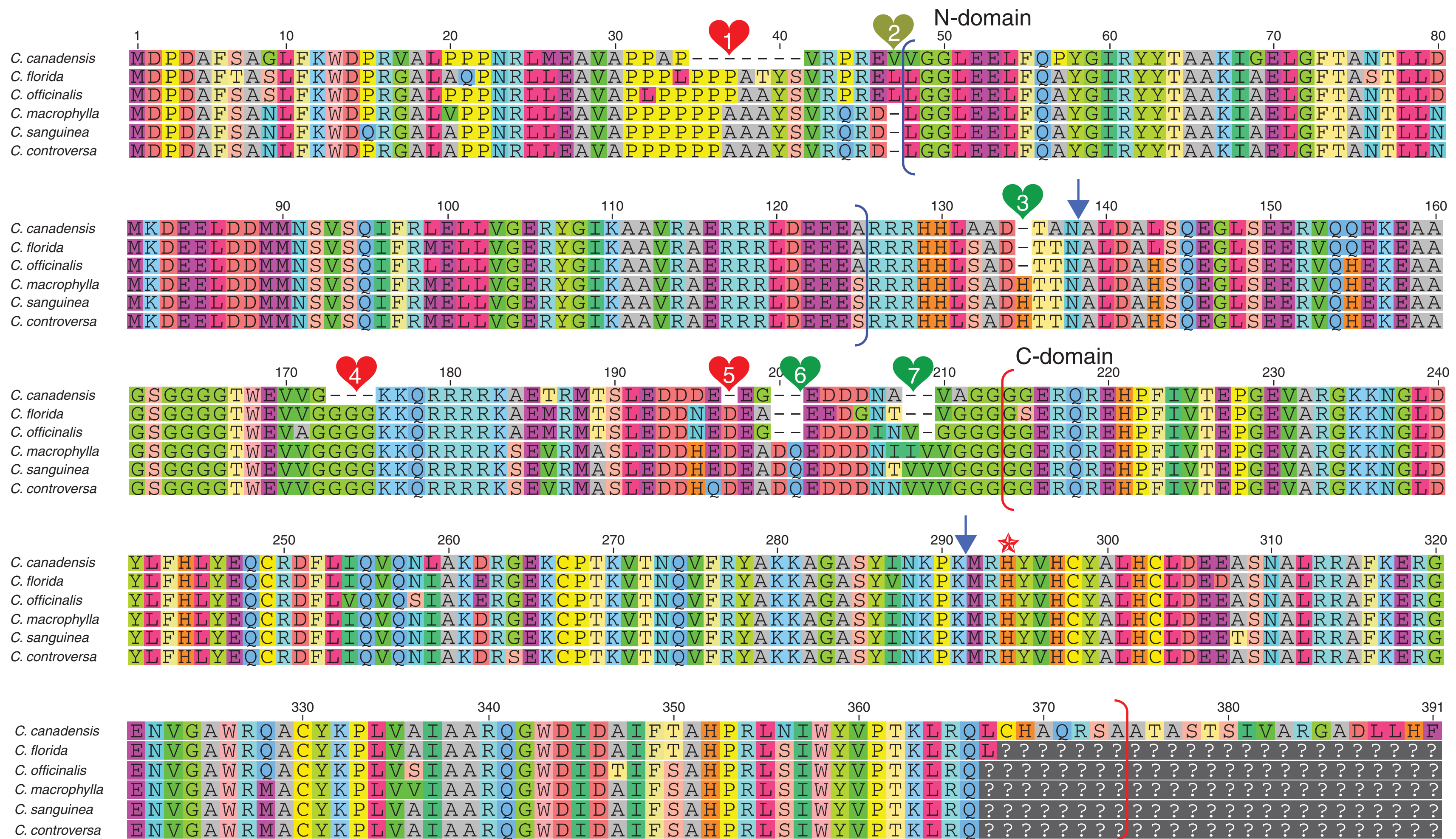

FIG. 2. Aligned amino acid sequences of *CorLFY* (*C. florida*, *C. canadensis*, *C. officinalis*, *C. macrophylla*, *C. sanguinea* and *C. controversa*). Identical amino acids are shown in the same colour. Composite numbering with gaps (dashes) is indicated at the top. Unless specified, composite numbering is used in descriptions. N-terminal domain: sites 48–126, marked by black brackets; C-terminal domain: sites 214–374, marked by red brackets. Two blue arrows indicate the boundary between exons 1 and 2 and the boundary between exons 2 and 3, respectively. The seven numbers (1–7) shown within coloured heart symbols indicate the seven indels. Red heart symbols represent deletions in *C. canadensis*, the yellow heart symbol represents a deletion in the BW group (*C. macrophylla*, *C. sanguinea* and *C. controversa*) and green heart symbols represent deletions shared by the DW (*C. canadensis*), BB (*C. florida*) and CC (*C. officinalis*) groups and by the DW (*C. canadensis*) and BB (*C. florida*) groups. The red star indicates the conserved histidine residue (His at site 294 of *CorLFY*) in the C-terminal domain, which was predicted to play an essential role in DNA-binding activity (Maizel *et al.*, 2005). The complete coding region was obtained only in *C. canadensis*; question marks indicate missing data in the other species.

Western blue (Promega, Madison, WI, USA). The *in situ* experiments for each probe were carried out at least twice with several biological replicates (different inflorescence buds) in each experiment. Both positive and negative controls were included in each experiment. We used the antisense probes of *CorAP3* from Feng *et al.* (2012) as positive control (data not shown). A sense strand probe was used as a negative control.

Gene and protein sequence analyses

We performed phylogenetic analyses to determine the evolutionary relationships of *CorLFY* genes and identify evolutionary changes in gene sequences that may be correlated with the gene expression pattern and inflorescence architectures. The phylogenetic analyses were conducted for both the cDNA sequences and the amino acid sequences of *CorLFY* with and without inclusion of some *LFY*-like sequences from the asterid and rosid clades (Table 3). These sequences were randomly selected among the completed cDNA pool of *LFY* in GenBank. The sequences were first downloaded from NCBI, then translated to the predicted amino acid sequences and aligned in Geneious v5·1 (http://www.geneious.com/; Drummond *et al.*, 2010), followed by manual adjustment. The *Cornus LFY* sequences were manually added to the matrix. The final matrices, with and without inclusion of some *LFY*-like sequences from the asterid and rosid clades respectively, contained 1320/1101 bp, 440/367 amino acids (gaps included) and 19/6 taxa. The cDNA sequence matrices were further adjusted based on the protein sequence alignments. Phylogenetic analyses were conducted using the maximum likelihood (ML) methods implemented in RAxML-HPC v7.2.8 (available at http://www.phylo.org/; Miller *et al.*, 2010). The ML analyses for cDNA matrices were performed using the GTR model with a gamma distribution for site variation, all parameter values as 'estimated' and rapid bootstrapping of 500 replicates. The JTT model was selected as the best model in the ML analyses of the protein sequences. Trees from ML analyses were rooted using the six rosid species and viewed and edited using MEGA 4.0.2 (Tamura *et al.*, 2007). Analyses including only *CorLFY* were not rooted.

TABLE 3. *Taxon sampling for phylogenetic analysis*

Lineage	Order	Family	Species
Rosids	Cornales	Cornaceae	*Cornus florida*
	Cornales	Cornaceae	*Cornus canadensis*
	Cornales	Cornaceae	*Cornus officinalis*
	Cornales	Cornaceae	*Cornus macrophylla*
	Cornales	Cornaceae	*Cornus sanguinea*
	Cornales	Cornaceae	*Cornus controversa*
	Ericales	Ericaceae	*Impatiens balsamina*
	Lamiales	Phrymaceae	*Mimulus lewisii*
	Lamiales	Phrymaceae	*Mimulus guttatus*
	Lamiales	Plantaginaceae	*Antirrhinum majus*
	Solanales	Solanaceae	*Nicotiana tabacum*
	Asterales	Asteraceae	*Helianthus annuus*
	Asterales	Asteraceae	*Chrysanthemum morifolium*
	Solanales	Solanaceae	*Solanum tuberosum*
	Lamiales	Scrophulariaceae	*Buddleja davidii*
Asterids	Fagales	Fagaceae	*Castanea mollissima*
	Fagales	Juglandaceae	*Juglans regia*
	Rosales	Rosaceae	*Cydonia oblonga*
	Rosales	Rosaceae	*Pyrus communis*

RESULTS

Sequence characteristics of LFY *homologues in* Cornus

The full-length cDNA sequence of *CorLFY* was obtained in *C. canadensis* (*C. canaLFY*) and cloned from products of single PCR amplification. For the other five species (*C. florida*, *C. officinalis*, *C. macrophylla*, *C. sanguinea* and *C. controversa*), approximately 70 bp of the 3′ end of the cDNA coding sequences were missing (*C. floLFY*, *C. offiLFY*, *C. macroLFY*, *C. sanLFY* and *C. conLFY*) due to failure to obtain the 3′ end of the coding sequence in PCR products using the 3′ RACE approach. Sequencing of multiple clones of PCR products from different species (see Materials and methods) did not detect paralogous sequences (no polymorphisms among clones were observed), indicating that *CorLFY* is likely to be a single-copy gene in *Cornus*. In *C. canadensis*, the *CorLFY* cDNA coding sequence was 1125 bp long and predicted to encode a protein of 375 amino acids that shared extensive sequence similarity with the *Arabidopsis* LFY protein. Three exons were predicted based on sequence similarity and conserved splicing sites in *LFY* of *Arabidopsis* and confirmed by sequencing of genomic DNA in *C. florida.* The sizes of the three predicted exons are shown in Table 4. The two conserved domains present in other LFY-like proteins were also found in *CorLFY*, with the N-terminal domain spanning from amino acid 48 to amino acid 126 and the C-terminal domain from site 214 to site 374 (Fig. 2). In addition, *CorLFY* was characterized by a proline-rich region (amino acid residues 1–47) and a highly acidic region (amino acid residues 80–88) within the N-terminal domain (amino acid residues 1–138), and another highly acidic region (amino acid residues 192–205) in the second exon (amino acid residues 139 to site 291) (Fig. 2). Thirteen of the 47 amino acid sites were prolines in the proline-rich region. Compared with the conserved N- and C-terminal domains, the proline-rich region and highly acidic region in the interdomain were more variable among *Cornus* species and contained seven indels (designated indels 1, 2, 3, 4, 5, 6 and 7 in Fig. 2). Three of the seven indels (1, 4 and 5) occurred uniquely in *C. canadensis* (DW group). Three other indels (3, 6 and 7) were shared by

TABLE 4. *Variation in sizes (bp) of the three predicted exons for* CorLFY *from different species*

Species	First exon	Second exon	Third exon*	Accession number
Cornus canadensis L. f.	414	347	364	KC332279
Cornus florida L.	435	359	294+ ?	KC332280
Cornus officinalis Seib. & Zucc.	435	362	290+ ?	KC332275
Cornus macrophylla Wall.	435	365	290+ ?	KC332278
Cornus sanguinea L.	435	371	290+ ?	KC332276
Cornus controversa Hemsl.	435	371	290+ ?	KC332277
Amino acid sites in Fig. 2	1–138	139–291	292–391	

* Values followed by '?' are estimated and may range up to approximately 70 bp larger.

C. canadensis (DW group), *C. florida* (BB group) and *C. officinalis* (CC group). Finally, indel 2 was a deletion of one amino acid (site 47) shared by the three species of the BW group (*C. macrophylla*, *C. sanguinea* and *C. controversa*).

Expression of CorLFY *detected by PCR analysis*

PCR using the species-specific primer sets (P-I) for the cDNA libraries derived from total RNAs resulted in a single DNA amplicon approximately150 bp in length in all six species during early and late inflorescence development (Fig. 3). The P-I primer pairs span the junction between exons 2 and 3 in the *CorLFY* homologues. The P-I primer pairs detected expression in young leaves, developing bracts, flowers and stage 0 inflorescence samples by PCR of 30 cycles (Fig. 3). However, due to the limited number of biological replicates and the non-quantitative nature of our assay we cannot rule out the possibility of differences in the expression levels of *CorLFY* homologues that we did not detect by the PCR analyses, either between species or within species, of different tissue or developmental samples.

Expression of CorLFY *detected by RNA* in situ *hybridization*

Our *in situ* hybridization experiments detected expression of *CorLFY* in the developing inflorescence meristem (IM in Fig. 4A, B, G, K), inflorescence branch meristem (IBM in Fig. 4C, D, H, L, N, Q), in the floral meristem (FM in Fig. 4I, J, M, O) and during early floral organogenesis (Fig. 4E, F, I, J, P, R) of all inflorescence types except the depauperate inflorescences of *C. canadensis*. Due to lack of materials, we were only able to observe expression in floral meristems and floral organ primordia of *C. canadensis* (Fig. 4K). For the three BW species, one or two of these stages are also missing due to lack of material (i.e. floral organ stage for *C. macrophylla*, IM stage for *C. sanguinea*, and IM and FM for *C. controversa*; the stages not shown in Fig. 4). Furthermore, we could not examine *CorLFY* expression at the earlier stages (i.e. when apical meristems are still vegetative) due to difficulty obtaining these samples. The *in situ* hybridization results obtained showed no apparent differences in the expression patterns of *CorLFY* among the head-like, umbel-like inflorescences and elongated inflorescence types found in the species studied. Sense-strand negative control probes were used to estimate the levels of non-specific background signal (Fig. 4A–F).

Gene genealogy and evolutionary changes

In rooted analysis of cDNA and protein sequences including *Cornus* and other taxa, the *CorLFY* sequences formed a well-supported monophyletic group closely allied with *LFY*

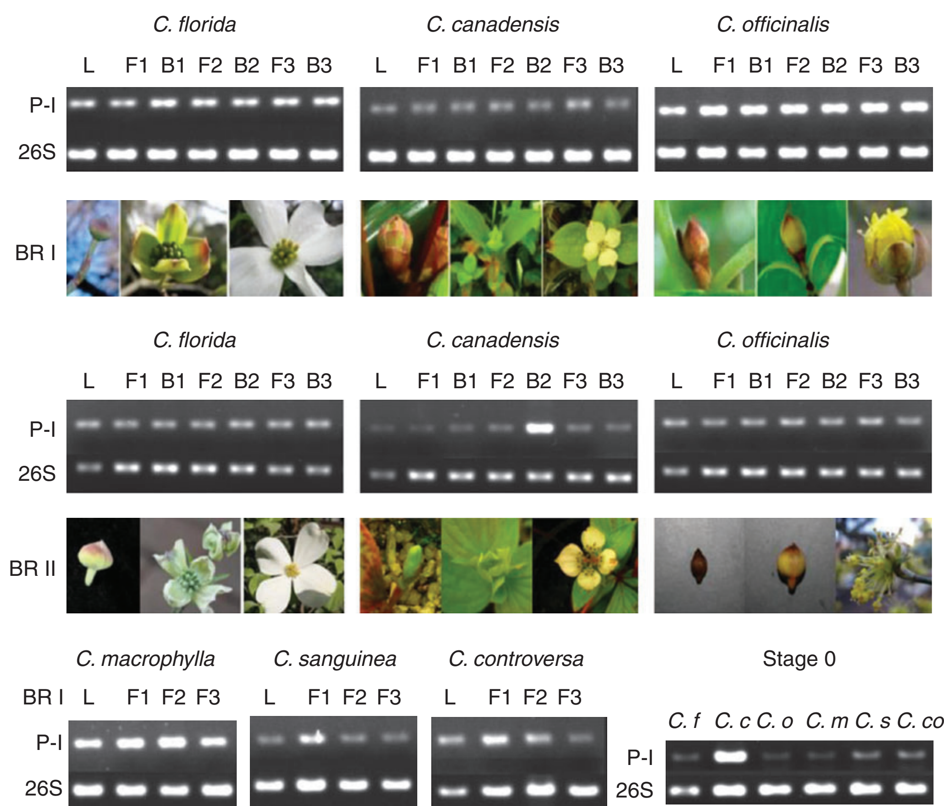

FIG. 3. Expression of *CorLFY* in late developmental stages of *Cornus* bracts and flowers and young leaves detected by RT–PCR. P-I, RT-PCR using six different pairs of species-specific primers developed for each *Cornus* species. BR I and BR II indicate two biological replicates; BR II included only the three species with involucral bracts (*C. florida*, *C. canadensis* and *C. officinalis*). For description of the three developmental stages, refer to Table 1. Inflorescence images represent the three stages analysed and are modified from Feng *et al.* 2012. L, leaf; B1–B3, three late developmental stages of bracts representing unopened bracts, expanding bracts and whitened bracts, respectively; F1–F3: three late developmental stages of flowers associated with the three bract developmental stages. 26S rDNA was used as the internal control. The cDNA concentrations used for PCR were the same as those for 26S, both of which were run at 30 cycles.

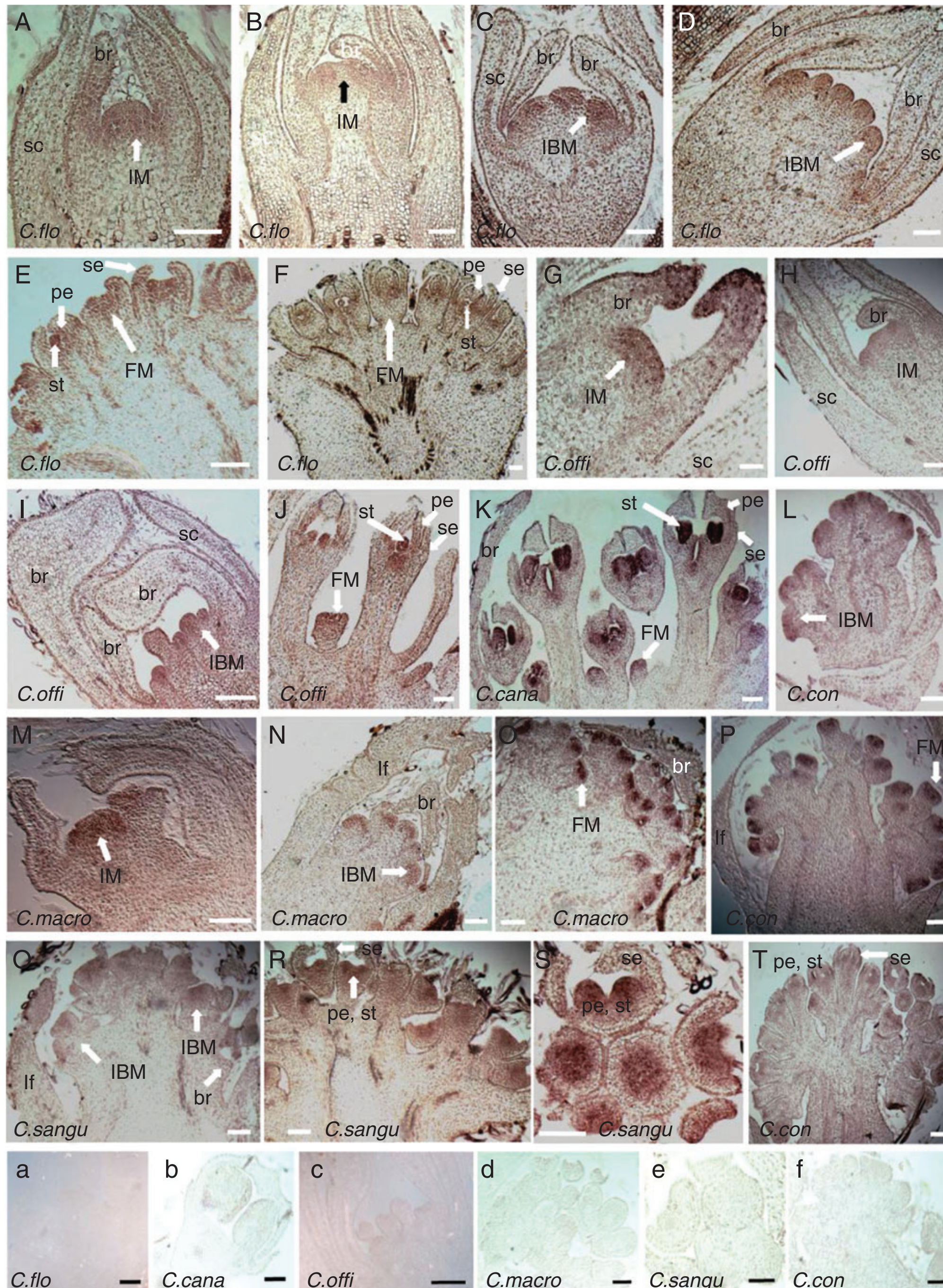

FIG. 4. Expression pattern of *CorLFY* in early developmental stages of *Cornus* inflorescences detected by *in situ* RNA hybridization. (A–F) Expression of *CorLFY* in developing inflorescence primordia (IM, IBM, FM, br, se, pe, st) of *C. florida*. (G–J) Expression of *CorLFY* in developing inflorescence primordia (IM, IBM, FM, br, se, pe, st) of *C. officinalis*. (K) Expression of *CorLFY* in developing inflorescence primordia (FM, se, pe, st) of *C. canadensis*. (M–O) Expression of *CorLFY* in developing inflorescence primordia (IM, IBM, FM) meristems of *C. macrophylla*. (L, P, T) Expression of *CorLFY* in developing inflorescence meristems (IBM, FM, se, pe, st) of *C. controversa*. (Q–S) Expression of *CorLFY* in developing inflorescence primordia (IBM, FM, br, se, pe, st) of *C. sanguinea*. (a–f) Negative control of *CorLFY* expression in the six *Cornus* species: (a) inflorescence meristem of *C. florida*, comparable to (E); (b) young flower bud of *C. canadensis*, comparable to one of those in (K); (c) inflorescence branch meristems, comparable to stage in (I); (d) young flower bud of *C. macrophylla*, comparable to stage in (O); (e) young flower bud of *C. sanguinea*, comparable to stage in (S); (f) floral meristem of *C. controversa*, comparable to stage in (P). VM, vegetative meristem; IM, inflorescence meristem; IBM, inflorescence branch meristem; FM, flower meristem; *C. flo*, *C. florida*; *C. cana*, *C. canadensis*; *C. offi*, *C. officinalis*; *C. macro*, *C. macrophylla*; *C. sangu*, *C. sanguinea*; *C. con*, *C. controversa*; lf, leaf; br, bract; se, sepal; pe, petal; st, stamen; sc, scale. Scale bars = 100 μm.

homologues from Solanales and Lamiales, respectively (Fig. 5A, B). The *LFY*-like genes from Solanaceae, Lamiales, Asteraceae and Rosids formed monophyletic clades, but relationships among these clades and *Cornus* remained unclear (weakly supported) (Fig. 5A, B). However, within the *Cornus* clade, relationships among the BB, DW, CC and BW lineages were different

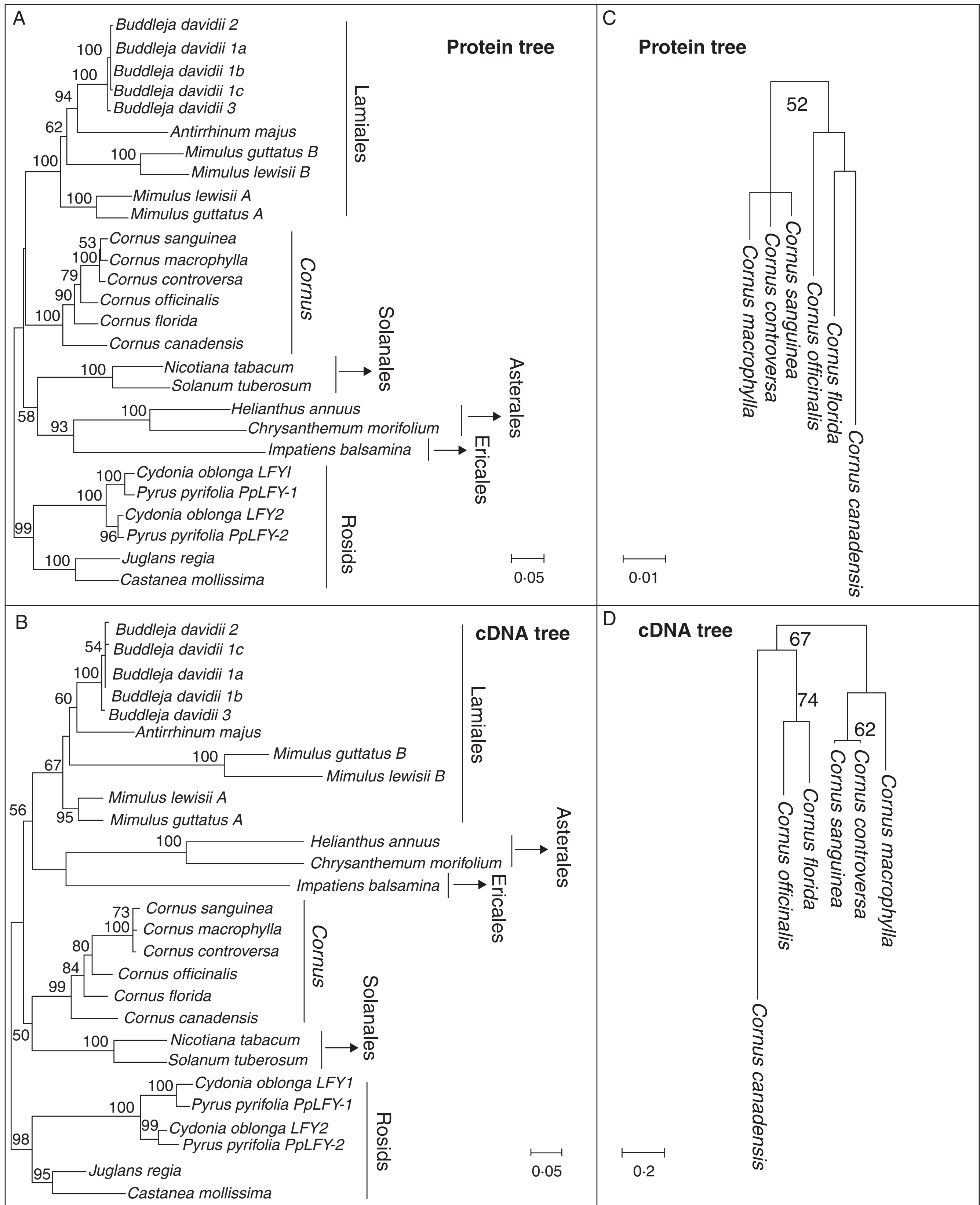

FIG. 5. Phylogram of *LFY* orthologues resulting from ML analyses of cDNA sequences and amino acid sequences. Numbers at nodes are bootstrap supports from ML analyses. Only bootstrap support ≥50 % is shown (italicized numbers). (A, B) Rooted trees for (A) proteins and (B)cDNA. (C, D) Unrooted trees for (C) proteins and (D) cDNA.

from those found in phylogenetic studies of *Cornus* using several molecular markers from plastic and nuclear genomes that included many to all species (Fan and Xiang 2003; Xiang *et al.*, 2006, 2008; Xiang and Thomas, 2008), which showed (BW(CC(BB, DW))). The *CorLFY* cDNA and protein phylogenies, in contrast, showed (DW(BB(CC,BW))) (Fig. 5A, B). This incongruence could be an artefact of the distant outgroup rooting, ambiguity in alignment between *Cornus* and the divergent outgroup taxa in variable portions of the interdomain region, or due to other unknown reasons. The sister of *Cornus*, Alangiaceae, and all other families of Cornales were missing in the phylogenetic analyses due to lack of data. Unknown factors in the molecular evolution of *LFY* may also contribute to this conflict, e.g. selection and functional constraint. A number of amino acid sites support a closer relationships of BB, CC and DW, as found in previous phylogenetic studies, but a few amino acid changes were shared by the BB and CC species, which bear head-like and umbel-like inflorescences, respectively (i.e. site 37 in indel 1 and site 47 in indel 2; Fig. 2). These changes support a closer sister relationship between BB and CC and are in agreement with the lack of elongation of IM and IBMs in these two lineages, but a closer relationship between lineages BB and CC is in conflict with the previous phylogeny, which showed a closer relationship between BB and DW.

Results of the unrooted phylogenetic analyses of cDNA and protein sequences including only *CorLFY* were consistent with the observed pattern of protein sequence variation described above (Fig. 5C, D). The alignment of amino acid sequences of *CorLFY* also showed that variation at a number of sites is correlated with the divergence of lineages with different inflorescence types (Fig. 2). Three deletions, of sites 35–41, 173–175 and 197, and 10 other unique substitutions were observed in *C. canadensis* of the DW group (four small minidichasia). Eight, six and 15 substitutions were observed for *C. florida* (head-like inflorescence), *C. officinalis* (umbel-like inflorescence) and the BW group with thyrsoids with elongated internodes, respectively. These substitutions can be observed in the aligned matrix shown in Fig. 2.

DISCUSSION

Expression of CorLFY *during inflorescence development and the evolution of* Cornus *inflorescence architectures*

Flowering plants exhibit a diverse array of inflorescence architectures. Despite the importance of the inflorescence in angiosperm reproduction and evolution, little progress has been made in understanding the developmental-genetic bases of inflorescence evolution. This is largely because the genetic programme controlling inflorescence development is complicated and remains unknown in most species. Nonetheless, available data have supported key roles of *LFY* and *TFL1* in structuring racemes and cymes (Souer *et al.*, 1998, 2008; Molinero-Rosales *et al.*, 1999; Ahearn *et al.*, 2001; Jack, 2004; Conti and Bradley, 2007; Prusinkiewicz *et al.*, 2007; Koes, 2008; Thouet *et al.*, 2012).

By *in situ* hybridization and PCR on cDNA clones, we detected expression of *CorLFY* mRNA in the IM, IBMs, FMs and developing floral organs in head-like inflorescences, umbel-like inflorescences and elongated inflorescences of different *Cornus* species examined (Figs 3 and 4). This expression of *CorLFY* is consistent with the expectation that *CorLFY* plays a role in the specification of inflorescence and floral meristem identity in *Cornus*. However, our experiments did not show any differences in the levels or patterns of *CorLFY* expression between the three different inflorescence types in *Cornus* that might have helped us to define the molecular mechanisms that underlie the divergent morphologies. A lower level of *CorLFY* activity in IM and emerging IBMs might be expected in the species with more highly branched inflorescence architectures and elongated inflorescence branches, as has been demonstrated in petunia and tobacco (Souer *et al.*, 1998; Amaya *et al.*, 1999; Ahearn *et al.*, 2001). Thus, one might predict that lower levels of *CorLFY* activity would be functioning in the IM and IBMs of the BW species (e.g. *C. macrophylla*, *C. controversa* and *C. sanguinea*) producing elongated inflorescences and more highly branched inflorescences, relative to those species producing head-like or umbel-like inflorescences (e.g. *C. florida* and *C. officinalis*). Further experiments are needed to test this hypothesis. Although the nature of our experiments did not allow us to observe differences in the *CorLFY* expression patterns in these different inflorescence types, it remains possible that quantitative differences in expression level exist among the species. It is also possible that the *CorLFY* gene products are regulated at a post-transcriptional level, and differences in *CorLFY* sequences among the *Cornus* species may play a role at this level. Additionally, the differential development of these inflorescence types could also be attributed to functions of TFL1 or other proteins (e.g. UFO homologues).

In petunia and tomato, the expression of floral meristem identity was found to be regulated via transcription of *DOUBLE TOP* (DOT)/*ANANTHA*, a homologue of *UNUSUAL FLORAL ORGANS* (*UFO*) rather than by the transcription of *LFY* (Souer *et al.*, 2008). *DOT* was found to activate *ALF* (homologue of *LFY*) by a post-translational mechanism in petunia (Souer *et al.*, 2008). The study by Souer *et al.* (2008) suggested that divergent expression patterns of *LFY* and *UFO* homologues supported differential spatiotemporal control of floral identity in these distinct species. The expression data we present here suggest that either (1) the expression of *CorLFY* at the transcription level does not play a key role in regulating the evolutionary shift from elongated, highly branched inflorescences to condensed head-like and umbel-like inflorescences in *Cornus*, or (2) the transcription level of *CorLFY* does play a role but at a quantitative level that was not detected in this study. The divergence in spatiotemporal control of floral identity in different dogwood lineages may also involve differences in the activity of the CorLFY protein or the expression or activities of *CorTFL1* and *CorUFO*. We hope to test this in the future using the genetic transformation system we have established in *C. canadensis* (Liu *et al.*, 2013).

Sequence characterization and evolution of CorLFY

LFY homologues exist as two copies in gymnosperm species (Mouradov *et al.*, 1998; Mellerowicz *et al.*, 1998; Frohlich and Parker, 2000) and as a single copy in most angiosperms (Coen *et al.*, 1990; Weigel *et al.*, 1992; Souer *et al.*, 1998; Molinero-Rosales *et al.*, 1999; Kyozuka *et al.*, 1998; Rottmann *et al.*, 2000; Carmona *et al.*, 2002; An *et al.*, 2011). The fact that two or more copies of *LFY* homologues found in a number

of distantly related angiosperm lineages, such as *Ionopsidium acaule* (violet cress; Shu *et al.*, 2000) and *Idaho ascapigera* (Sliwinski *et al.*, 2007) (Brassicaceae), *Nicotiana* (Ahearn *et al.*, 2001) (Solanaceae), several fruit tree species of Rosaceae (Wada *et al.*, 2002; Esumi *et al.*, 2005), *Allium sativum* (Rotem *et al.*, 2007) (Alliaceae) and *Zea mays* (Bomblies *et al.*, 2003) (Poaceae), demonstrates that duplication of *LFY* occurred multiple times during angiosperm evolution. Our sequencing of cloned PCR products (>20 clones from the three fragments) from degenerate primers (designed from aligned sequences of asterids) failed to detect any paralogous sequences within a species, indicating that *CorLFY* likely exists in *Cornus* as a single copy, as it does in most angiosperms.

Like most *LFY* homologues found in other angiosperm species, the six *CorLFY* genes were predicted to have three exons and the predicted proteins have two conserved regions, an N-terminal domain and a C-terminal domain (Fig. 2). The predicted amino acid sequences of *CorLFY* share some other motifs with previously characterized *LFY* proteins, including a proline-rich region and a highly acidic region in the second exon (Fig. 2). The proline-rich region is well conserved in *LFY* homologues of angiosperms but is not well conserved in those of gymnosperms (Frohlich and Meyerowitz, 1997; Carmona *et al.*, 2002). However, the proline-rich motif was later found to be absent from *FLO*/*LFY* homologues of some angiosperm species, such as *Malus domestica* (apple; Wada *et al.*, 2002) and *Eucalyptus* (Dornelas *et al.*, 2004). In *Eucalyptus*, homologues lacking the proline-rich region are able to restore the wild type phenotype of *lfy* mutants, which suggests that the proline-rich region may not be functionally significant in this species (Dornelas *et al.*, 2004). Maizel *et al.* (2005) have demonstrated that the C-terminal domain of LFY functions as a DNA-binding domain. In the present study, we also observed a conserved histidine residue in the C-terminal domain of *CorLFY* (His at site 294; Fig. 2) previously shown to be important for LFY DNA-binding activity (Maizel *et al.*, 2005). A number of amino acid changes were observed in the C domain (Fig. 2). It is unclear whether these changes may affect the role of the protein in its regulatory function on inflorescence development in the different dogwood lineages. In addition to the proline-rich and DNA-binding domains described above, there is a highly acidic region in the N-terminal domain of the first exon of *CorLFY*. Our alignment of *CorLFY* with previously identified LFY-like proteins confirmed that the N-terminal domain is conserved throughout angiosperm *LFY* homologues (data not shown).

Among the six *CorLFY* sequences, many changes in the gene and protein sequences were found, with the *C. canadensis* sequences showing the greatest number of changes (Fig. 2). These changes were dispersed in different regions, with the indels restricted to the interdomain and proline-rich regions, while amino acid substitutions were observed in all regions.

Expression of CorLFY *in vegetative organs and in flowers*

Expression of *LFY* homologues in the vegetative shoot apical meristem and leaf primordia has previously been reported in many angiosperms, such as *Nicotiana tabacum* (Kelly *et al.*, 1995), *Arabidopsis* (Blázquez *et al.*, 1997; Hempel *et al.*, 1997), *Impatiens* sp. (Pouteau *et al.*, 1997), *Solanum lycopersicum* (Molinero-Rosales *et al.*, 1999), *Ionopsidium acaule* (Shu *et al.*, 2000), *Populus* (Rottmann *et al.*, 2000), *Malus domestica* (Wada *et al.*, 2002), *Carica papaya* (Yu *et al.*, 2005), *Idahoa* (Sliwinski *et al.*, 2007), *Silene* (Allnutt *et al.*, 2007), *Oryza sativa* (Rao *et al.*, 2008) and *Dendranthema* (Ma *et al.*, 2008), which suggests a role of *LFY* in leaf organogenesis. However, the exact mechanism is not yet completely understood. Li *et al.* (2013) demonstrated that *LFY* stimulates the formation of floral and leaf primordia through auxin-regulated pathways. Expression of *LFY* homologues in developing leaves has also been reported in many investigated angiosperm species (Kelly *et al.*, 1995; Bradley *et al.*, 1997; Blázquez *et al.*, 1997; Hofer *et al.*, 1997; Pouteau *et al.*, 1997; Molinero-Rosales *et al.*, 1999; Rottmann *et al.*, 2000; Souer *et al.*, 1998; Southerton *et al.*, 1998; Walton *et al.*, 2001; Carmona *et al.*, 2002; Dong *et al.*, 2005; Ma *et al.*, 2008), including *Cornus* in this study (Fig. 3). In pea and tomato plants, *lfy* mutants showed changes in leaf morphology, confirming the function of *LFY* homologues in leaf development (Hofer *et al.*, 1997; Molinero-Rosales *et al.*, 1999). Interestingly, expression of *LFY* homologues in vegetative meristems was not detected in the snapdragon (Coen *et al.*, 1990), rubber tree (Dornelas and Rodriguez, 2005) and *Populus tomentosa* (An *et al.*, 2011). However, the lack of expression of *LFY* homologues in vegetative meristems of rubber tree was attributed to a season-dependent mechanism that regulates its expression (Dornelas and Rodriguez, 2005). Seasonally dependent expression of *LFY* homologues has also been reported in other woody species, such as kiwifruit, grape and apple (Walton *et al.*, 2001; Carmona *et al.*, 2002; Wada *et al.*, 2002; Almada *et al.*, 2009). Therefore, *LFY* homologues may have a role in leaf development that is largely conserved across angiosperm lineages. It has been suggested that *LFY* orthologues have a general role in regulating indeterminacy in lateral primordia derived from apical meristems, and that this role may reflect an ancestral function of the gene (Hofer *et al.*, 1997).

LFY-like genes have been found to play key roles in the transition from inflorescence meristem to floral meristem in a diversity of plants (Weigel *et al.*, 1992; Weigel and Nilsson, 1995; Blázquez *et al.*, 1997; Nilsson *et al.*, 1998; Molinero-Rosales *et al.*, 1999; Pidkowich *et al.*, 1999; Peña *et al.*, 2001; Wada *et al.*, 2002; Bomblies *et al.*, 2003; Kim *et al.*, 2005; Zhang *et al.*, 2010). In our study, expression in floral meristems and during floral organogenesis was observed for all species studied (Figs 3 and 4). These results and the expression of *CorLFY* throughout the development of inflorescences are consistent with the expectation that activity of *CorLFY* is likely required in floral and inflorescence development in *Cornus*, as has been suggested in other plants.

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