ABSTRACT

ADKINS, JEFFREY ALAN. Characterization and expression of *FLORICAULA/LEAFY* homologues in *Buddleja davidii* (Under the direction of Dr. Dennis J. Werner.)

Over a decade of intense research efforts, primarily in the model plants *Arabidopsis thaliana*, *Antirrhinum majus* and *Petunia ×hybrida*, have augmented our understanding of physiological and anatomical processes in flower development with insights into their molecular underpinnings. Elucidation of sequences and functions of numerous genes and gene products involved in floral induction and development has added to our overall understanding of the molecular genetic control of meristematic phase change and inflorescence development in flowering plants. Insights into these components of plant development have the potential to greatly impact our ability to modify and cultivate plants for the nutritional, economical, social and emotional benefit of humans.

FLORICAULA (*FLO*) in *Antirrhinum majus* and *LEAFY* (*LFY*) in *Arabidopsis thaliana* are floral meristem identity genes that signal the transition from indeterminate inflorescence meristems to determinate floral meristems. *LFY* is expressed in both vegetative and reproductive tissues, and low expression during the vegetative phase prevents premature flowering. *LFY* encodes a DNA-binding transcription factor shown to localize to the nucleus and interact directly with floral organ development genes. Upregulation of *FLO/LFY* serves as a reliable indicator of the transition to a floral meristem from an inflorescence meristem with the associated cessation of further shoot elongation. With this in mind, it is clear that spatial and temporal expression of *LFY* plays a central role in the degree of inflorescence branching.

Buddleja, a cosmopolitan taxon of roughly 100 species, provides a unique model for studying inflorescence development at the molecular level. Great diversity in inflorescence architecture exists among *Buddleja* species, and numerous hybrids exist between and among these taxa. Breeding goals have included enhancement of floral architecture through increased panicle branching and total flowers per inflorescence. The *B. davidii* inflorescence is an indeterminate panicle of racemes, and several clones exhibiting enhanced inflorescence branching are known.

Homologues of *FLO/LFY* have been isolated from *B. davidii* in an effort to facilitate our understanding of the molecular contribution to inflorescence branching. Five full-length cDNAs were identified as *FLO/LFY* homologues. Although *FLO/LFY* homologues exist as a single copy in most diploid higher plants, we anticipated finding cDNAs representing at least two gene copies in the tetraploid *B. davidii*. Nucleotide sequence identity among the five clones was at least 96%. Three clones shared 100% identity at both the nucleotide and deduced amino acid sequence level with the exception of gaped regions. These three appear to represent alternative splice forms of a single allele (*BdFL1*α, *BdFL1*β and *BdFL1*γ) and two others (*BdFL2* and *BdFL3*) represent separate alleles. Nucleotide sequence homology of *BdFL* clones was 86% to 88% with *FLO* and 62% to 68% with *LFY*.

Five unique cDNA isoforms of *FLO/LFY* homologues were isolated from *Buddleja davidii*. Analysis of the nucleotide and presumed amino acid sequences suggest that the five cDNAs are products of at least two different coding sequences. In addition, three of the five may be due to alternative splicing based on comparisons to similar isoforms in *Arabidopsis*. One additional clone is unique due to the absence of a proline-rich region near the N-terminal that is common among most *FLO/LFY* homologues reported to date. Expression analysis of

the *Buddleja FLO/LFY* homologues showed similar expression patterns in seven different samples among four of the five clones. A fifth clone was undetectable in any of the samples.

CHARACTERIZATION AND EXPRESSION OF *FLORICAULA/LEAFY* HOMOLOGUES IN *BUDDLEJA DAVIDII*

by
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DEDICATIONS

I dedicate this to my wife Carrie and my beautiful little ladies:

Miranda, Piper, Meadow and Willow

BIOGRAPHY

Jeffrey A. Adkins grew up in an Atlanta, Georgia suburb the youngest of five children. Following high school, he worked for ten years in the landscape and nursery industry. Jeff served as the Assistant to the Director of the Criminal Justice Task Force in Atlanta, Georgia and the National Conference on Crimes Against Children in Washington, D.C. where he met his future wife Carrie who was a conference volunteer. Following the birth of their first child, Miranda, Jeff continued his formal education in earnest. He earned a B.S.A. degree in Horticulture from the University of Georgia in 1998 followed by an M.S. degree in Horticulture in 2001 under the direction of Dr. Michael Dirr. During his time at the U. of Georgia, two additional daughters, Piper and Meadow, were added to the family. Seeking new challenges in research and academics, he enrolled in a doctoral program in Horticultural Science at North Carolina State University. A fourth daughter, Willow, was born soon after arriving in Raleigh. In February, 2004 Jeff was offered and accepted the position of Assistant Professor of Horticulture and Molecular Biology at the University of Rhode Island in Kingston, Rhode Island. At URI, Jeff will teach Plant Propagation and other horticulture courses and conduct horticulture related research in molecular biology and breeding. Jeff and his wife Carrie currently live in Wakefield, RI with their four daughters.

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Time and personal circumstances prevented me from developing as many meaningful interactions as I would have liked. However, there are those whose involvement, encouragement and patience made my program and overall experience at NC State gratifying. Dr. Dennis Werner's willingness to support my research for the sake of scientific intrigue was critical, and I thank him for that. I also appreciate the willingness of Drs. Steve Clouse, Ralph Dewey and Tom Ranney to serve as committee members and for their genuine interest in my personal and professional success. I particularly appreciate the guidance and support of Dr. John Williamson. A late comer to the committee, he was a vital resource from the beginning. A master of devils advocacy, Dr. Williamson helped to pry loose ideas when the research got stuck. Thanks also to my fellow graduate students for the warm and friendly environment to work in day to day, and thanks to Ms. Rachel McLaughlin for the always smiling face and willingness to assist.

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CHAPTER 1

Introduction

Floriculture and nursery crops are important agricultural commodities worldwide. In the United States, cash receipts from nursery and floriculture enterprises increased about 34% between 1991 and 1998 (U.S.D.A., 1999), and demand for new and improved varieties has increased concomitantly. Genetic variation within ornamental species is often limited compared with other agricultural crop species presenting challenges for breeders and plant collectors. This limited variation is most evident with non-native taxa that often originate from only a few genotypes. Plants originating from foreign expeditions are often cultivated in small numbers at select universities, arboreta, and botanic gardens. Typically, these plants are directly introduced to industry following evaluation, or seedlings are cultivated and evaluated for potential introduction. In either case, limited genetic backgrounds often reduce their usefulness for breeding. For this reason, characterization of genetic diversity is an essential component of many breeding programs.

Finances, time, and increasingly stringent import regulations make large scale germplasm expansion of exotic plants impractical. However, DNA fingerprinting technologies such as restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLP) have increased our ability to assess genetic diversity and has allowed breeders to take full advantage of intra- or interspecies genetic potential. Furthermore, the ability, through genetic engineering, to reach beyond species or even family levels to different kingdoms for genetic variation offers seemingly endless potential for developing novel traits in plants.

Biological and mechanical systems for incorporating novel genes into diverse

organisms have already greatly impacted agricultural crops through transgenic expression of genes conferring beneficial traits. Additionally, rapid improvement of techniques for isolating and characterizing novel genes in model organisms, and the development of sequence databases provide enormous resources for scientific inquiry. In addition to crop improvement, molecular analysis of genetic diversity among divergent organisms has increased our basic understanding of gene conservation and evolution. Genetic diversity and novel sources of genetic variation are the focus of research reported here.

Buddleja History, Distribution, and Taxonomy

The genus *Buddleja* L. (syn. Buddleia) Buddlejaceae, formerly Scrophulariaceae and Loganiaceae, has received significant attention from horticulturists and botanists for its unique garden qualities and intriguing phylogeny. New World species possess yellow, orange, or white often sweetly scented flowers. Old world species flower colors are typically white or many shades of pink, purple or blue. *Buddleja*, a cosmopolitan taxon native to South Africa and Madagascar, southeast Asia and North and South America, is comprised of about 100 species (Moore, 1960; Norman, 2000). The largest area of intraspecific diversity, found in North and South America in tropical, sub-tropical and temperate regions, includes 63 native tree and shrub species. Additionally, Norman (2000) lists four Old World adventives: *B. davidii* Franch., *B. lindleyana* Fort. ex Lindl., *B. madagascariensis* Lam. and *B. polystachya* Fresen. *B. davidii*, the most widely cultivated of the four, has a broad subtropical and temperate distribution.

The majority of classical taxonomic work has focused on Old World species. African species, numbering about 13, are less often cultivated than many of the popular Asian species.

However, some researchers have placed great evolutionary importance on African taxa. Moore (2001) hypothesized Africa as the generic center of origin. Although greater diversity of *Buddleja* species exists in Asia and the Americas, Moore points out that greater generic diversity exists in Africa indicating a long history of genetic divergence. Recent taxonomic treatments reduce the African genera *Adenoplusia* and *Nicodemia* to synonyms of *Buddleja*. Still, the greatest generic diversity still remains in Africa (Norman, 2000). No better hypothesis has emerged over the past 40 years, and experts tend to accept the plausibility of Moore's proposal (Norman, 2000; Oxelman et al., 1999).

About 21 *Buddleja* species are indigenous to Asia and are widely distributed in central China, Tibet and Japan (Norman, 2000). High intraspecies phenotypic diversity exists in these regions including *B. alternifolia*, *B. japonica* and *B. lindleyana*; the three most cold hardy taxa (Moore, 1961). *Buddleja davidii* is widely distributed in central China, Tibet and Japan. This species has small light seeds with prominent wings which likely contribute to its colonizing ability (Norman, 2000). Indeed *B. davidii* has rapidly naturalized in areas of introduction including tropical and subtropical South America, Europe, and New Zealand (Norman, 2000).

Buddleja **Cytology and Evolution**

The basic chromosome number n=19 is considered to reflect alloploidy (Moore, 1947). About 48% of *Buddleja* species are known based on differences in cytology, and both New World and Old World polyploids have been identified (Moore, 1947). Polyploidy appears most frequently in Asia, and includes diploid, tetraploid, hexaploid, 12-ploid and 16-ploid species (Norman, 2000). Intraspecies ploidy variation has been reported in some taxa

including the 8–24-ploid *B. covilei* (Norman, 2000). Chromosome number characterization indicates smaller chromosomes in polyploids versus diploids (Moore, 1947).

Moore (1961) attempted to correlate photoperiodism with ploidy and phylogenetic level. Phylogenetic levels of primitive, intermediate or advanced were defined based on inflorescence morphology. In general, primitive types have a dichasium of flowers in few leaf axils along the terminal stem position and are subtended by non-reduced leaves (Moore, 1960). Indices of evolutionary change in inflorescence morphology included greater flower numbers per node, increases in number of flowering nodes per stem, elongation of inflorescence to a more spicate form, inflorescence branching and size reduction of leaves subtending the inflorescence (Moore, 1960). Evolutionary advancement is often positively correlated with polyploidy, and here all polyploid species were phylogenetically more advanced except the intermediate *B. delavayi*. Results indicate all diploid species, except *B. lindleyana*, are spring flowering short day plants while most polyploids are summer flowering. Short day flowering plants were represented by species at all three phylogenetic levels while most summer flowering types were phylogenetically advanced (Moore, 1961).

Molecular components of inflorescence development

Elucidation of genetic control mechanisms in angiosperm flower development has provided new prospects for plant improvement. The identification and characterization of floral organ identity genes, first from *Arabidopsis thalianus* and *Antirrhinum majus*, led to the ABC model of angiosperm flower development (Fig. 1) (Bradley et al., 1993; Coen and Meyerowitz, 1991) . Three floral homeotic gene classes (A, B and C) alone and combined direct development of the four whorls in typical angiosperm flowers. Class A genes are

involved in sepal formation. Class B and A together direct petal formation and B and C together direct stamen formation. Class C alone is involved in Carpel formation. Although additional genes necessary for proper flower development in *Arabidopsis* have been recently identified, the original model remains applicable.

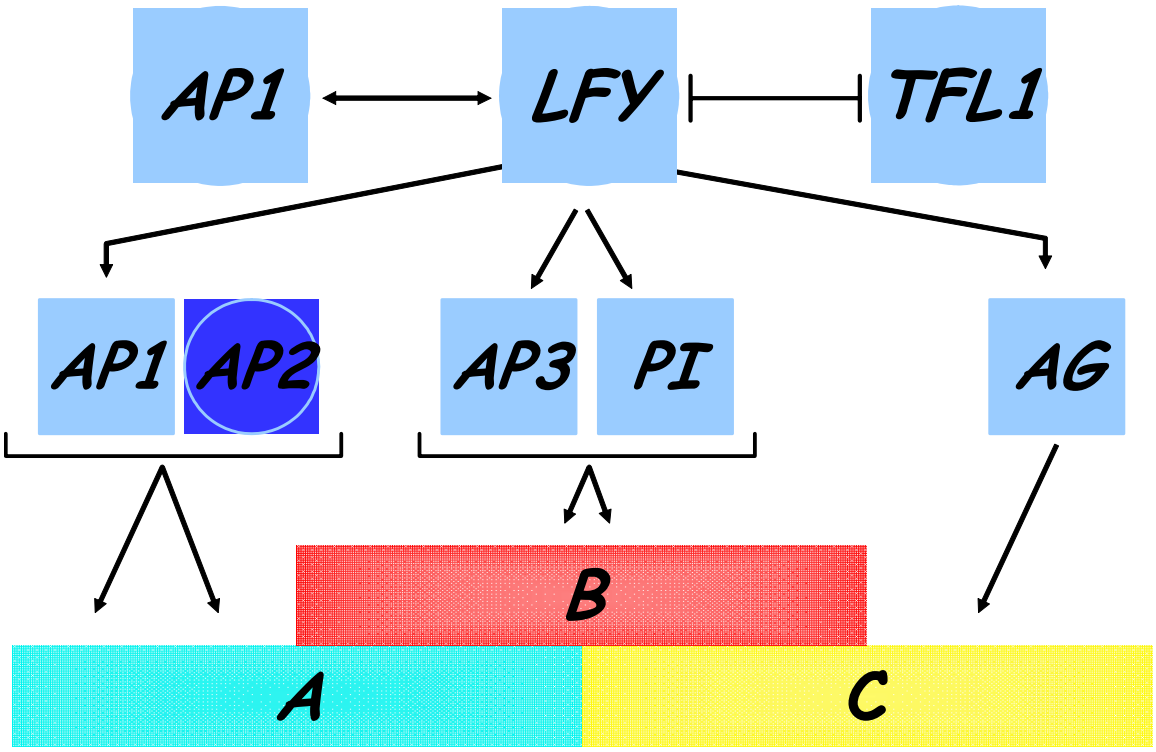

Fig. 1. Schematic representation of the interaction of the floral meristem identity genes and floral homeotic genes of the ABC model of flower development in *Arabidopsis thaliana* (Bradley et al., 1993; Coen and Meyerowitz, 1991).

Floral homeotic genes are members of the MADS-box gene family (Schwarz-Sommer et al., 1990). MADS-box genes encode proteins containing a highly conserved 56 amino acid (aa) MADS domain involved in DNA binding and occur in a diverse group of

eukaryotic organisms including yeasts, mammals, insects, amphibians and plants (Shore and Sharrocks, 1995). Floral homeotic genes in *Arabidopsis* and *Antirrhinum*, including the ABC genes, were the first MADS-box genes identified in plants (Jack, 2001). However, plant MADS-box genes are not limited to floral development, and are involved in diverse roles within various organs including fruit (Boss et al., 2001; Yao et al., 1999), tubers (Kang and Hannapel, 1996), leaves (Brunner et al., 2000) and embryonic tissues and roots (Perry et al., 1999; Purugganan et al., 1995).

The gene specifying C function in *Arabidopsis* flowers [*AGAMOUS* (*AG*)] was the first floral homeotic gene isolated and characterized. *AG* encodes a transcription factor of the MADS-box family, and possesses a conserved N-terminal region (Riechmann and Meyerowitz, 1997; Yanofsky et al., 1990). A second C class gene, isolated from *Antirrhinum* [*PLENA* (*PLE*)] using an *AG* probe, shows high sequence and functional homology with *AG* (Bradley et al., 1993). *AG* expression, occurring at stage 3 of flower development in *Arabidopsis*, is initially restricted to the central apices of the floral meristem (Deyholos and Sieburth, 2000; Yanofsky et al., 1990). As flower development progresses, *AG* expression can be detected throughout the third and fourth whorls, and late in development *AG* expression is limited to specific cell types in stamens and carpels (Bowman et al., 1991). Two MADS genes, *LEUNIG* (*LUG*) and A-class *APETELA*2 (*AP*2), negatively regulate *AG* expression in stamens and carpels (Deyholos and Sieburth, 2000). Conversely, *AG* negatively regulates *AP*2 in developing stamens and carpels restricting its expression to whorls one and two (Riechmann and Meyerowitz, 1997).

Early *AG* expression is under the direct control of the floral meristem identity gene *LEAFY* (*LFY*) (Parcy et al., 1998). *LFY*, along with the A-class gene *APETALA*1, control

meristem identity and homeotic gene expression during early flower development. Floral evocation in plants involves complex genetic interactions much of which remains unclear. However, over the past decade several classes of genes involved in the developmental switch from a vegetative meristem to a floral meristem have been identified in both model plant systems and agronomic species (Ahearn et al., 2001; Coen et al., 1990; Shindo et al., 2001; Weigel et al., 1992). A general model for the relationship between floral meristem identity genes and flowering time genes has been recently reviewed (Pidkowich et al., 1999; Soltis, 2002). *LEAFY* (*LFY*) in *Arabidopsis thaliana* and *FLORICAULA* (*FLO*) in *Antirrhinum majus* are expressed in meristems prior to and during the transition from a floral to vegetative state, exist as a single copy gene, and act as upstream regulators of floral organ identity genes (Coen et al., 1990; Weigel et al., 1992).

LFY expression also occurs at low levels during vegetative growth in both meristematic regions and in leaves unlike *FLO* whose expression is detectable only in meristems during phase transition and reproductive development (Coen et al., 1990; Weigel et al., 1992). *AP1*, an *Arabidopsis* A-class floral organ identity gene, is also expressed early in meristems transitioning to a floral state and can partially compensate for *LFY* loss-of-function (Hempel et al., 1997). In addition, *Arabidopsis* plants homozygous for the *lfy* mutant lack normal flowers (Weigel et al., 1992). However, the *lfy* phenotype can be partially compensated for in plants overexpressing *AP1* (Liljegren et al., 1999). *AP1* expression follows *LFY* expression in wild-type plants, and has been demonstrated to be transcriptionaly regulated by *LFY* (Wagner et al., 1999). Similarly, *ap1* homozygous mutant plants lack normal flowers (Mizukami and Ma, 1997). In contrast, overexpression of *LFY* in *Arabidopsis* and ectopic expression of *LFY* in other plants results in precocious flowering

(Hempel et al., 1997). Another meristem identity gene in *Arabidopsis*, *TERMINAL FLOWER 1* (*TFL1*), acts to maintain the inflorescence meristem identity causing continued inflorescence elongation by preventing *LFY* expression in the meristem cells (Liljegren et al., 1999). Mutant *tfl* plants show a phenotype similar to plants overexpressing *LFY*.

***FLO/LFY* homologous**

The role of *FLO/LFY* homologues in determining meristem fate is well established in several plant species, and its interactions with both upstream and downstream genes involved in regulation and signal transduction in meristems and floral organs has demonstrated (Parcy et al., 2002; Wagner et al., 1999; William et al., 2004). Although *FLO/LFY* and homologues appear functionally conserved, some variation has been reported. For instance, expression of *NFL* in tobacco is not restricted to the meristematic region as is *LFY*, but is also expressed in leaves (Ahearn et al., 2001). However, *NFL* still functions as a transitional switch to a floral meristem in tobacco. The functional conservation of *FLO/LFY* is well illustrated by the ability of the orthologue *NEEDLY* (*NLY*) in *Pinus radiata*, to restore function in *Arabidopsis lfy* mutants (Mouradov et al., 1998). This provides good evidence that *FLO/LFY* divergence occurred prior to the split of angiosperms and gymnosperms. Numerous additional *FLO/LFY* homologues have been reported in higher plants and basal angiosperms, and homologues in higher plants display strong conservation of two features: 1) a proline-rich region near the N-terminus and 2) an acidic central region (Frohlich and Meyerowitz, 1997; Weigel et al., 1992). The functional role of floral meristem identity genes such as *FLO* and *LFY* as central regulators floral meristem identity makes them key in uncovering the molecular genetic aspects of inflorescence development and diversity.

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CHAPTER 2

CLONING AND EXPRESSION ANALYSIS OF *FLORICAULA/LEAFY* HOMOLOGUES IN *BUDDLEJA DAVIDII*

(Formatted for submission to the Journal of the American Society for Horticultural Science)

Cloning and expression analysis of *FLORICAULA/LEAFY* homologues in *Buddleja davidii*

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Molecular Biology-Biotechnology

Cloning and expression analysis of *FLORICAULA/LEAFY* homologues in *Buddleja davidii*

Additional index words. *Arabidopsis*, *Antirrhinum majus*, inflorescence architecture, flower development, polyploidy.

Abstract.

Five cDNAs with homology to the floral meristem identity genes *FLORICAULA* (*FLO*) from *Antirrhinum majus* and *LEAFY* (*LFY*) from *Arabidopsis thaliana* have been isolated from *Buddleja davidii*. The cDNAs were RT-PCR amplified from total RNA extracted from young developing inflorescences. The five clones shared 96% or greater nucleotide identity and were more similar to *FLO* than *LFY*. Strong sequence similarity and the existence of gaps at possible alternative splice sites suggest alternative splicing may account for three clones designated *BdFL1*α, *BdFL1*β and *BdFL1*γ. Expression patterns of all five clones were examined by RT-PCR. Expression of *BdFL1*α, *BdFL1*β, *BdFL2* and *BdFL3* was detected in terminal inflorescences, lateral inflorescences, and in meristems of shoots with 1−3, 4−6 or 7−9 nodes, but not in leaves or whole flowers. Expression of *BdFL1*α was not present at detectable levels in any of the samples.

Introduction

Rapid progress has been made over the past 15 years in uncovering the molecular genetic control of meristematic phase change and inflorescence development in flowering plants. Several pathways for floral induction and development have been extensively characterized in model plant species, and these findings have provided increasing insights into the molecular components of inflorescence development and diversity. Furthering our knowledge of the molecular aspects of plant development has the potential for greatly impacting our ability to modify and cultivate plants for the nutritional, economical, social and emotional benefit of humans.

FLORICAULA (*FLO*) in *Antirrhinum majus* (Coen et al., 1990) and *LEAFY* (*LFY*) in *Arabidopsis thaliana* (Weigel et al., 1992) are floral meristem identity genes that signal the transition from an indeterminate inflorescence meristem to a determinate floral meristem (Mandel and Yanofsky, 1995). *LFY* encodes a DNA-binding transcription factor, and during the transition to a floral meristem LFY directly activates *APETALA1* (*AP1*) (Wagner et al., 1999), a second floral meristem identity gene and floral organ development gene, *CAULIFLOWER* (*CAL*), the *AP1* homologue, and other *AP1* related genes (William et al., 2004). In addition to a role as a meristem identity gene, *LFY* is directly involved in floral organ development through the activation of several floral homeotic genes (Busch et al., 1999). *LFY* expression is low during the vegetative phase and upregulated following reception of inductive signals (Blazquez et al., 1997). In *Arabidopsis* and other plants, overexpression of *LFY* leads to the premature conversion of vegetative meristems and early flower development, whereas, mutations in *LFY* and *FLO/LFY* homologues results in an

prolonged vegetative state and incomplete conversion of secondary meristems into flowers (Coupland, 1995; Rottmann et al., 2000; Schultz and Haughn, 1991).

FLO/LFY genes make useful markers for identifying floral meristems because of very early expression in the transitioning meristem which signals a commitment to the floral organ development program. Also, because *FLO/LFY* expression leads to loss of indeterminacy resulting in termination of shoots with flowers, these genes play an important role in determining inflorescence architecture. The expression timing and location of *FLO/LFY* genes in both terminal and lateral inflorescence meristems determines the degree of branching found. In pea, expression of the *FLO/LFY* homologue *ALF* in one half of the bifurcating inflorescence meristem results in flower formation in the *ALF* expressing meristem and continued elongation in the second meristem resulting in continued inflorescence elongation (Souer et al., 1998).

Buddleja, a cosmopolitan taxon of roughly 100 species, provides a unique model for studying inflorescence development at the molecular level. Great diversity in inflorescence architecture exists among *Buddleja* species, and numerous hybrids exist between and among many of these taxa. Breeding goals have included the enhancement of floral architecture through increased panicle branching and total flowers per inflorescence. *Buddleja davidii*, a popular ornamental landscape plant, is a tetraploid of unknown genetic origin. The *B. davidii* inflorescence is an indeterminate panicle of racemes. Several clones exhibiting enhanced inflorescence branching are known, and the trait has been the focus of breeding efforts. We were interested in identifying *Buddleja FLO/LFY* homologues as a means of investigating the molecular genetic changes that result in the enhanced branching architecture. In this report we describe the isolation of *B. davidii FLO/LFY* homologues and the analysis of expression

patterns in various tissues. We will also discuss our findings as they relate to the polyploid nature of *B. davidii*.

Materials and Methods

Plant Materials. Tissues were collected from multiple clones of *Buddleja davidii* 'Nanho Purple' maintained in containers using standard cultural practices. Young inflorescences were collected prior to visible pigmentation, and leaves were collected prior to full expansion. All materials were frozen immediately in liquid nitrogen and stored at −80 ºC until used.

Nucleic acid extraction. Frozen tissues were ground to a fine powder in liquid nitrogen with a pre-chilled mortar and pestle prior to extraction. Total RNA was extracted from 1 to 2 cm long developing inflorescences using Tri-Reagent following the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). About 1 g of ground tissue was homogenized in 10 ml of Tri-Reagent. Purified RNA was solubilized in nuclease free water and stored at − 80 ºC until used. Genomic DNA was isolated from expanding leaves following Dellaporta et al. (1983). About 5 g of ground tissue was added to 15 ml of miniprep extraction buffer (0.1 M Tris-HCL pH 8.0, 50 mM EDTA, 0.5 M NaCl, and 10 mM 2-mercaptoethanol) and 20 % SDS. Following incubation for 10 min at 65 ºC, 5 ml of 5 M KOAc was added, and the solution was incubated for 20 min on ice followed by centrifugation at 25000 g_n for 20 min. The supernatant was filtered through cheese cloth, mixed with one volume isopropanol and incubated for 30 min at −20 ºC. The solution was centrifuged at 20000 g_n for 15 min, and the resulting pellet was resuspended in 2.4 ml $T_{10}E_{10}$. DNA was precipitated with 0.24 ml 3 M NaOAc and 1.25 ml isopropanol and pelleted by

centrifugation at 12000 g_n for 5 min. The resulting pellet was washed twice with 70% EtOH, resuspended in 1 ml $T_1E_{0.1}$ and stored at 4 ºC.

cDNA cloning of *FLO*/*LFY* homologues. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to isolate partial cDNA fragments homologous to *FLO/LFY*. All PCR products were cloned directly using the pGEM-T Easy Vector System (Promega, Madison, Wis.), and inserts were sequenced at the University of Georgia Sequencing and Synthesis Facility using an Amersham Biosystems model 3700 sequencer. For first strand cDNA synthesis, 5 µg of total RNA was primed with an oligo d(T) primer and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) in a 20 µl reaction. Degenerate primers, based on conserved amino acid *FLO/LFY* homologue sequences reported for six diverse angiosperm species, were designed with CODEHOP (Rose et al., 1998). Degenerate oligonucleotide primers BLFY2 (5'-ATATGAAGGATGAGGAGCTGGA**Y**GA**N**ATGATGA-3') and BLFY4 (5'-AACAGGTAATCCAGGCCGTT**Y**TT**Y**TTN**N**CC-3') corresponding to conserved regions of the amino acid sequences of *FLO* and *FLO*/*LFY* homologues *ALF* from *Petunia* (Souer et al., 1998), *FALSIFLORA* from tomato (Molinero-Rosales et al., 1999), *NFL1* from tobacco (Kelly et al., 1995) and *PTLF* from *Populus* (Rottmann et al., 2000) were used in a 50 µl PCR to amplify the initial internal cDNA fragments. The cycling conditions included an initial Amplitaq Gold (Applied Biosystems, Foster City, Calif.) activation step at 95 ºC for 10 min followed by 50 cycles of 94 ºC for 45 s, 60 ºC for 45 s, and 72 ºC for 45 s.

The resulting partial cDNA sequences were used to design gene specific primers 5SP3 (5'-CCACCACCATCTCCCACACTCCTCCA-3') and 3P2 (5'-TGGCTTCGATGGAGGAGGAGGATGACG-3') for 5'− and 3'− RACE respectively.

Amplification of the 5'− and 3'− ends was carried out using the Smart RACE cDNA Amplification kit (Clontech, Palo Alto, Calif.) following the manufacturers protocols. The RACE products were cloned and sequenced as above. Oligonucleotide primers BdF5 (5'-AATGGATCCTGATGCCTTCTCAGCAAG-3') and BdF3 (5'-CAAGTTTCCATTCCATTGGGCAAAGTG-3') were used to amplify full length cDNAs of *FLO*/*LFY* homologues. A 50 µl PCR was carried out using Amplitaq Gold under the following cycling parameters: 95 ºC for 10 min followed by 35 cycles of 94 ºC for 45 s, 60 ºC for 45 s, and 72 ºC for 45 s. A cDNA control containing no RT was included for each template. PCR products of about 1.3 kbp were cloned and 25 cloned cDNAs were sequenced as above. Five unique cDNAs were designated *BdFL1*α, *BdFL1*β, *BdFL1*γ, *BdFL2* and *BdFL3*. Identification of the *BdFL* clones was determined based on homology searches and alignment of the nucleotide and deduced amino acid sequences with other angiosperm *FLO*/*LFY* homologues using Clustal W.

Expression analysis. Qualitative expression analysis of the *BdFL* clones was conducted using RT-PCR and primers BdX1 (5'-AATGGATCCTGATGCCTTCTC-3') and BdX2 (5'-GCACTGGCTCCTCTGACAAC-3') designed to amplify all five cDNAs. Total RNA (~5 µg) extracted from meristems of shoots bearing 1−3, 4−6 or 7−9 nodes, terminal or lateral inflorescences (~2 cm), whole flowers or vegetative leaves was used to generate first-strand cDNA as above. A PCR was carried out using Amplitaq Gold and 2 µl of the RT reaction. Cycling parameters included 95 ºC for 10 min followed 40 cycles of 95 ºC for 30 s, 58 ºC for 30 s and 72 ºC for 1 min. Using the shared primers BdX1 and BdX2, amplicons of all five clones together would resolve as only two bands following gel electrophoresis because of the identical or nearly identical number of base pairs in the amplified region.

However, three out of the five clones possessed a single *NgoMIV* restriction site 105 bp downstream of the BdX1 priming site. Therefore, to resolve individual fragments, 10 µl of each RT-PCR product was restricted with *NgoMIV* for 3 h in a 20 µl total volume reaction. Following restriction, products were analyzed on an ethidium bromide stained 10% polyacrylamide Ready Gel TBE gel (Bio-Rad, Hercules, Calif.).

Results

Isolation of cDNA clones. Homology searches of the GenBank database revealed three cDNA fragments with high *FLO* sequence identity generated from degenerate primer PCR. The 384, 453 and 492 base pair (bp) fragments were 71, 85 and 85 % identical to *FLO* at the nucleotide level (data not shown). Primers designed based on the above sequences were used for RACE−PCR to amplify the 5'−, and 3'− flanking regions. The 3'−RACE produced fragments (~700 bp) that included the putative translation stop site and a portion of the poly−A tail. The sequences were at least 94% identical at the nucleotide level but differed at the polyadenylation sites. Two kinds of cDNA fragments were isolated from the 5'−RACE. The 262 bp and 299 bp fragments were nearly identical at the nucleotide level except for the presence of a 36 bp gap in one sequence. The gap corresponded to a proline rich region common in other angiosperm *FLO*/*LFY* homologues.

To generate full-length cDNAs, a sense primer (BdF5) targeted to a region overlapping the predicted translation start site and an antisense primer (BdF3) targeted to a region downstream of the presumed translation stop site were used to amplify about 1.3 kbp fragments. Twenty five cloned fragments were sequenced in both directions, and five unique clones were identified as *FLO*/*LFY* homologues based on computer homology searches. Sequence identity among the five clones was at least 96% at both the nucleotide and deduced

amino acid level (Fig. 1). Three clones shared 100% identity at both the nucleotide and deduced amino acid sequence level with the exception of gapped regions (Fig. 2A). The gaps appear to result from the existence of an additional splice donor site and two additional splice acceptor sites (Fig. 2A). The three putative alternative splice forms were designated *BdFL1*α, *BdFL1*β and *BdFL1*γ. The two other clones were designated *BdFL2* and *BdFL3*. *BdFL2* has a 36 bp gap identical to one of the 5'−RACE products resulting in the deletion of most of the polyproline coding region (Fig. 2B). With the exception of the missing polyproline region, *BdFL2* is identical to the *BdFL1* clones at both the nucleotide and deduced amino acid sequence level. *BdFL3* contained 9 single base pair differences and exhibited a splicing pattern identical to *BdFL1*β. Of the 9 polymorphisms, eight were silent substitutions and only one at nucleotide position 56 results in the substitution of a leucine for a proline residue. Nucleotide sequence homology of *BdFL* clones was 86% to 88% with *FLO* and 62% to 68% with *LFY*. The deduced amino acid sequence of *BdFL1*α was further analyzed and showed homologies of 80% with *ALF*, 77% with *FALSIFLORA*, 87% with *FLO*, 66% with *LFY*, 80 % with *NFL1* and 78% with *PTLF* (Fig. 3).

Qualitative expression of *BdFL* clones. RT-PCR was carried out to investigate differences in temporal and spatial expression among the *BdFL* clones. Total RNA from the seven different tissues was RT-PCR amplified. RT-PCR products from tissues containing all five transcripts were expected to resolve as two bands. Therefore, RT-PCR products were first digested with *NgoMIV* to facilitate resolution of individual cDNAs. Using a 10 % polyacrylamide gel, we were able to resolve all five products in a preliminary study (data not shown). *BdFL* transcripts were present at detectable levels in RNA from five out of the seven samples (Fig. 4). Only vegetative leaves and whole flowers contained no *BdFL*

transcripts at detectable levels. Where *BdFL* expression was found, four out of five expected bands could be visualized. Only *BdFL1*γ was not present at detectable levels.

Discussion

Homologues of the meristem identity genes *FLO* and *LFY* were isolated from *B. davidii* using degenerate RT-PCR and total RNA from young developing inflorescences. The 1235 bp to 1274 bp clones and their deduced amino acid sequences were more similar to *FLO* than *LFY*. This is not surprising given the close phylogenetic relationship of *Antirrhinum* to *Buddleja* (Backlund et al., 2000). Two highly conserved features present in most *FLO*/*LFY* homologues reported in higher plants are a proline rich region near the N-terminal and an acidic central region (Weigel et al., 1992). Both features were present in the *BdFL* clones except for the polyproline region in *BdFL2*. The absence of a proline rich region has been reported in other flowering and non-flowering plants including the gymnosperm orthologue *NEEDLY* from *Pinus radiata* (Mouradov et al., 1998) and the apple *FLO*/*LFY* homologues *AFL1* and *AFL2* (Wada et al., 2002). Both regions are associated with transcription factor DNA binding and nuclear localization. Indeed, *LFY* has been shown to transcriptionally activate *APETALA1* (*AP1*), a second *Arabidopsis* floral meristem identity gene (Wagner et al., 1999). The acidic region was strongly conserved in all *BdFL* clones. If the proline rich region is critical for downstream gene activation by *BdFL*, relative abundance of *BdFL2* may play a role in timing the conversion of inflorescence meristems to floral meristems.

Without data based on functional analysis, the exact role of the individual *BdFL* clones remains uncertain. Whether or not the transcripts represent isoforms of one or more

alleles is also incomplete without such data. However, it appears that the sequence polymorphisms present in *BdFL3* are real and not the result of PCR or sequencing artifacts. We sequenced five different *BdFL3* clones in both directions with the polymorphisms present in each case. Also, the same polymorphisms were present in some fragments generated from the degenerate PCR and RACE procedures. The three *BdFL1* isoforms, *BdFL2* and *BdFL3* appear as unique based on the sequence polymorphisms and appear to represent alleles of at least two different genes.

FLO/LFY gene products play a critical role in the vegetative to reproductive transition within the inflorescence meristem. In *Arabidopsis*, ectopic expression of *LFY* is sufficient for specifying floral meristems. In addition to specifying a meristem as floral, *LFY* has a second role in directing the expression of floral homeotic genes. *LFY* expression occurs early in transitioning meristems, and increasing *LFY* RNA/protein levels serves as a useful marker for the transition to a floral meristem. *FLO/LFY* genes in most diploid angiosperm plants are represented by a single gene copy. However, two *FLO*/*LFY* homologues *AFL1* and *AFL2* were found in apple and a third possible homologue was identified based on southern blot analysis (Wada et al., 2002). Considering apples likely polyploid ancestry, it would seem feasible to find two copies of apple *FLO*/*LFY* homologues. However, the question remains whether *AFL1* and *AFL2* represent two gene copies or allelic variation. In contrast, *FLO*/*LFY* homologues *NFL1* and *NFL2* in the allotetraploid *Nicotiana tobacum* do represent two unique gene copies in a single genome (Kelly et al., 1995). Single copies of *NFL1* and *NFL2* were present in the diploid *N. tabacum* progenitor species *N. tomentosiformis* and *N. sylvestris* respectively. The progenitor species and the nature of the

duplication event that resulted in the formation of *B. davidii* are unknown. Interestingly, like the *BdFL* clones *NFL1* and *NFL2* share a high degree of homology.

We were interested in investigating whether differences in the *BdFL* amino acid sequences are indicative of functional differences as well. Unfortunately, genetic transformation or similar molecular methods that would facilitate such investigations have not yet been developed for *Buddleja*. However, functional differences are sometimes correlated with spatial and development related expression patterns. Therefore, *BdFL* expression in three tissue types and three meristematic stages of development was analyzed. *B. davidii* inflorescences develop continuously throughout the growing season on relatively short shoots. Therefore, it is reasonable to expect *BdFL* expression during early stages of shoot development. Differences in expression among the clones in early developing versus later developing meristems may indicate divergent roles among the clones. However, four out of five clones were always present where expression was detected. While *LFY* expression is detectable in vegetative tissues, *FLO* expression is not. *BdFL* expression, like *FLO*, appears limited to transitioning meristems and developing reproductive tissues, and the lack of expression found in flowers correlates with a diminishing role of *LFY* and *FLO* in later stages of flower development.

Weigel et al. (1992) reported the presence of *LFY* isoforms resulting from the presence of alternative splice donor and splice acceptor sites. The alternative splice sites in *LFY* transposed onto the *BdFL* sequence would account for the different *BdFL1* isoforms. Much of the current knowledge regarding alternative splicing is derived from studies of animal systems which has revealed that many alternatively spliced genes serve as on/off switches in a signal transduction pathway (Kazan, 2003). Computational analysis of the

recently completed *Arabidopsis* genome sequence has begun to reveal a potentially large role for alternative splicing in proteome diversity. The function of *LFY* alternative splice forms has not been investigated, and the specific influences of alternative splicing on plant development and diversity remains unclear. A role for alternative splicing in flower development is being uncovered. *FCA* is an autonomous flowering pathway gene represented by three isoforms in *Arabidopsis* (Macknight et al., 1997). The action of the autonomous pathway results in eventual flowering under non-inductive conditions. The role of *FCA* and the autonomous pathway is the repression the flowering repressor *FLC* (Macknight et al., 2002; Michaels and Amasino, 2001). Two alternative splice sites in the *FCA* pre-mRNA results in four different *FCA* isoforms only one of which contains the complete open reading frame, and it is the relative levels of the different isoforms that contributes to the control of flowering (Macknight et al., 1997). Whether *LFY*, and by comparison *BdFL*, alternative splice forms serve a similar function is unknown. A major difference between the *LFY*/*BdFL* and *FCA* is that all isoforms of the former maintain a complete open reading frame.

Five unique cDNAs of *B. davidii FLO/LFY* homologues have been described. The overall goal is to augment breeding efforts for this important ornamental plant. In addition, the identification of additional *FLO/LFY* homologues from diverse plant species will aid in the broader effort to reveal the origins of flowers and inflorescences. In addition, discovery of the molecular underpinnings of plant developmental processes will ultimately enhance our ability to develop and cultivate plants.

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BdFL1γ  MDPDAFSASLFKWDPRGVPPPPNRLLEAVVPPPPPAPPAAAAYSMRPRELGGLEEMFQAY 60
BdFL2   MDPDAFSASLFKWDPRGVPPPPNRLLEA------------AAYSMRPRELGGLEEMFQAY 48
BdFL3   MDPDAFSASLFKWDPRGVLPPPNRLLEAVVPPPPPPPPAAAAYSMRPRELGGLEEMFQAY 60
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Fig. 1. Alignment of the deduced amino acid sequence of *FLO/LFY* homologues from *Buddleja davidii*. *BdFL1*α, *BdFL1*β and *BdFL1*γ probably represent alternative splice forms of a single transcript. *BdFL2* lacks a proline-rich region near the N-terminus and a single base pair polymorphism in *BdFL3* result in amino acid substitution at residues 19.

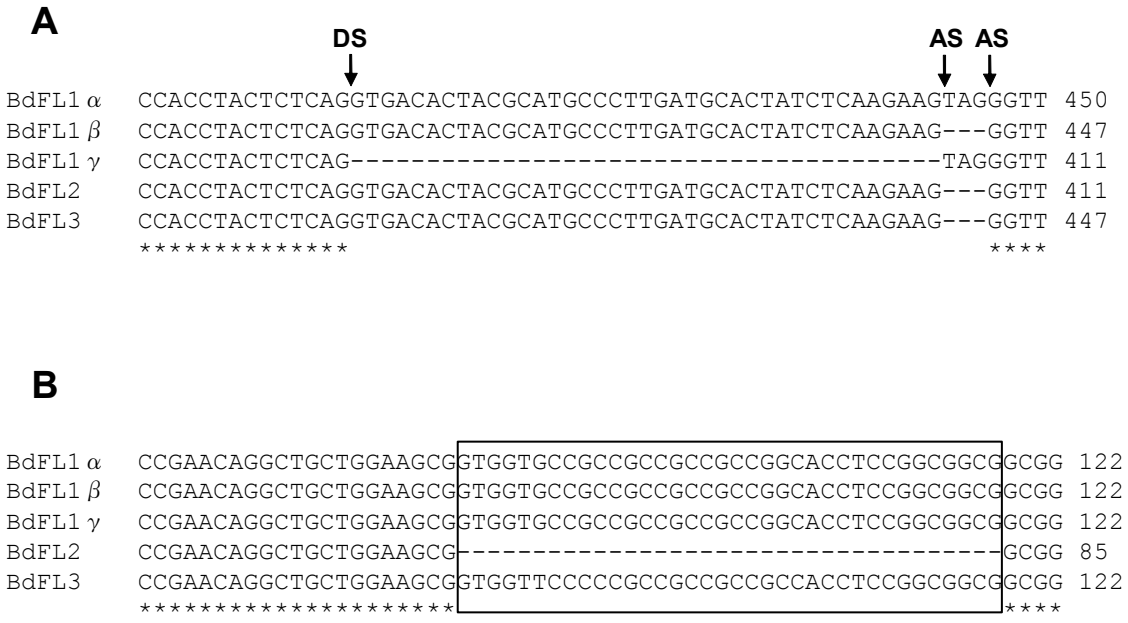

Fig. 2. Nucleotide sequence alignment of the polymorphic regions of the *BdFL* clones. (**A**) *BdFL1* is represented by three isoforms resulting from the presence of one alternative splice donor site (DS) and two alternative splice acceptor sites (AS). Clones *BdFL2* and *BdFL3* exhibit a splicing pattern identical to *BdFL1*β. (**B**) Boxed region encodes seven (*BdFL1*) or eight (*BdFL3*) proline residues out of 12 total. The proline rich region is absent in *BdFL2*.

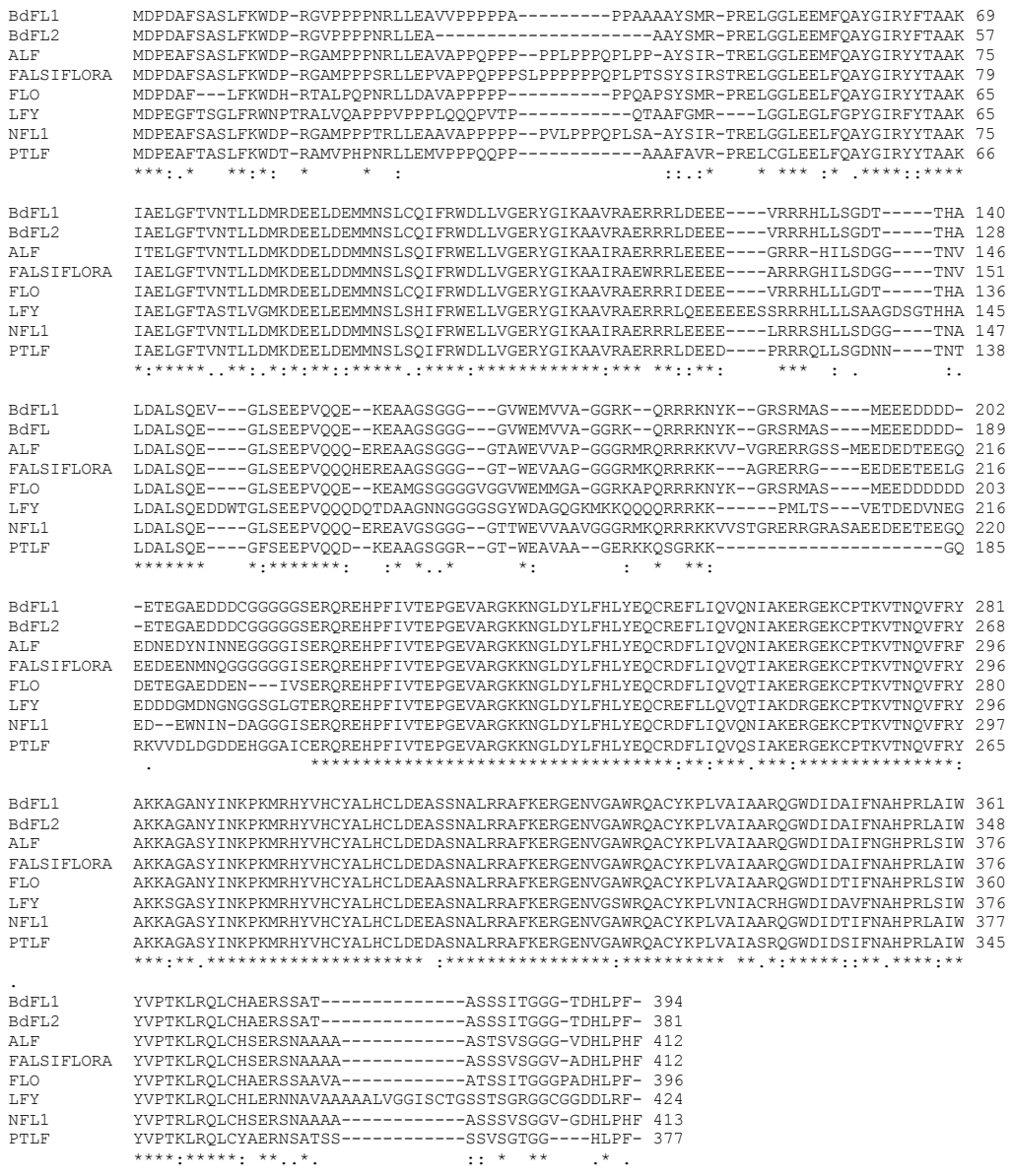

Fig. 3. Alignment of the *BdFL1*α and *BdFL2* deduced amino acid sequences with floral meristem identity genes *ALF* from *Petunia ×hybrida*, *FALSIFLORA* from *Lycopersicon esculentum*, *FLO* from *Antirrhinum majus*, *LFY* from *Arabidopsis thaliana*, *NFL1* from *Nicotiana tabacum* and *PTLF* from *Populus trichocarpa*. The *, double dot and single dot symbols below the sequences represent identical residues, conserved substitutions and semi-conserved substitutions respectively.

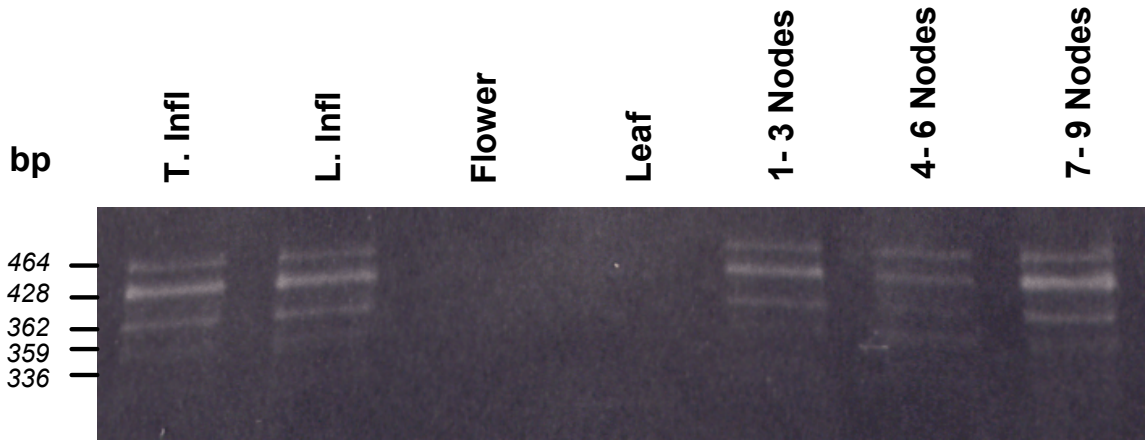

Fig. 4. Qualitative expression analysis of *Buddleja davidii FLORICAULA/LEAFY* homologues in seven different tissue samples by RT-PCR with shared gene specific primers. Expected positions of the *BdFL1α* (362 bp), *BdFL1β* (359 bp), *BdFL1γ* (336 bp), *BdFL2* (428 bp) and *BdFL3* (464 bp) fragments are indicated at left. Samples were loaded on an ethidium bromide stained 10% polyacrylamide gel. Tissue samples included: Terminal inflorescences (T. Infl.), lateral inflorescences (L. Infl.), flowers, leaves (Leaf), and meristems of shoots bearing 1−3, 4−6 or 7−9 nodes.

APPENDIX

B. lindleyana inflorescences are similar to those of *B. davidii*. They are composed of simple dichasia of three flowers on an indeterminate thyrse. A single interspecific hybrid between these two species has inflorescences with architecture essentially identical to *B. lindleyana* and with little apparent genetic influence from *B. davidii*. A reasonable hypothesis can be developed predicting a *FLO/LFY*-like genes in *Buddleja* that are involved in the observed variation in floral architecture based on current knowledge of *FLO/LFY*-like genes in other plant species.

Partial cDNA sequences representing possible *FLO/LFY* homologues from *Buddleja lindleyana* have been isolated by degenerate primer PCR and 3'−RACE. Two fragments 245 bp and a 330 bp long represent a region about 80 bp from the possible translation start site based on alignment with *FLO/LFY* homologues (Fig. 1). Two additional 753 bp and 755 bp fragments were generated by 3'−RACE and contain a poly A tail (Fig. 2).

```
BLIC_5 TATATGAAGGATGAGGAGCTGGATGATATGATGAACAGCCTGTGTCAGATTTTCAGGTGG 60
BLIC_9 TATATGAAGGATGAGGAGCTGGATGACATGATGAACAGCCTGTGTCAGATTTTCAGGTGG 60
       ************************* ********************************

       GACCTACTTGTTGGGGAGAGGTATGGTATCAAGGCCGCCGTCAGAGCTGAGCGCCGCCGC 120
       GACCTACTTGTTGGGGAGAGGTATGGTATCAAGGCCGCCGTCAGAGCTGAGCGCCGCCGC 120
       ************************************************************

       CTTGAGGAGGAGGAAGTGAGGCGGCGCCACCTACTCTCAGGGTTGTCAGAGGAGCCGGTG 180
       CTTGAGGAGGAGGA---------------------------------------------- 134
       **************

       CAACAAGAGAAGGAGGCAGCGGGAAGCGGTGGCGGAGGATGACGATTGTGGCGGTGGTGG 240
       ------------------------------------TGACGATTGTGGCGGTGGTGG 155
                                           ********************

       AGGAAGCGAGCGGCAGAGGGAGCATCCGTTCATCGTGACGGAGCCCGGTGAGGTGGCGCG 300
       AGGAAGCGAGCGGCAGAGGGAGCATCCGTTCATCGTGACGGAGCCCGGTGAGGTGGCGCG 215
       ************************************************************

       CGGAAAGAAAAACGGCCTGGATTACCTGTT 330
       CGGTAAAAAAAACGGCCTGGATTACCTGTT 245
       *** ** ************************
```

Fig. 1. Alignment of partial sequences of possible *FLO/LFY* homologues from *Buddleja lindleyana* amplified by PCR using degenerate primers BLFY2 (5'-ATATGAAGGATGAGGAGCTGGA**Y**GA**N**ATGATGA-3') and BLFY4 (5'-AACAGGTAATCCAGGCCGTT**Y**TT**Y**TT**N**CC-3').

```
BL372  TGAAGCGAGCGGCAGAGGGAGCATCCGTTCATCGCGACGGAGCCCGGTGAGGTGGCGCGT 60
BL375  TGAAGCGAGCGGCAGAGGGAGCATCCGTTCATCGTGGCGGAGCCCGGTGAGGTGGCGCGT 60
       ********************************** * ************************

       GGGAAGAAGAACGGCCTCGATTATCTCTTCCATCTCTATGAGCAATGCCGTGAGTTCTTG 120
       GGGAAGAAGAACGGCCTTGATTATCTCTTCCATCTCTATGAGCAATGCCGTGAGTTCTTG 120
       ***************** ****************************************

       ATCCAAGTTCAAAACATAGCCAAGGAGAGGGGAGAAAAATGTCCCACCAAGGTGACGAAC 180
       ATCCAAGTTCAAAACATAGCCAAGGAGAGGGGAGAAAAATGTCCCACCAAGGTGACGAAC 180
       ************************************************************

       CAAGTGTTCCGGTACGCAAAGAAAGCCGGAGCGAACTACATAAACAAGCCCAAAATGAGG 240
       CAAGTGTTCCGGTACGCAAAGAAAGCCGGAGCGAACTACATAAACAAGCCCAAAATGAGG 240
       ************************************************************

       CACTACGTGCACTGCTACGCGCTCCACTGCCTCGACGAGGCATCATCCAACGCGCTGCGT 300
       CACTACGTGCACTGCTACGCGCTCCACTGCCTCGACGAGGCATCATCCAACGCGTTGCGT 300
       ************************************************** ******

       AGAGCTTTCAAGGAGAGAGGAGAGAACGTTGGCGCGTGGAGGCAGGCCTGCTACAAGCCT 360
       AGAGCTTTCAAGGAGAGAGGAGAGAATGTTGGCGCGTGGAGGCAGGCCTGCTACAAGCCT 360
       ************************* ********************************

       CTAGTGGCCATAGCAGCAAGGCAGGGATGGGATATCGACGCCATATTCAATGCTCATCCA 420
       CTTGTGGCCATAGCAGCAAGGCAGGGATGGGATATCGATGCCATATTCAATGCTCATCCA 420
       ** ********************************** *********************

       CGCCTCGCCATCTGGTACGTCCCCACCAAGCTCCGCCAGCTCTGCCATGCTGAGAGGAGC 480
       CGCCTCGCCATCTGGTACGTCCCCACCAAGCTCCGCCAGCTCTGCCATGCTGAGAGGAGC 480
       ************************************************************

       AGCGCTACTGCATCCAGCTCCATTACCGGTGGTGGGACTGATCACTTGCCTTTCTAGAGC 540
       AGCGCTGCCGCACCCAGCTCCATTACCGGTGGTGGGACTGATCACTTGCCTTTCTAGTGC 540
       ****** * *** ****************************************** **

       GTAATCAGTGTTATGGAAATTTAAGTAGATTTGAGTTGTGAGATTGTAATGTTCCTTTCA 600
       GTAATCAGTGTTATGGAA-TTTTAGTAGATTTGAGTTGTGAGATTGTAATGTTCCTTTCA 599
       ****************** *** ************************************

       CTTTGCCCAATGTAATGGGAACTTGATCTTGTAGTAGTAAGGGTACTAGTATTTTTCCCT 660
       ATTTGGCCA-TGTAATGGTAACTTGATCTTGTAGTAGTAAGAGTACTAGTATTTTTCCCT 658
       **** *** ******** ********************** ******************

       TTTTATGTTTGTTAGCATGATAACCAATATATGTTAACTATAATGACCATAATTTCTAAT 720
       TTTTGTGTTTGTTAGCATGATAACCAATATATGTTAGCTATAATGACCGTAATTTCGAAT 718
       **** ******************************* *********** ******* ***

       TTCTATCACAAAAAAAAAAAAAAAAAAAAAAAA---- 753
       ATCTATCTCAAAAAAAAAAAAAAAAAAAAAAAAAAA 755
       ****** ************************
```

Fig. 2. Alignment of partial sequences of possible *FLO/LFY* homologues from *Buddleja lindleyana* amplified by 3'−RACE.