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Characterization and Heterologous Expression of *SLF*, a Functional Homolog of the Floral Regulator *LEAFY/FLORICAULA* from *Salix discolor*

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ABSTRACT

This study was done to contribute to our understanding of the *LFY/FLO* gene activity in willow, a dioecious woody plant. *SLF* is the *Salix discolor* homolog of the *LFY/FLO* gene which was cloned from the reproductive buds of a male individual, clone S365. *In situ* hybridization revealed that *SLF* is strongly expressed in the inflorescence meristems, bracts, and floral meristems, but only weakly expressed in the vegetative meristems and leaf primordia. Since a genetic transformation system coupled with *in vitro* regeneration is currently not available for willow, *Arabidopsis thaliana* was used to analyze the functions of *SLF*. Transformed *A. thaliana* produced flowers more than two weeks earlier than the controls; furthermore, they produced terminal and solitary flowers instead of inflorescence branches. The phenotypes of the transgenic lines were dominant and heritable, demonstrating that *SLF* was functional and participated in the flowering of *A. thaliana*. Many of these phenotypes are being described for the first time from a *LFY*/*FLO* homolog from a dioecious plant. Complementation test showed that *SLF* was able to restore the wild-type phenotype of the *lfy-6* mutant. This study revealed that *SLF* affected various aspects of floral development in transgenic *A. thaliana* and therefore, suggested that *SLF* is the functional homolog of the *LFY/FLO* gene.

Keywords: *Arabidopsis thaliana*, flowering, leafy, willow, woody plant

INTRODUCTION

The switch from vegetative to reproductive stage is one of the remarkable processes in the development of plants. Of the genes that are involved in this event, the *LEAFY/FLORICAULA* (*LFY/FLO*) gene performs one of the most crucial functions. Therefore, functional homologs of this master regulatory gene have been isolated and characterized from a variety of plants ranging from bryophytes to flowering plants (Himi *et al*. 2001; Carlsbecker *et al*. 2004; Dornelas and Rodriguez 2005; Tanahashi *et al*. 2005; Dornelas *et al*. 2006; Allnutt *et al*. 2007). The homologs from seed plants have been almost exclusively obtained from bisexual species, which are either herbaceous (Kelly *et al*. 1995; Hofer *et al*. 1997; Bomblies *et al*. 2003; Busch and Gleissberg 2003; Allnutt *et al*. 2007) or woody (Southerton *et al*. 1998; Rottman *et al*. 2000; Walton *et al*. 2001; Wada *et al*. 2002; Carmona *et al*. 2002; Carlsbecker *et al*. 2004; Dornelas and Rodriguez 2005; Dornelas *et al*. 2006). To date, the only *LFY/FLO* homolog described from a dioecious plant is *PTLF* from *Populus trichocarpa* (Rottmann *et al*. 2000). Therefore, there is a need for more studies in this area to expand our knowledge of the activities of the *LFY/FLO* gene in this type of plants.

Although willow and poplar belong to the same family (*Salicaceae*), there are marked differences in the morphology and development of their reproductive structures (Zhang and Fernando 2005; Fernando and Zhang 2006). A willow flower is typically composed of a nectary and two anthers or a single ovary that arises from the axil of the bract; the unisexual flowers are clustered in an upright catkin (Zhang and Fernando 2005). Willow flowers are initiated one to two years after seed germination (Gullberg 1993; Taylor 2002). In the flowers of poplars, a periant cup exists, there are three carpels or 40 to 60 anthers per flower, flowers are clustered in a hanging catkin, and flowering generally occurs after five years (Boes and Strauss 1994). These morphological and developmental differences suggest that there may also be some differences at the molecular level as regards the mechanism of flowering in willow and poplar.

Except for our report on *SAP1*, the *Salix discolor* homolog of the *AP1* gene (Fernando and Zhang 2006), there is no other information currently available on the pattern of gene expression during the initiation of the inflorescences and flowers in willow. The present study is based on a male plant and therefore, represents the first step towards our understanding of the overall genetic and molecular mechanisms of flowering in willow. This study aims to: 1) isolate the homolog of the *LFY/FLO* gene from a male willow individual, 2) analyze the sequence and structure of the *SLF* gene, 3) characterize the temporal and spatial expression patterns of *SLF*, and 4) analyze the functions of *SLF* through over-expression using a heterologous host, *Arabidopsis thaliana*.

MATERIALS AND METHODS

Plant materials and growth conditions

Twigs of *S. discolor* (clone S365) bearing vegetative and male reproductive buds at various developmental stages were collected from late July to early August in 2003 and 2004. The collections were done from SUNY-ESF's Genetics Field Station in Tully, NY. The vegetative and reproductive bud scales were removed, the buds were either immediately frozen in liquid nitrogen, and stored at -80°C for RNA isolation, or fixed in 4% (v/v) paraformaldehyde in 1X phosphate buffered saline (PBS) buffer for *in situ* hybridiza-

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tion. Young leaves were also collected from the field in July and August 2004, and frozen and stored as above for DNA extraction. Wild-type *A. thaliana* (ecotype Columbia) and 35S::*SLF A. thaliana* transgenic lines were sown on an enriched potting mix (Miracle-Gro, Marysville, OH). The seeds were grown in the greenhouse at 22°C with 16 h of light exposure. The plants were illuminated with a mixture of cool white and plant growth fluorescent lamps.

Isolation, cloning, and sequencing

Total RNA was isolated from 0.3 g reproductive buds at inflorescence meristem stage using an improved RNA isolation method by Salzman *et al*. (1999). Total RNA was resuspended in 25 µL of diethyl pyrocarbonate (DEPC)-treated water. First-strand cDNA was obtained through reverse transcription PCR using CLONTECH SMART PCR cDNA Synthesis Kit (Clontech, San Jose, CA). A pair of degenerate primers (LFY101 and LFY306) specific to the two highly conserved regions of various *LFY/FLO* homologs (Coen *et al*. 1990; Weigel *et al*. 1992; Southerton *et al*. 1998; Rottmann *et al*. 2000) was designed (**Table 1**). Gradient PCR was done and a 551-bp PCR product was obtained and cloned into pCR2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). DNA sequencing was done at the BioResource Center, Cornell University, Ithaca, NY. The DNA sequence obtained was analyzed by submission to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) followed by manual inspection of sequences. To obtain the complete coding sequence of *SLF*, rapid amplification of cDNA ends (RACE) technique was used. Two gene-specific primers (SLF213GSP1 and SLF165GSP2) were designed based on the partial sequence of *SLF* (**Table 1**). 5′-RACE and 3′-RACE PCR products were obtained using SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA). The PCR product was sequenced and analyzed as above. The full-length cDNA was amplified by PCR with primers 5SLF1 and 3SLF1 (**Table 1**) using the first strand cDNA as the template. To confirm the sequence of the full-length cDNA, DNA sequencing was repeated from five different pure colonies.

SLF genome structure analysis

To identify the introns in *SLF*, a 2.2 kb DNA fragment was obtained by PCR with primers 5SLF1 and 3SLF1 (**Table 1**) using genomic DNA as the template. The amplified product was cloned into pCR2.1-TOPO vector and sequenced separately with primers 5SLF1, 3SLF1, SLF213GSP1 and SLF165GSP2 (**Table 1**). The sequences were analyzed and compared with the full-length cDNA to determine the location and length of the introns in the *SLF* genome structure.

Sequence alignment

The coding sequence of *SLF* was aligned with the nucleotide sequences of *LFY/FLO* homologs from several species. The alignments were done through BLAST. Multiple alignments based on deduced amino acid sequence were done using UNIPROT (http://www.pir.uniprot.org/search/blast.shtml).

Constructs and plant transformation

The primers 5SLF3 and 3SLF3 (**Table 1**) containing *Xho*I restriction enzyme site were used to amplify *SLF* for integration into the pCAMBIA3300 construct. The primers 5SLF3 and TER1 (**Table 1**) were used to confirm the orientation of *SLF* in the pCGSLF construct. Another construct (pCGFP) containing only *mgfp5-er* driven by the CaMV 35S promoter was made and used as the negative control for plant transformation. The plasmid DNAs were introduced into *Agrobacterium tumefaciens* strain EHA105 by CELL-Porator Electroporation System (GibcoBRL, Gaithersburg, MD). *A. tumefaciens*-mediated transformation of *A. thaliana* was performed by floral dip technique according to Clough and Bent (1998) and Desfeux *et al*. (2000). T_1 seeds on a moist filter paper were screened under a fluorescence microscope (Leica DMLB, San Jose, CA) based on GFP expression. GFP expressing seeds were sown in potting mix and grown in the greenhouse under long-day condition. All organs including leaves, roots, and flowers were further screened through GFP expression. T_2 and T_3 seeds were screened from the transgenic lines based on GFP expression. To further examine the function of *SLF*, pollen grains from homozygous T_3 lines were crossed with the strong *lfy-6* mutant allele. Early-formed flowers of *lfy-6* have pistils but do not have petals and stamens, while late-formed flowers are normal. Therefore, early-formed flowers were pollinated and other flowers that developed later were cut out. The resulting seeds were harvested and grown to observe the floral phenotypes. In F_2 individuals, the number of plants showing normal and *lfy-6* phenotypes was counted. The ratio of these two floral phenotypes was calculated and compared with Mendel's segregation patterns.

DNA isolation and Southern analysis

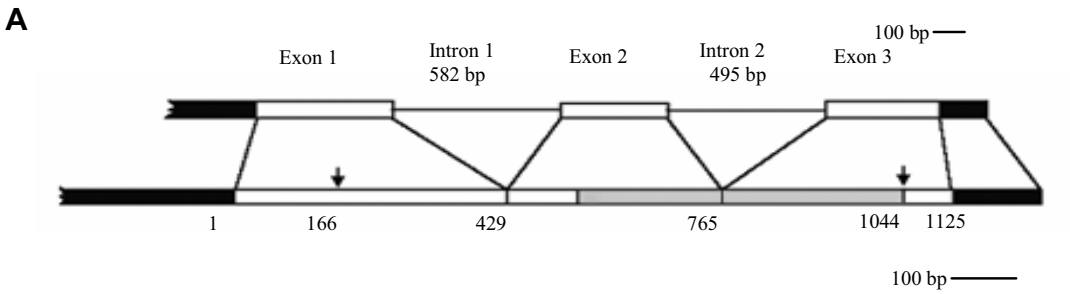
Genomic DNA was extracted from young leaves of *S. discolor* using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). For Southern blot analysis, the genomic DNA was purified three times with phenol/chloroform and digested separately with *Eco*RI and *Hin*dIII at 37°C for 15 h. Approximately 10 µg of the digested DNA was subjected to electrophoresis through 0.8% agarose gel and blotted to positively charged nylon membranes (Roche, Indianapolis, IN) (Sambrook *et al*. 1989). The primers RNSLF3 and RNSLF4 (**Table 1**) were designed to amplify a 500-bp DNA fragment (**Fig. 1A**), which is one of the highly conserved regions of *SLF* (**Fig. 1B**). The amplified PCR product was used as a probe and labeled with digoxigenin-11-UTP using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Indianapolis, IN). Hybridization and immunological detection were done according to the manufacturer. The membranes were incubated with gentle agitation in the probe/hybridization solution at 40°C for 12 h. Stringency washes with 0.5 × SSC/0.1% SDS were performed after hybridization. Anti-digoxigenin-alkaline phosphatase (AP) was used as the antibody. Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color substrate solution was used to visualize the hybridization on the membrane. The membrane was scanned using the GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA).

In situ hybridization

The 500 bp RNSLF3/RNSLF4 PCR product (which is the same fragment used as probe in Southern blot) was cloned into pSPT18 and pSPT19 using the DIG RNA Labeling Kit (Roche, Indianapolis, IN). The digoxigenin-labeled antisense and sense RNA probes were obtained with T7 and SP6 RNA polymerases, respectively. Vegetative and reproductive buds of *S. discolor* were fixed in 4% (v/v) paraformaldehyde in 1 × PBS buffer (pH 7), dehydrated through a graded series of ethanol, infiltrated, and embedded in paraffin. Sectioning and slide pretreatment, *in situ* hybridization, and post-hybridization were performed as described by Jackson (1991). Using thin sections from 6 different buds, RNA *in situ* hybridizations with nonradioactive probes were incubated at 53°C for 18 h. A series of washes with 0.2 × SSC and NTE were performed after hybridization. The samples were treated with anti-digoxigenin-AP antibody and NBT/BCIP color detection was performed in the dark for 2-3 days. The slides were examined under a light microscope (Leica DMLB, San Jose, CA) and representative images were obtained using a DEI-7500 CE digital video camera (Optronics, Goleta, CA).

Northern analysis

Total RNA was extracted from leaves of six 35S::*SLF A. thaliana* transgenic lines and one transgenic control line (sans the *SLF* gene) using RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Twenty µg of total RNA for each sample was subjected to electrophoresis through 1.2% formaldehyde agarose gel. Total RNA was blotted on positively charged nylon membranes (Roche, Indianapolis, IN). The 500-bp PCR product amplified from *SLF* cDNA, which is the same fragment used in Southern blot, was used as a probe and labeled with digoxigenin-11-UTP using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Indianapolis, IN). Hybridization and immunological detection were done



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SLF       MDP-EAETAS-LFKWDTRAMVPHPNR-LLEMVAPPQ------QPPAAAFAVRPRE-------LCGLEELFQAYGIRY  61
PTLF      MDP-EAETAS-LFKWDTRAMVPHPNR-LLEMVPPPQ------QPPAAAFAVRPRE-------LCGLEELFQAYGIRY  61
VFL       MDP-DAETAS-LFKWDPRGAVAPPNR-LLEAVAPPPPGAAAPAPVAAAYAIRPRE-------LGGLEDLFQEYGVRY  67
AFL1      MDP-DAESAN-LFKWDLRGMVVPTNRVQLEAAVPPAAT---AGGAAAGYTLRPSREL----GLGGLEDLFQAYGVRY  68
FLO       MDP-DAE----LFKWDHRTALPQPNR-LLDAVAPPPPP----PPQAPSYSMRPRE-------LGGLEELFQAYGIRY  60
LFY       MDP-EGETSG-LFFWNPTRALVQAPP-----PVPPPLQQQPVTPQTAAFGMR----------LGGLEGLFGFYGIRE  60
ZFL1      MDPNDAESAAHPFRWDLGPPAHAAPA-----PAPPP-------PPLAPLLLPPHAP-------RELEDLVAGYGVRP  58
PRFLL     MDP-ESESAA-FFKWDQRPPALAPPQMQRSAGLEAQRIFHDFGVPNAAAMAASNNSSSCRKELNCLEELFRNYGVRY  75
NEEDLY    MDA-EHEPVG-FFRWDQRP----APVVAAAAAPTTTVFNKDHGRPLEVILPMNG-----RKDLKSLEDLFKEYGVRY  66

SLF       YTAAKIAELGFTVNTLLDMKDEELDEMMNSLSQIFRWDLLVGERYGIKAAVRAERRRLDEED----PRRRQLLSGD-  133
PTLF      YTAAKIAELGFTVNTLLDMKDEELDEMMNSLSQIFRWDLLVGERYGIKAAVRAERRRLDEED----PRRRQLLSGDN  134
VFL       YTAAKIAELGFTVSTLLDMKDEELDDMMNSLCQIFRWDLLVGERYGIKAAVRAERRRLDEEE----SRRRHLLSADT  140
AFL1      YTAAKIAKLGFTVNTLLDMKDDELDDMMSSLSQIFRWELLVGERYGIKAAVRAERRRFEEED----SRRRNPVSGDT  141
FLO       YTAAKIAELGFTVNTLLDMRDEELDEMMNSLCQIFRWDLLVGERYGIKAAVRAERRRIDEEE----VRRRHLLLGDT  133
LFY       YTAAKIAELGFTASTLVGMKDEELEEMMNSLSHIFRWELLVGERYGIKAAVRAERRRLQEEEEEESSRRRHLLLSAA  137
ZFL1      STVARISELGFTASTLLGMTERELDDMMAALAGLFRWDVLLGERFGLRAALRAERGRVMSLG------ARCFHAGST  129
PRFLL     ITLTKMVDMGFTVNTLVNMTEQELDDLVRTTVEIYRVELLVGEKYGIKSAIRAEKRRLEEAER---KRMEQLFVDVD  149
NEEDLY    VTLAKMTEMGFTANTLVNMTEEEIEDLMKTLVELYHMDLLIGERYGIKSAIRAEKKRLQDSLE---MQRLEILSEAE  140

SLF       ---------TNTLDALSQE----GFSEEPVQQD--KEAAGSGGR---GTWEAVTAGER-KKQPGRK--KGHRKVVDL  189
PTLF      N---------TNTLDALSQE----GFSEEPVQQD--KEAAGSGGR---GTWEAVAAGER-KKQSGRK--KGQRKVVDL  191
VFL       ---------ANAIDALSQE----GLSEEPVQQE--KEAGGSGGV---GTWEVVVAGER-KKQQRRK--KGKTRMGSA  196
AFL1      T---------TNALDALSQE----GLSEEPVQQE--KEMVGTGVG---MAWEVVTAGERRKKQRRMK--KGQYRNCSA  199
FLO       ---------THALDALSQE----GLSEEPVQQE--KEAMGSGGGGVGGVWEMMGAGGRKAPQRRRKNYKGRSRMASM  195
LFY       GDS----GTHHALDALSQEDDWTGLSEEPVQQQDQTDAAGNNGGGGSGYWDAGQGKMKKQQQQRRRK-KPMLTSVET  209
ZFL1      LD-------AASQEALSDERDAAASGGGMAEGEAGRRMVTTTAG---KKGKKGVVGTRKGKKARRK--KELRPLNVL  194
PRFLL     GKRKIDEN---ALDTLSQE----GLSVEEPQGDNAIILSQNNTSANFPLNLNAGMDPVLILQNSGHLGTTVSGLIGM  219
NEEDLY    RKRILHDDQNTFAAAMASE----GTSK-ELRANDPLIFPESTSADHAPMNIASCKDSTLILQNSNQAQFCGSGLIGV  212

SLF       DG----DDEHGG--------------------AICERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQ  242
PTLF      DG----DDEHGG--------------------AICERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQ  244
VFL       DDNMNEDDNEGGDEDDD----------KGSGERGSERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQ  263
AFL1      GGGHDNDHNEGVDDKDDDMDNMNGQGNGGGGGLLGERQREHPFIVTEPGEVARGKKNGLDYLFYLYELCRDFLIQVQ  276
FLO       EEDDDDDDDETEGAEDD------------ENIVSERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQ  259
LFY       DEDVNEGEDDDGMDNGN-----------GGSGLGTERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCREFLLQVQ  275
ZFL1      DDENDGDEYGGGSESTES-----------SAGGSGERQREHPFVVTEPGEVARAKKNGLDYLFHLYEQCRVFLLQVQ  260
PRFLL     PDTNYGSEQTKACK-KQKRRR-----SKDSGEDGEERQREHPFIVTEPGELARGKKNGLDYLFDLYEQCGKFLLDVQ  290
NEEDLY    PEHSSESDERKADTNKQKRRR-----SKEPGEDGEDRPREHPFIVTEPGELARGKKNGLDYLFDLYEQCGKFLLEVQ  284

SLF       NIAKERGEKCPTKVTNQVFRYAKKAGASYINKPKMRHYVHCYALHCLDEDASNALRRAFKERGENVGAWRQACYKPL  319
PTLF      SIAKERGEKCPTKVTNQVFRYAKKAGASYINKPKMRHYVHCYALHCLDEDASNALRRAFKERGENVGAWRQACYKPL  321
VFL       NIAKERGEKCPTKVTNQVFRYAKKAGASYINKPKMRHYVHCYALHCLDEEASNALRRAFKERGENVGAWRQACYKPL  340
AFL1      NIAKERGEKCPTKVTNQVFRYAKKSGASYINKPKMRHYVHCYALHCLDVEASNVLRRAFKERGENVGAWRQACYKPL  353
FLO       TIAKERGEKCPTKVTNQVFRYAKKAGANYINKPKMRHYVHCYALHCLDEAASNALRRAFKERGENVGAWRQACYKPL  336
LFY       TIAKDRGEKCPTKVTNQVFRYAKKSGASYINKPKMRHYVHCYALHCLDEEASNALRRAFKERGENVGSWRQACYKPL  352
ZFL1      SIAKLGGHKSPTKVTNQVFRYANKCGASYINKPKMRHYVHCYALHCLDEEASNALRRAYKSRGENVGAWRQACYAPL  337
PRFLL     HIAKERGEKCPTKVTNQVFRHAKHSGAGYINKPKMRHYVHCYALHCLDIEQSNRLRRAYKERGENVGAWRQACYYPL  367
NEEDLY    RIAKEKGEKCPTKVTNQVFRHAKHNGAVYINKPKMRHYVHCYALHCLDSEQSNHLRRLYKERGENVGAWRQACYYPL  361

SLF       VAIASRQGWDIDSIFNAHPRLAIWYVPTKLRQLCYAERN------GATAS------SSVSGTG----VHLPF-     375
PTLF      VAIASRQGWDIDSIFNAHPRLAIWYVPTKLRQLCYAERN------SATSS------SSVSGTG----GHLPF-     377
VFL       VALAARQGWDIDAIFNAHPRLAIWYVPTRLRQLCHSERSNAAAAAAAAAS------SCISGGA----DHLPF-     402
AFL1      VVIAAAQGWDIDAIFNSHPRLSIWYVPTKLRQLCHAERHN-----ATASS------SASGGGG----EHLPY-     410
FLO       VAIAARQGWDIDTIFNAHPRLSIWYVPTKLRQLCHAERSS----AAVAAT------SSITGGGPA--DHLPF-     396
LFY       VNIACRHGWDIDAVFNAHPRLSIWYVPTKLRQLCHLERNNAVAAAAALVGGISCTGSSTSGRGGCGGDDLRF-     424
ZFL1      VEIAARHGFDIDAVFAAHPRLAVWYVPTRLRQLCHQARGS----------------HAHAAAG---LPPPPMF     391
PRFLL     VAMAKDNGWDIEGVFNKHEKLRIWYVPTKLRQLCHLEKSK----------------QSHL-------------     411
NEEDLY    VAIARENNWDIEGIFNRNEKLKIWYVPTKLRQLCHMERSK----------------ECQ--------------     404
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Fig. 1 Genome structure, cDNA and deduced amino acid sequences of SLF. (**A**) Genome structure of *SLF* (top) and *SLF* cDNA (bottom). The *SLF* locus has three exons (boxes) and two introns (lines). Gray box (from nucleotides 550 to 1044) in the cDNA region indicates location of the probe used in Southern and northern blots. Arrows indicate the *Hin*dIII restriction sites in the genomic *SLF* clone. There is no *Eco*RI restriction site in the genomic SLF clone. (**B**) Alignment of the deduced amino acid sequences of SLF, LFY, FLO, and eight other LFY/FLO homologs (accession numbers in parentheses): PTLF from *Populus trichocarpa* (U93196), VFL from *Vitis vinifera* (AF450278), FLO from *Antirrhinum majus* (M55525), AFL1 from *Malus* × *domestica* (AB056158), LFY from *Arabidopsis thaliana* (M91208), ZFL1 from *Zea mays* (AY179882), PRFLL from *Pinus radiata* (U92008), and NEEDLY from *Pinus radiata* (U76757). Black shadows indicate identical amino acids; dashes indicate gaps introduced to optimize the alignment. The acidic central domain is located between the two arrows.

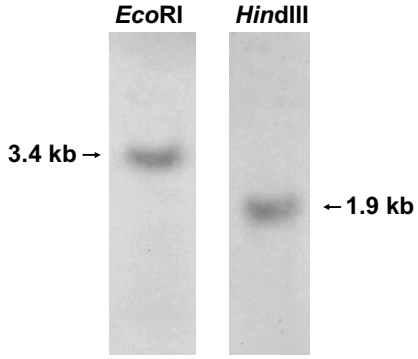

Fig. 2 Southern blot of genomic DNA from *Salix discolor* probed with *SLF* partial sequence. Ten µg genomic DNA was loaded per lane. Stringency washes with 0.5 × SSC/0.1% SDS were performed. NBT/BCIP color substrate solution was used for 12 h to visualize the hybridization on the membrane. MW was determined through comparison with the 1 kb DNA ladder.

according to the manufacturer. The membranes were incubated with gentle agitation in the probe/hybridization solution at 50°C for 12 h. Stringency washes with 0.1 × SSC/0.1% SDS were performed after hybridization. Anti-digoxigenin-AP was used as the antibody. NBT/BCIP color substrate solution was used to visualize the hybridization on the membrane. The membrane was scanned using the GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA).

RESULTS

SLF cDNA and amino acid sequence analyses

Using degenerate PCR primers designed from the conserved regions of *LFY*, a 551-bp partial sequence of *SLF* cDNA was obtained. The coding region of *SLF* was 1125 bp (GenBank Accession No. AY230817). The *SLF* genome structure was made up of three exons and two introns (**Fig. 1A**). The introns were 582 bp and 495 bp long. The deduced amino acid sequence of *SLF* was compared with the LFY and FLO proteins, LFY/FLO homolog proteins from several woody species, and other recently reported LFY/FLO homologs from flowering plants (**Fig. 1B**). The alignment confirmed two highly conserved regions, one containing 77 residues from amino acid 47 to 123, and the other containing 157 residues from amino acid 201 to 357. There were three regions with lower similarity (**Fig. 1B**). The proline-rich region in SLF had six proline residues between amino acid 20 and 36. As compared to other LFY/FLO homologs, SLF had a smaller number of acidic residues. FLO had 14 acidic residues but SLF had only four in the acidic central domain (between the two arrows in Fig. 1B). The alignment of sequences revealed that SLF had 97% identity with PTLF (*P. trichocarpa*), 78% with VFL (*Vitis vinifera*), 76% with FLO (*Antirrhinum majus*), 72% with AFL1 (*Malus* × *domestica*), 67% with LFY (*A. thaliana*), 56% with ZFL1 (*Zea mays*), 52% with PRFLL (*Pinus radiata*), and 49% with NEEDLY (*P. radiata*).

Southern hybridization using a PCR product amplified from the second highly conserved region of *SLF* revealed a single hybridizing *Eco*RI band of 3.4 kb. A single 1.9-kb *Hin*dIII band was also detected (**Fig. 2**). In the genomic *SLF* clone, there is no *Eco*RI restriction site but there are two *Hin*dIII restriction sites. However, the probe used is between the two *Hin*dIII restriction sites (**Fig. 1A**). This suggests that there is only one copy of *SLF* in *S. discolor*.

In situ expression patterns of *SLF* in *S. discolor*

The expression patterns of *SLF* in *S. discolor* were analyzed using male reproductive buds bearing inflorescence meristems and developing male flowers. Strong *SLF* expression was localized in the inflorescence meristems as well as in the developing flowers along the flanks of the inflorescence meristems (**Fig. 3A**). *SLF* was strongly detected in all the layers of the inflorescence meristems and bract primordia (**Fig. 3B**), young bracts (**Fig. 3D**), and floral meristems (**Fig. 3D, 3E**). Using lateral vegetative buds, *SLF* RNA was also detected, but in low levels in the vegetative meristems and leaf primordia (data not shown). Using the sense probe, no detectable hybridization was observed in any part of the male reproductive and vegetative buds (**Fig. 3C**).

Functional analysis of *SLF* in *A. thaliana*

To test the functions of *SLF*, a construct (pCGSLF) to overexpress the gene in *A. thaliana* was made. In addition to the *SLF* gene, the construct contains *mgfp5-er* driven by the CaMV 35S promoter. Several 35S::*SLF A. thaliana* transgenic lines were successfully screened based on GFP expression (**Table 2**). To analyze the phenotypes of homozygous transgenic lines, two negative controls were used including wild type and 35S::*GFP A. thaliana* (**Table 2**). The T_1 seeds were collected in separate pools, germinated,

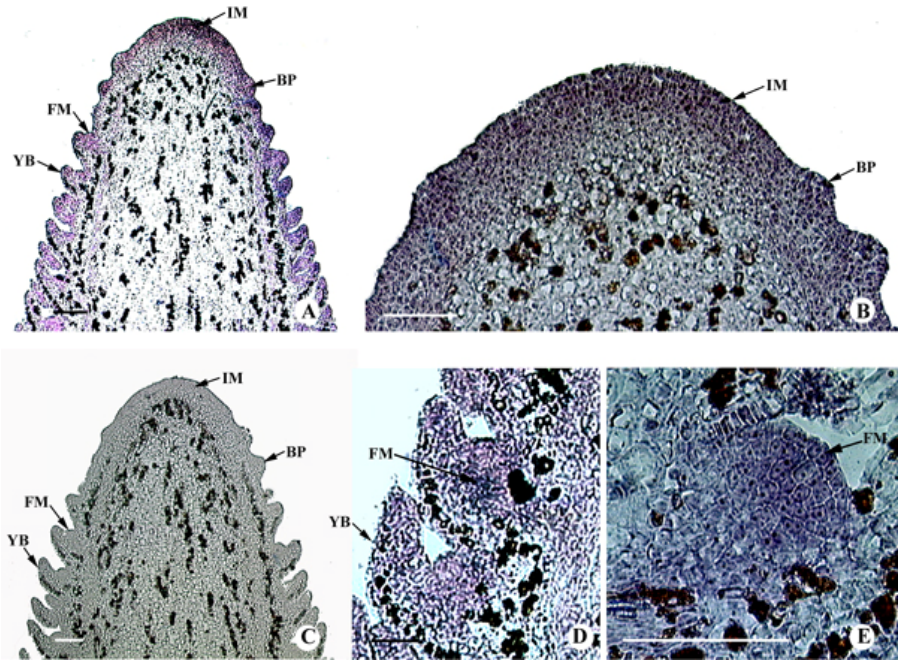

Fig. 3 *In situ* hybridization analysis of *SLF* expression in male reproductive buds of *Salix discolor*. All images are longitudinal sections. (**A**) Early male inflorescence, antisense probe. (**B**) Magnified inflorescence meristem, antisense probe. (**C**) Early male inflorescence, sense probe. (**D**) Young bracts and floral meristems, antisense probe. (**E**) Magnified floral meristem, antisense probe. BP, bract primordia; FM, floral meristem; IM, inflorescence meristem; YB, young bract. Bars = 100 µm.

Table 2 Phenotypes of various T_3 homozygous 35S::*SLF* transgenic lines of *Arabidopsis thaliana* and controls under long-day condition.

Genotypes	n	Days to flowering	Rosette leaves at flowering	Inflorescence branches after six weeks	Solitary flowers	Terminal flowers
35S::*SLF*1	40	13.2 ± 0.7*	5.9 ± 0.5*	1	+	+
35S::*SLF*2	39	13.3 ± 1.2*	6.2 ± 0.7*	1	+	+
35S::*SLF*3	40	11.7 ± 0.9*	4.0 ± 0.0*	1	+	+
35S::*SLF*4	37	13.4 ± 1.0*	5.7 ± 0.9*	1	+	+
35S::*SLF*5	38	10.3 ± 0.8*	2.0 ± 0.0*	7.4 ± 1.3	-	-
35S::*SLF*6	39	13.2 ± 0.8*	5.8 ± 1.1*	1	+	+
35S::*GFP*	36	29.6 ± 1.1	14.4 ± 0.7	3.3 ± 0.9	-	-
Wild type	40	28.7 ± 1.3	13.9 ± 1.2	3.2 ± 0.7	-	-

* indicates significance at 5% level (P value < 0.05) compared to the controls

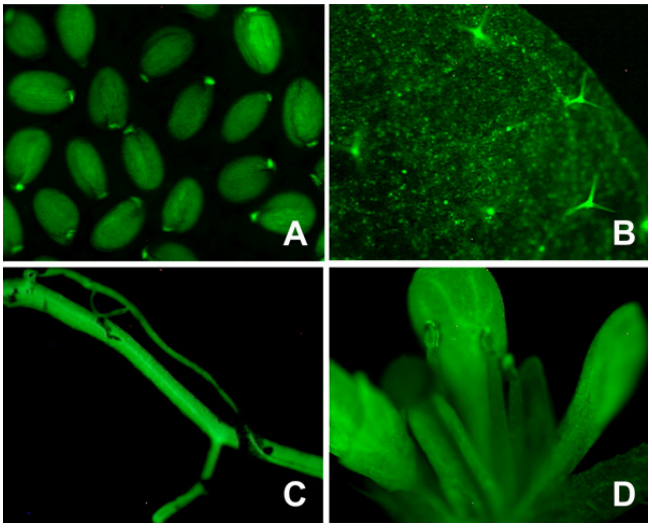

Fig. 4 GFP screens of different developmental stages and organs in T_3 35S::*SLF* *Arabidopsis thaliana* transgenic line. (**A**) Seeds. (**B**) Leaf. (**C**) Roots. (**D**) Flower.

and screened using GFP expression. A total of 45 positive transgenic T_1 lines were obtained, and all these were considered as independent transformation events. A total of ten T_2 lines were chosen based on the lines with brightest GFP expression and phenotypes such as early flowering, formation of terminal flowers, and conversion of inflorescence branches to solitary flowers. A total of six homozygous T_3 lines were isolated based on the number of seeds expressing GFP and their segregation ratios. For each of the six homozygous T_3 lines, 40 seeds were planted and grown under long-day condition. All organs including leaves, roots, and flowers were further screened for GFP expression (**Fig. 4**). There was no difference observed in the phenotypes of the wild-type and transgenic control line (35S::*GFP*, transformed with *GFP* only). This demonstrated that *GFP* was not responsible for the flowering response that was observed from the *SLF* transgenic lines. Compared to wild-type and the transgenic control line (**Fig. 5A**), the transgenic *SLF* lines displayed early flowering, formation of terminal flowers, conversion of inflorescence branches to solitary flowers, and formation of more inflorescence branches (**Fig. 5B-E**), indicating reiteration of *SLF* function.

Under long-day condition, floral buds in lines 35S::*SLF*1, 2, 3, 4, 5 and 6 were produced much earlier and with smaller number of rosette leaves compared to the controls (**Fig. 5B, Table 2**). Conversions of inflorescence branches to solitary flowers were also observed in these lines (**Fig. 5E, Table 2**), except in 35S::*SLF*5 where more inflorescences branches were produced compared to the controls (**Fig. 5D, Table 2**). Early flowering in lines 35S::*SLF*3 and 35S::*SLF*5 occurred with only four and two rosette leaves, respectively (**Fig. 5C, Table 2**).

To determine whether *SLF* can rescue the phenotype of a *lfy* mutant of *A. thaliana*, pollen grains were collected from each of three homozygous T_3 lines (35S::*SLF*) and used to pollinate several *lfy-6* individuals. The early-formed flowers of *lfy-6* have pistils but no petals and stamens (**Fig. 5F**). Compared to the wild-type, the shape of rosette leaves in *lfy-6* plants was relatively round with smooth margins (**Fig. 5G**). The phenotypes produced by the F_2 individuals demonstrated that *SLF* was able to rescue the floral phenotype of *lfy-6*. In F_2 plants, there were 11 out of 200 individuals showing the phenotype of *lfy-6*. All other plants had normal flowers (**Fig. 5H, 5I**). As we have predicted, many of the individuals form terminal flowers or more inflorescence branches. These results confirm that almost 1/16 individuals still display the phenotype of *lfy-6* because *SLF* gene is not expressed in their progenies. This is consistent with Mendel's rule of independent assortment.

To determine the *SLF* expression level in T_3 transgenic lines, northern analysis was done using a digoxigenin-11-UTP labeled probe. *SLF* was expressed at similar levels in all six T_3 transgenic lines (**Fig. 6**). In addition, based on the segregation of the T_2 generation (**Table 3**), the ratio of GFP expression was 3:1 in these transgenic lines. This confirms that only a single T-DNA insertion locus was present in each transgenic line.

DISCUSSION

Sequence analysis of *SLF*

The LFY/FLO protein has a proline-rich terminus region and an acidic central domain, which are involved as transcriptional activators (Coen *et al*. 1990; Weigel *et al*. 1992). Sequence analysis of LFY/FLO homologs from various plants also shows the presence of these two regions. PRFLL and NEEDLY from *P. radiata* have fewer proline residues in their proline-rich terminus region than any of the LFY/FLO proteins from angiosperms. Furthermore, the acidic region is absent in PRFLL and NEEDLY (Mellerowicz *et al*.

Table 3 Segregation ratios of GFP expression in the T_2 generation of six 35S::*SLF* transgenic lines.

Transgenic line	GFP positive	GFP negative	Segregation ratio	95% CI	p-value*
35S::*SLF*1	476	159	2.99:1	0.714, 0.782	1.000
35S::*SLF*2	602	199	3.03:1	0.720, 0.781	0.935
35S::*SLF*3	634	211	3.00:1	0.720, 0.779	1.000
35S::*SLF*4	491	162	3.03:1	0.717, 0.785	0.928
35S::*SLF*5	512	170	3.01:1	0.716, 0.783	0.965
35S::*SLF*6	487	161	3.02:1	0.716, 0.784	0.928

*Test of p = 0.75 vs p not = 0.75

The test is for the proportion of positive GFP expression that is equal to 0.75 vs not equal, and the results in all transgenic lines are not significant (p-value>0.888), which indicates that the data are consistent with the hypothesized 3:1 segregation ratio.

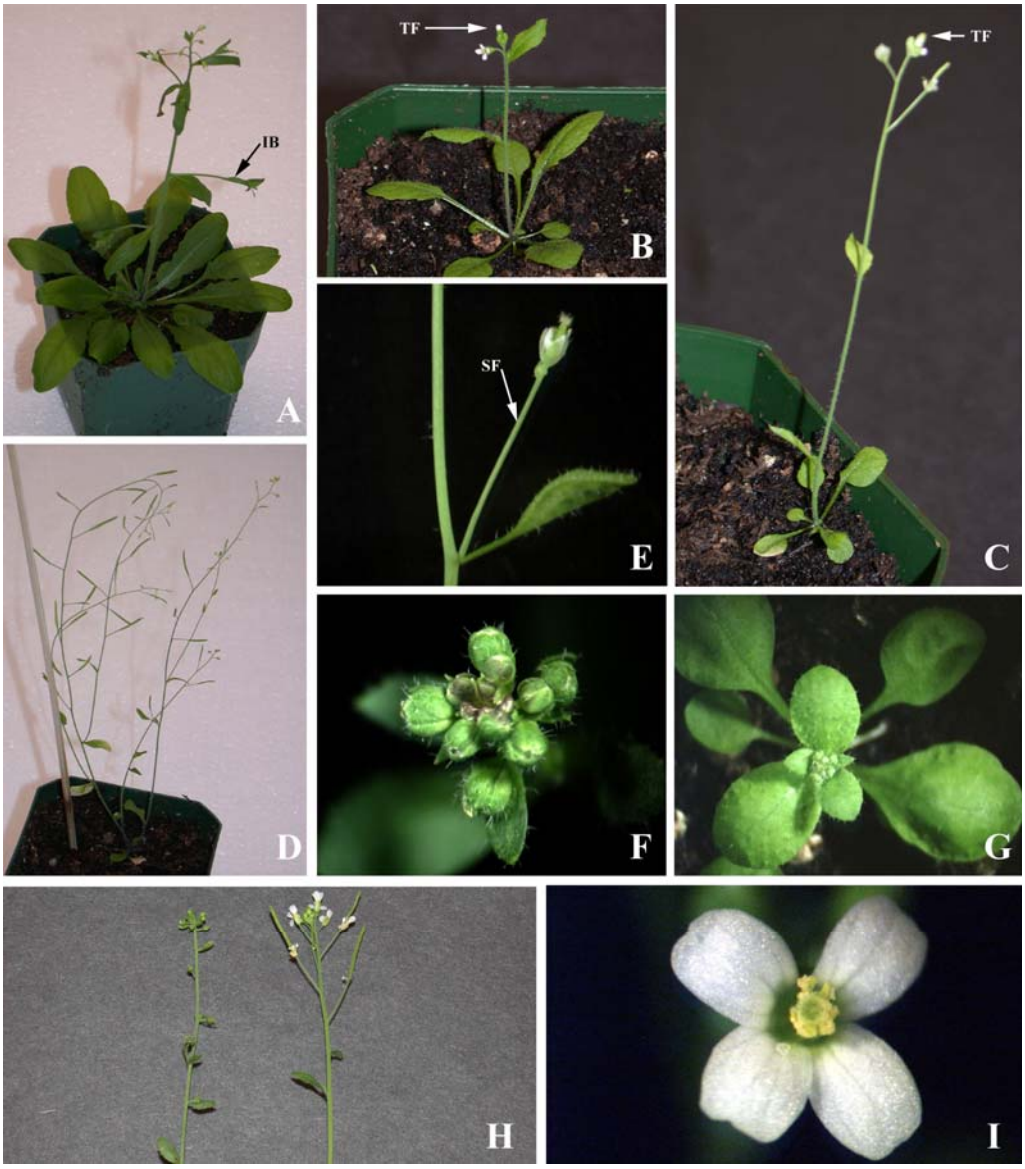

Fig. 5 Heterologous expression of *SLF* in *Arabidopsis thaliana* transgenic lines. (**A**) wild-type *Arabidopsis*. (**B**) 35S::*SLF*1 showing the formation of terminal flower and 5 rosette leaves before flowering. (**C**) Transgenic line 35S::*SLF*3 showing the formation of terminal flower and 4 rosette leaves before flowering. (**D**) Transgenic line 35S::*SLF*5 showing more number of inflorescence branches and early flowering. (**E**) The conversion of inflorescence branch to solitary flower. (**F**) The early formed flowers of *lfy-6* with no p*etal*s and stamens. (**G**) The rosette leaves of *lfy-6* showing round shape with smooth margins. (**H**) Comparison of floral phenotypes between *lfy-6* (left) and rescued plant (right) at F_2. (**I**) Early formed flowers of F_2 bearing petals and stamens. IB, inflorescence branch; TF, terminal flower; SF, solitary flower.).

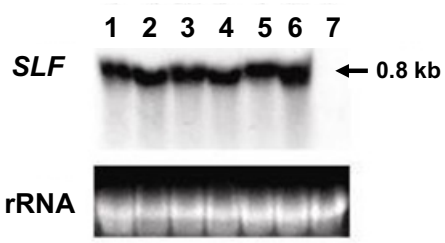

Fig. 6 Northern analysis in six T_3 *Arabidopsis thaliana* transgenic lines. Lane: 1, 35S::*SLF*1; 2, 35S::*SLF*2; 3, 35S::*SLF*3; 4, 35S::*SLF*4; 5, 35S::*SLF*5; 6, 35S::*SLF*6; 7, 35S::*GFP* (transgenic control line). Twenty µg total RNA was loaded per lane. Non-radioactive probe was used. NBT/BCIP color substrate solution was used for detection. The lower panel displays the rRNA of each sample to indicate equal loading amounts.

1998; Mouradov *et al*. 1998). The proline-rich region of LFY, FLO, and other angiosperm LFY/FLO homolog proteins has nine residues (Coen *et al*. 1990; Weigel *et al*. 1992; Kelly *et al*. 1995; Busch and Gleissberg 2003). However, SLF only has six proline residues. Seven proline residues were reported in PTLF (Rottmann *et al*. 2000). SLF and PTLF also both have a smaller number of acidic residues as compared to LFY/FLO. The alignment of the predicted amino acid sequences of SLF and PTLF shows that they share 97% similarity. SLF has a total of 375 residues, while PTLF had 377 residues. The difference is due to the presence of two asparagine residues at amino acid 134 and 135 in PTLF. Based on gene structure, the number and location of introns in *SLF* and *PTLF* are similar. However, the sequences and sizes of the introns are different.

SLF expression patterns in *S. discolor*

The localization of *SLF* expression in various parts of the male reproductive buds of willow shows that this gene is strongly involved in the formation of the inflorescence meristems, bracts, and floral meristems. *PTLF* is also strongly expressed in developing flowers of poplar (Rottmann *et al*. 2000). The expression of *SLF* was detected at low levels in the vegetative meristems and leaf primordia from lateral vegetative buds. *PTLF* was also detected at low levels in the vegetative meristem from terminal buds (Rottmann *et al*. 2000). It appears that gene expression between the lateral and terminal buds are similar. Both *LFY* and *FLO* are strongly expressed in the floral meristems prior to the initiation of floral organ primordia (Coen *et al*. 1990; Weigel *et al*. 1992). In leaf primordia, expression of *LFY/FLO* homologs has also been detected at low levels in *A. thaliana*, *Nicotiana tabacum*, and *Petunia* × *hybrida* (Weigel *et al*. 1992; Kelly *et al*. 1995; Souer *et al*. 1998), but not in *A. majus* (Coen *et al*. 1990) and *Cedrela fissilis* (Dornelas *et al*. 2006). In *P. radiata*, *NEEDLY* and *PRFLL* are expressed in the vegetative meristems, but not in the female cones (Mellerowicz *et al*. 1998; Mouradov *et al*. 1998). These studies show and confirm that there is variation in the expression patterns of *LFY/FLO* homologs in the vegetative and reproductive meristems among species.

SLF expression in *A. thaliana*

The functions of *LFY*/*FLO* homologs from several woody plants have been assessed through heterologous expression using *A. thaliana* or *N. tabacum* (Mellerowicz *et al*. 1998; Mouradov *et al*. 1998; Southerton *et al*. 1998; Rottmann *et al*. 2000; Wada *et al*. 2002; Carlsbecker *et al.* 2004; Dornelas and Rodriguez 2005; Dornelas *et al*. 2006). In willow, an *in vitro* regeneration protocol that is coupled with genetic transformation is currently not available. Therefore, *A. thaliana* was used to generate transgenic lines to characterize the functions of *SLF*. Overexpression of *SLF* in *A. thaliana* produced a total of six homozygous T_3 transgenic lines. The phenotypes of these transgenic lines are dominant and heritable. These demonstrate that *SLF* is functional and affects various aspects of flower development in transgenic *A. thaliana*.

Arabidopsis thaliana transformed with *PTLF* flowered an average of five days earlier with one fewer rosette leaf than the wild-type (Rottmann *et al*. 2000). Other than these, no marked difference was observed in the phenotypes of the transformed *A. thaliana* and the wild type (Rottmann *et al*. 2000). Using *SLF*, transformed *A. thaliana* lines showed dramatic changes in their flowering response, including the formation of terminal flowers and conversion of inflorescence branches to solitary flowers. Also, in all six 35S::*SLF A. thaliana* transgenic lines, flowering occurred more than two weeks earlier than the control plants. The discrepancy in the results between *SLF* and *PTLF* is surprising considering their very high sequence similarity. However, in a few *LFY/FLO* homologs with a high degree of amino acid sequence similarity different phenotypic effects have also been reported. *AFL1* and *AFL2* are 90% similar, but fewer solitary flowers occurred in *AFL1* transgenic *A. thaliana* than in *AFL2* (Wada *et al*. 2002). It is also possible that the presence of two 35S promoters in the pCGSLF binary vector increased *SLF* expression. Furthermore, it was reported that the 35S promoter driving the selectable marker gene could alter the expression of transgenes (Yoo *et al*. 2005).

Conversion of inflorescence branches to solitary flowers and formation of terminal flowers were produced in 35S::*SLF*1, 2, 3, 4, and 6 transgenic lines. These floral phenotypes were not observed in *A. thaliana* transformed with *PTLF* according to the report of Rottmann *et al*. (2000). However, transgenic *A. thaliana* expressing *NEEDLY* from *P. radiata* and *ELF1* from *Eucalyptus globulus* produced solitary flowers from axils of rosette leaves and terminal flowers from primary shoots (Mouradov *et al*. 1998; Southerton *et al*. 1998). Therefore, *SLF* appears to produce phenotypes typical of a *LFY/FLO* gene. More branches of primary inflorescence and inflorescence branches were produced in the 35S::*SLF*5 transgenic line. Since *SLF* is involved in inflorescence and flower development, overexpression of *SLF* can produce more inflorescences and flowers. Although these transgenic lines show different phenotypes, northern analysis demonstrated that *SLF* is expressed at similar levels in all these six transgenic lines. Based on the segregation analysis of the T_2 generation, the 3:1 ratio of seeds expressing GFP implies that the T-DNA was inserted in a single locus in all these lines; and such an approach is typically used in *A. thaliana* (Berbel *et al*. 2001; Honda *et al*. 2002). Although this still needs to be pursued, it is possible that the location of the T-DNA integration in the *A. thaliana* genome might be responsible for such a dramatic phenotype in the 35S::*SLF*5 transgenic line.

Introduction of *SLF* from T_3 homozygous *A. thaliana* transgenic lines into *lfy-6* mutants resulted in the rescue of the *lfy-6* floral phenotypes. The mutant *lfy-6* lacks both petals and stamens in the early-formed flowers. The rescue experiment showed a high degree of functional conservation between LFY/FLO and SLF. These results, and those of Dornelas and Rodriguez (2005) on rubber tree, are the only reports that described LFY/FLO homologs that are able to rescue the floral phenotype of a *lfy*mutant. On the other hand, various complementation tests using *LFY* homologs from herbaceous flowering plants (Weigel *et al*. 1992; Wada *et al*. 2002) and conifers (Mouradov *et al*. 1998; Carlsbecker *et al*. 2004; Dornelas *et al*. 2006) showed similar positive results when crossed with the *lfy-6* null allele.

This study has revealed that *SLF* from a male individual of *S. discolor* affects various aspects of flower development in transgenic *A. thaliana*. In fact, many of these phenotypes have not been reported from the study using a *LFY/FLO* homolog from a closely related dioecious species, *P. trichocarpa*. These results expand our understanding of the functions of *LFY/FLO* homologs and suggest that the *LFY/FLO* homolog from a male dioecious plant is equally functional to those from bisexual species. Unfortunately, female *S. discolor* was not available during our study.

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