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Research Article

A *FLORICAULA/LEAFY* gene homolog is preferentially expressed in developing female cones of the tropical pine *Pinus caribaea* var. *caribaea*

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Abstract

In angiosperms, flower formation is controlled by meristem identity genes, one of which, *FLORICAULA* (*FLO*)/*LEAFY* (*LFY*), plays a central role. It is not known if the formation of reproductive organs of pre-angiosperm species is similarly regulated. Here, we report the cloning of a conifer (*Pinus caribaea* var. *caribaea*) *FLO/LFY* homolog, named *PcLFY*. This gene has a large C-terminal region of high similarity to angiosperm *FLO/LFY* orthologs and shorter regions of local similarity. In contrast to angiosperms, conifers have two divergent genes resembling *LFY*. Gymnosperm FLO/LFY proteins constitute a separate clade, that can be divided into two divergent groups. Phylogenetic analysis of deduced protein sequences has shown that *PcLFY* belongs to the *LFY*-like clade. Northern hybridization analysis has revealed that *PcLFY* is preferentially expressed in developing female cones but not in developing male cones. This expression pattern was confirmed by *in situ* hybridization and is consistent with the hypothesis of *PcLFY* being involved in the determination of the female cone identity. Additionally, mutant complementation experiments have shown that the expression of the *PcLFY* coding region, driven by the *Arabidopsis LFY* promoter, can confer the wild-type phenotype to *lfy-26* transgenic mutants, suggesting that both gymnosperm and angiosperm *LFY* homologs share the same biological role.

Key words: *LEAFY*, plant reproduction, development, gene expression, flowering.

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Introduction

The molecular mechanisms underlying both intrinsic and extrinsic controls of pine shoot development are largely unknown. For both practical and scientific reasons, we are interested in mechanisms that bring about the formation of reproductive organs rather than vegetative shoots. The practical goal is to control flowering in this commercially important tropical forestry species. The knowledge of fundamental aspects of the reproductive development will allow us to genetically modify this process and to produce trees that flower earlier or are sterile. Genetic engineering of tropical pine, including regeneration of transgenic plants, has already been achieved (Walter *et al.*, 1998). A further scientific goal is to uncover the regulation of reproduction in pre-angiosperm species and gain understanding of the evolution of this process in plants.

Several genes involved in the vegetative-to-reproductive transition have been cloned in angiosperm annual species (Levy and Dean, 1998; Lohmann and Weigel, 2002; Izawa *et al.*, 2003). These genes can be loosely classified into one of the following three groups: (i) flowering-time genes that are involved in the vegetative-to-reproductive phase change by acting globally or locally in the shoot apical meristem; (ii) locally acting meristem identity genes that regulate the establishment of the floral meristem identity; and (iii) locally acting floral organ identity genes that regulate the identity of floral organs. These genes and their functions appear to be conserved in angiosperms, including monocotyledons (An *et al.*, 1994; Pnuelli *et al.*, 1994; Colombo *et al.*, 1996; Mena *et al.*, 1996). In contrast, there is very little information concerning pre-angiosperm plant species, in which only a few corresponding genes have been cloned (*Picea abies*, Tandre *et al.*, 1995; *Gnetum gnemon*, Münster *et al.*, 1997; *Pinus radiata*, Mouradov *et al.*, 1998; *Pinus radiata*, Mellerowicz *et al.*, 1998). The *LEAFY* (*LFY*) gene of *Arabidopsis*, or its -*Antirrhinum* ortholog *FLORICAULA* (*FLO*), is one of the key regulatory genes involved in both the control of the vegetative-to-reproductive phase change (Blázquez *et al.*, 1997) and the acquisition by axillary meristems of the floral meristem identity (Huala and Sussex, 1992; Weigel *et al.*, 1992; Weigel and Nilsson, 1995). Plants overexpressing

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LFY flower early, produce flowers in the place of lateral vegetative shoots and convert their otherwise indeterminate terminal shoot meristems to flowers (Weigel and Nilsson, 1995; Blázquez *et al*., 1997). These effects can also be observed in heterologous angiosperm species (Nilsson and Weigel, 1997), indicating that the function of *LFY* is largely conserved. Mutations in *FLO/LFY* cause the conversion of flowers into shoots. In *Arabidopsis*, male-sterile flowers are occasionally produced even in mutants homozygous for the strongest *lfy* alleles, indicating that a parallel pathway may specify floral meristem identity. In contrast, in *Antirrhinum*, mutations in *FLO* abolish flowering (Coen *et al*., 1990). Thus, the activity of the parallel pathway specifying floral meristem identity can vary among species. This paper reports cloning and expression studies of a *Pinus caribaea* var. *caribaea FLO/LFY* homolog named *PcLFY*. In *Pinus*, genes *NEEDLY (NLY)* and *PRFLL* were reported, representing two divergent *Pinus radiata LFY* homologs (Mouradov *et al*., 1998; Mellerowicz *et al*., 1998). Sequence comparisons indicate that PRFLL and NLY represent divergent proteins, and that conifers, in contrast to angiosperms, have two *LFY*-like paralogs. *PRFLL* is largely expressed in buds and male cones but not in female cones or other somatic tissues, while *NLY* is preferentially expressed in female cones (Mouradov *et al*., 1998; Mellerowicz *et al*., 1998). We performed a detailed spatial and temporal analysis of the *P. caribaea PcLFY* gene, using Northern and *in situ* hybridization analysis, and our results were consistent with *PcLFY* being involved in female cone identity determination. Additionally, our experiments demonstrated that *PcLFY* can complement the *lfy-26 Arabidopsis* mutant, which shows the strongest mutant phenotype, suggesting that both pine and *Arabidopsis LFY* homologs may share the same biological role.

Material and Methods

Plant material

Material for genomic DNA and RNA extraction and for microscopy of vegetative and reproductive tissues of *Pinus caribaea* var. *caribaea* was collected in the fields of Escola Superior de Agricultura Luiz de Queiroz, of the University of São Paulo (Piracicaba, SP, Brazil). Young expanding needles were collected for the extraction of genomic DNA. RNA-blot, *in situ* hybridization and SEM analyses were performed on plant tissues collected and fixed at different developmental stages during two growing seasons corresponding to the years 2000 to 2003.

DNA and RNA extraction, library construction and gene cloning

Total RNA for cDNA library construction and Northern experiments was isolated from young pine leaves (needles), vegetative apices, developing male and female cones at different developmental stages and roots of recently germinated seeds (germinated on wet paper in the dark for 4 days), using the RNeasy plant Minikit (QIAGEN) according to the manufacturer's instructions. Genomic DNA for PCR amplification, Southern analysis and construction of genomic libraries was isolated by the traditional CTAB-based method (Sambrook *et al*., 1989).

The genomic clones of *PcLFY* were isolated by screening 300,000 plaques from a *P. caribaea* genomic library (76 x 10^{-6} pfu) constructed with partially *Sau3*A-digested genomic DNA, using the Packagene Lambda Packing Systems (Promega). This screening was performed with a biotin-labeled probe (North2South chemiluminescent system, Pierce), using the entire *Arabidopsis LFY* cDNA from plasmid pDW124 (Weigel *et al*., 1992) as template. Two adjacent *Bam*HI fragments (P45B with 3.35 kbp, and P65B with 6.4 kbp) containing the genomic *PcLFY* sequence were subcloned into pBluescriptKS (Clontech). Subclones were prepared by nested deletions (as described by Zhu and Clark, 1995) and sequenced on an ABI Prism 377 automated sequencer (Perkin-Elmer/Applied Biosystems), using the DYEnamic ET terminator Cycle Sequencing Kit (Amersham/ Pharmacia Biotech, USA) coupled with M13 reverse and forward primers according to the manufacturer's instructions. The complete *PcLFY* genomic sequence was deposited in the GenBank under the accession number AY640315.

A cDNA library was constructed using total RNA from a mix of male and female cones at different developmental stages. The poly-A fraction RNA was isolated (Sussman *et al*., 2000), and the first strand of cDNA was synthesized using the SuperScript cloning system (Life Technologies). The cDNA library screening was performed using a PCR-based strategy (Sussman *et al*., 2000) and the *LFY*-specific degenerated primers L1: 5'-CGGAY ATIAAYAARCCIAARATGMGICAYTA-3'and L4: 5'-C GGATCCGTGICK-IARIYKIGTIGGIACRTA-3' (Frohlich and Meyerowitz, 1997). The insert sizes of the positive clones were determined by PCR using the M13 forward and reverse primers, and the five positive clones were sequenced on both strands. The longest *PcLFY* cDNA sequence was deposited in the GenBank under the accession number AY640316.

Southern and Northern hybridization

Southern blotting was performed as described in Sambrook *et al*. (1989), using genomic DNA digested with *Xho*I and *Pst*I and blotted on a Hybond-N Plus membrane (Amersham). Northern experiments were performed using 10 μg of total RNA extracted from male and female developing cones (a mix of different developmental stages), vegetative apices and young needles, separated in a denaturating agarose gel (Sambrook *et al*., 1989) and hybridized to a *PcLFY* probe.

The *PcLFY* probe used in both Southern and Northern experiments was a 235bp PCR product obtained from the 3'

transcribed region of the gene, using the primers P13: 5'-CTCCAAGTGACAGAGCTGACG-3' and P25: 5'-CT GCTGGATGTGCAACAT-3', and a *PcLFY* cDNA clone as template. PCR reactions were performed in a final volume of 25 µL with an initial 3 min denaturation cycle at 96 °C, followed by 40 cycles of 96 °C for 40 s; 45 °C for 30 s, and 72 °C for 2 min. The PCR product was purified using the CONCERT Rapid PCR Purification System (Gibco-Life Sciences). The probe was labeled with fluorescein, using the DCP-Star GeneImage System (Pharmacia-Amersham). Hybridization conditions, washing stringencies and detection were those suggested by the kit manufacturer. As a control for gel loading in Northern experiments, the stripped membrane was re-hybridized with a heterologous probe for a constitutively expressed gene, under low stringency, using cDNA for an *Arabidopsis* ubiquitin as template (GenBank accession AB5432).

In situ hybridization

Preparation of slides, digoxigenin-labeling of RNA probes, and hybridization were performed as described elsewhere (Dornelas *et al*., 1999; 2000). A high-stringency hybridization condition was achieved using 50% formamide in the hybridization solution and washes with up to 0.1 % SSC at 55 °C. The template for the *PcLFY* digoxigenin-labeled probe was the 1,498 bp cDNA fragment containing the complete coding region, cloned in pGEM-T vector. The hybridized sections were viewed immediately and photographed under a Zeiss Axiovert 35 microscope.

Microscopy

All plant material collected for microscopy was immediately fixed in 4% paraformaldehyde under vacuum for 24 h and dehydrated to absolute ethanol, where it was stored at 4 °C until needed. For light microscopy, the dehydrated samples were embedded in Historesin (Leica, 2-hydroxyethyl-methacrylate). Resin polymerization was carried out at room temperature for 48 h. After polymerization, serial sections of 5-8 µm were obtained and stained with 0.05% toluidine blue (Dornelas *et al*., 1992). The histological sections were observed and photographed under a Zeiss Axiovert 35 microscope.

Alternatively, the plant material was initially dissected in absolute ethanol under an Olympus dissecting microscope. The resultant material was critical point-dried with CO_2 in a Balzer's drier and further dissected, when necessary. The samples were mounted in metallic stubs with carbon conductive adhesive tape, coated with colloidal gold and observed at 10-20 kV using a ZEISS DSM 940 A or a LEO 435 VP scanning electron microscope.

Sequence comparisons

The partial *PcLFY* sequences obtained were manipulated in a standard word processor and aligned using ClustalW (Thompson *et al*., 1994), before being checked for similarity with sequences already deposited in public databases, using BLASTX (Altschul *et al*., 1997). The complete nucleotide and protein sequences of different *LFY* homologs were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and aligned with the entire nucleotide and deduced protein sequences of *PcLFY*, using ClustalW (Thompson *et al*., 1994). Distance matrixes were obtained from the alignments, and comparative trees were built using TreeView (Page, 2000). Alternatively, parsimony analysis was performed using PAUP (Swofford, 1998).

Complementation of the *Arabidopsis lfy-26* mutant

The *Xba*I–*Sma*I *PcLFY* fragment, carrying the coding region of *PcLFY*, with its endogenous start and stop codons, was obtained from plasmid pPCLFY and blunt-ended using DNA polymerase I (Klenow fragment). An intermediate pDW132P vector was prepared by cloning the polished fragment described above into the *Sma*I site of pDW132, containing the *Arabidopsis LFY* promoter (a gift from D. Weigel, Salk Institute, LA Jolla CA, USA). The correct orientation of the cloning process was checked by endonuclease digestion. The *Pst*I-*Spe*I fragment from the resultant pDW132P (*LFY::PcLFY*) vector was blunt-ended with Klenow and cloned into the plant transformation vector pSKI015 (a gift from D. Weigel, Salk Institute, LA Jolla CA, USA), that contains the bar gene, allowing selection with the herbicide Basta (Sylvet), constituting the pSKI015P vector. *Arabidopsis* plants (Columbia ecotype) transgenic for pSKI015E T-DNA were generated by using *Agrobacterium tumefacien*s-mediated *in planta* transformation, as described by Bechtold and Pelletier (1998). Putatively transformed seeds were selected upon germination on sand wetted with a Basta (Sylvet) solution at 500 $\mu L.mL^{-1}$. Homozygous (Basta-resistant) lines were created by selfing. The resistant:sensitive segregation ratio was used to estimate the number of transformed T-DNA loci. T2 lines, homozygous for the *LFY*::*PcLFY* T-DNA loci, were identified by sowing 200-300 T2 seeds, derived from different T1 plants under selective conditions. Transgenic and non-transgenic plants were grown in growth chambers at 23 °C under illumination with fluorescent light: long-day (LD) conditions (16 h of light/8 h of darkness) or short-day (SD) conditions (8 h of light/16 h of darkness). Finally, *LFY::PcLFY* transformants in the Columbia ecotype were crossed to the strong *lfy-26* mutant allele in the Landsberg *erecta* background (wild-type and mutant *Arabidopsis* seeds were obtained from the ABRC seed stock at the Ohio State University facility at Columbus, Ohio, USA). To genotype F2 plants at the *LFY* locus, CAPS (Cleared Amplified Polymorphic Sequences; Konieczny and Ausubel, 1993) markers that distinguished between Columbia and Landsberg were used (URL: http://www.salk.eduyLABSypbio-wycaps.html). Transgenic and

non-transgenic *Arabidopsis* flowers and inflorescences at different developmental stages were photographed under a stereomicroscope or analyzed by SEM.

Results

Shoot and female cone development in *Pinus caribaea* var. *caribaea*

Vegetative and reproductive development in *Pinus* species is regulated by an integrated system of ontogenetic, positional and environmental factors. These factors determine the rate of formation, the type and the differentiation of primordia produced at the flanks of the apical meristem and in axils (Doak, 1935). In the juvenile stage, the meristem continuously produces primary needle primordia that elongate and form photosynthetic organs (Figure 1A). Some of the primary needle primordia are accompanied by axillary meristems that form either long or short shoot primordia (Riding, 1972). Long shoot primordia give rise to branches, whereas short shoot primordia form secondary needles while their apical meristems cease to function. As pine seedlings pass into the adult stage, the primary needles shorten and develop into scale-like cataphylls (Doak, 1935). Some cataphylls become bud scales, enclosing both apical and axillary meristems together with associated primordia and forming closed buds (Figure 1B and E). Bud growth becomes cyclic, with sequential formation of various types of primordia within each cycle. The number of cycles produced annually and bud morphology vary among pine species (Doak, 1935). The apical meristem produces bud scales and bracts subtending axillary short and long shoot primordia (Figure 1B and E). In addition, two new kinds of axillary primordia are formed: the male and female cone primordia. Male cone primordia usually develop in buds on subordinate branches located within the lower crown. These buds produce only one growth cycle during a year. Female cone primordia develop in buds on dominant branches located within the upper crown. These buds produce up to five growth cycles each year (Doak, 1935). Typically, female cones (Figure 1D and F) are found in the first two cycles initiated in the summer (Bollman, 1983). During each cycle, the meristem produces primordia in a defined sequence (Doak, 1935). Male and female cone primordia are not present in all buds, indicating that the formation of reproductive primordia in mature plants is regulated by some other factors, in addition to the ontogenetic stage and position. These factors probably include environment and gibberellins (Cecich *et al.*, 1994).

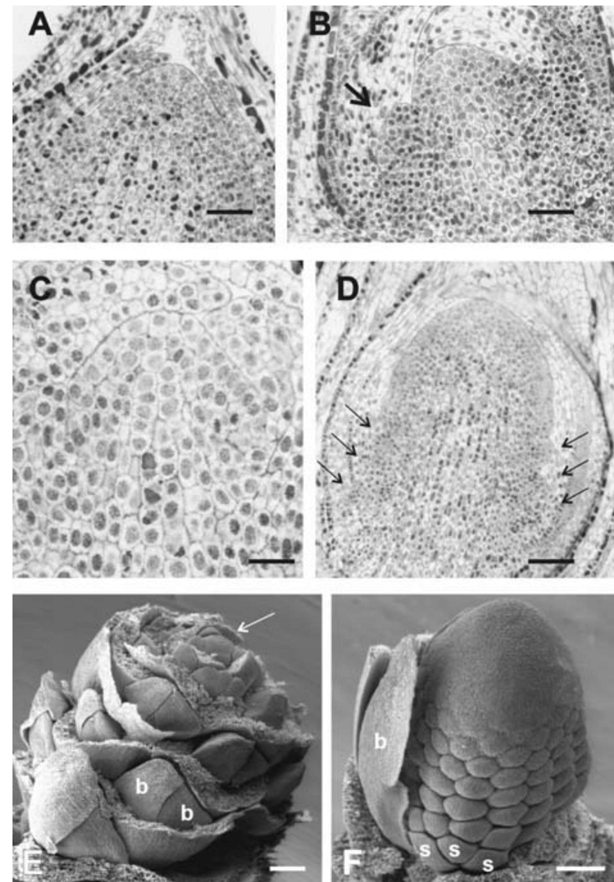

Figure 1 - *P. caribaea* var. *caribaea* vegetative and reproductive meristem development. A-D: Toluidine blue-stained longitudinal sections. E and F: SEM. Apical vegetative meristem of a juvenile (1 year-old) plant. B: Apical meristem of an adult (15 year-old) plant. The arrow indicates a lateral meristem. C: Early-developing female cone. The oviliferous scale primordial still not visible. D: Developing female cone at the onset of the oviliferous scales (arrows) E: Long-shoot apex of an adult (15 year-old) plant. The arrow points to the terminal meristem. Bracts (b) cover and protect the subordinate branch lateral meristems. F: Developing female cone at the onset of the oviliferous scales, at approximately the same stage as that shown in D. b: bract primordium s: oviliferous scale primordium. Bars: A and B: 150 μm; C: 100 μm; D and F: 200 μm.

Cloning and sequence analysis

Primary screening of 15,000 cDNA clones of a *P. caribaea* var. *caribaea* reproductive tissue library using a PCR approach (Sussman *et al.*, 2000) identified five positive clones that were sequenced on both strands. All the five clones contained inserts of over 1.5 kb, with identical open reading frames of 1233 bp coding for a 411-amino acid protein. BLASTX searches with PcLFY against public database identified a group of highly similar proteins, including PRFLL and NLY from radiata pine (Mouradov *et al.*, 1998; Mellerowics *et al.*, 1998), an LFY-like sequence of *Populus balsamifera* (L.) (U 93196), NFL1 and NFL2 from *Nicotiana tabacum* (L.) (Kelly *et al.*, 1995), LFY from *A. thaliana* (Weigel *et al.*, 1992), FLO from *Antirrhinum majus* (Coen *et al.*, 1990), and BOFH from *Brassica oleracea* (L.) (Anthony *et al.*, 1993). Some other gymnosperm sequences were also retrieved from the databases and from published work (Frohlich and Parker, 2000). Alignments of the deduced amino acid sequence of PcLFY and other LFY-like proteins revealed that PcLFY shared with these sequences two major regions of conservation: amino acids 61-126 and amino acids 247-406 (50% and 81% identity, and 75% and

88% similarity, respectively, to the LFY protein). It also had short regions of local similarity (Figure 2). The overall resemblance to the LFY protein was 53% identity and 58% similarity. The overall resemblance to the NLY and PRFLL protein was 72% identity and 94% similarity. Despite these similarities, the *P. caribaea* LFY-like protein clearly differed from its angiosperm counterparts. All pine sequences (PcLFY, PRFLL and NLY) had fewer proline residues in their proline-rich region (ca. first 40 amino acids) than any of the angiosperm proteins. The acidic region composed primarily of glutamic acid (E) and aspartic acid (D) residues (amino acids 207-219 of the LFY protein) was absent in pine. The less conserved 5' region was longer and the more conserved 3' region was shorter in pine species when compared to angiosperms.

Evolutionary relationships

A maximum parsimony consensus tree was constructed to access the phylogenetic relationships of PcLFY with its other gymnosperm and angiosperm counterparts (Figure 3). PcLFY is closely related to *P. radiata* PRFLL, but the distance between PcLFY and NLY exceeded the distance between most divergent angiosperm LFY orthologs. PcLFY was slightly closer to angiosperm proteins than NLY (distances 60-69% *vs.* 64-72%). When we considered only the gymnosperm LFY-like homologs, it became apparent that the gymnosperm sequences formed two groups: one containing NLY, and the other containing PcLFY and PRFLL. The phylogenetic relations of PcLFY with other plant LFY-related proteins revealed three facts. First, the gymnosperm sequences formed a group distinct from angiosperms. Second, the sequences of PcLFY and NLY were more divergent than any two angiosperm LFY orthologs. Third, the gymnosperm sequences formed two groups, named LFY-like and NLY-like. The mostly male theory of the origin of angiosperm flowers (Frohlich, 2003) assumes that the co-expression of genes responsible for the identity of male and female organs in a same structure would be sufficient to produce bisexual flowers in the angiosperm ancestor. Thus, the presence of two homologs of LFY in gymnosperms and of only one in most angiosperms (probably due to the loss of the NLY-like lineage) would be

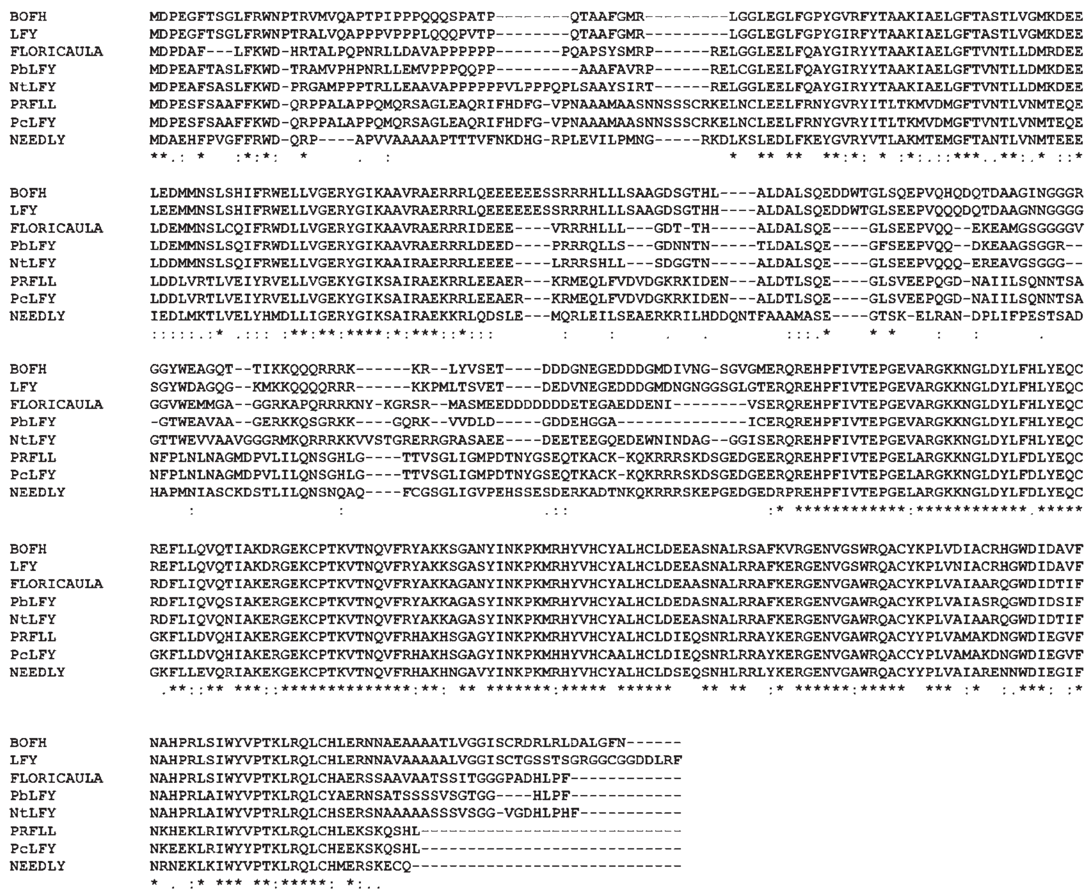

Figure 2 - Alignment of deduced amino acid LFY-like complete sequences with PcLFY. Sequences include: NEEDLY and PRFLL from radiata pine (Mouradov *et al.*, 1998; Mellerowicz *et al.*, 1998); PbLEAFY: LFY-like protein of *Populus balsamifera* (GenBank accession number U93196); NtLFY from *Nicotiana tabacum* (Kelly *et al.*, 1995); Leafy from *Arabidopsis thaliana* (Weigel *et al.*, 1992); FLO from *Antirrhinum majus* (Coen *et al.*, 1990), BOFH from *Brassica oleracea* (Anthony *et al.*, 1993). Identical residues are marked by asterisks, similar residues are marked by dots, gaps are marked by dashes.

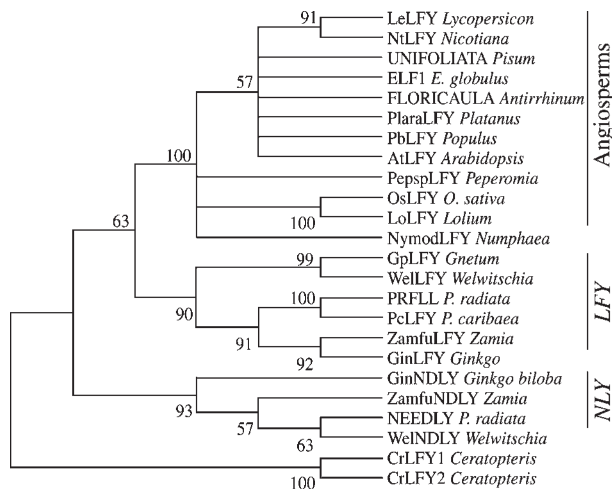

Figure 3 - Maximum parsimony consensus tree, built with the PAUP program, of deduced amino acid sequences of the complete LFY-like proteins. Numbers above the nodes indicate percent of trees with each node in a bootstrap analysis of 1000 random trees. Only the nodes present in 50% or more trees are drawn.

a key observation to corroborate the mostly male theory (Frohlich and Parker, 2000; Frohlich, 2003)

Gene copy number determination and expression analyses

To establish whether the *PcLFY* gene is a single-copy gene or part of a multigene family, we performed Southern Blot hybridization using digested *P. caribaea* genomic DNA. The hybridization pattern obtained using low stringency washes (2xSSC, 42 °C) suggested that an additional *PcLFY*-like gene may exist in the *P. caribaea* genome (Figure 4A). However, using more stringent washes (*i.e.*, 0.1 x SSC, 65 °C), a single band was detected in each lane (data not shown). We also used the higher stringent conditions (*i.e.*, 0.1 x SSC, 65 °C) to perform a Northern Blot analysis (Figure 4B), aiming to determine the steady-state *PcLFY* mRNA level in vegetative and reproductive tissues of *P. caribaea*. A single band corresponding to the transcript size of about 1.5 kb was observed, predominantly in developing female cones. This size corresponded to the cloned cDNA size and the transcript size of angiosperm *LFY* orthologs (Coen *et al*., 1990; Weigel *et al*., 1992; Kelly *et al.*, 1995; Anthony *et al*., 1993).

The ontogenetic pattern of *PcLFY* expression was further studied by *in situ* hybridization, in longitudinal sections of shoot apices of adult (15 years-old) plants (Figure 5). Low levels of the *PcLFY* transcript were found in all types of meristems, but the hybridization signals were higher in developing female cones (Figure 5). With the Northern hybridization analysis, where the level of steady-state RNA was much lower in developing male cones and vegetative buds than in the developing female cones, some degree of expression was observed in vegetative tissues and male cones by *in situ* hybridization. The reason for that was

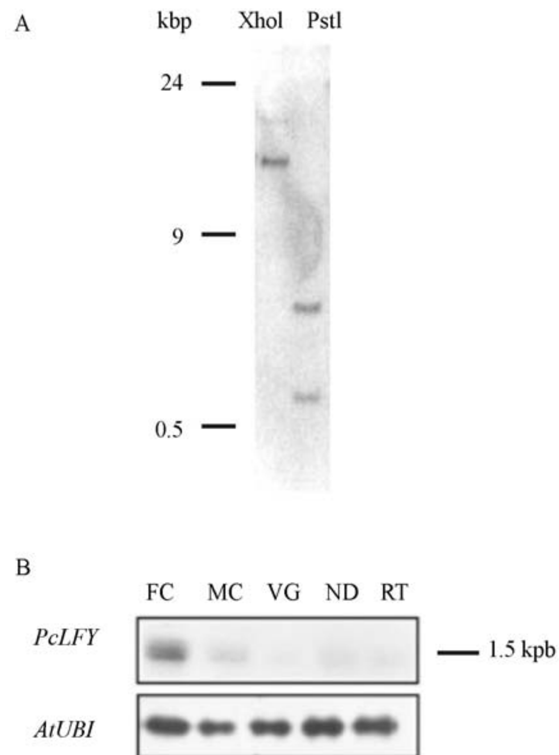

Figure 4 - A. Southern blot of genomic DNA from *P. caribaea* var. *caribaea* probed with *PcLFY*. Lane 1: digested with *Xho*I; lane:, digested with *Pst*I. **B**. Northern blot made with total RNA extracted from developing female cones (FC), developing male cones (MC), vegetative apices of juvenile plants (VG), young leaves (needles) of adult plants (ND), and seedling roots (RT), probed with *PcLFY*. The same blot was re-probed at low stringency with a heterologous *Arabidopsis* ubiquitin sequence (AtUBI) to show uniform loading and transfer of all lane contents.

probably a dilution of the peripheral bud tissues by very large pith and vascular tissue in the dominant mature buds, or maybe the probe and the stringency used in the hybridizations were not specific enough. No *PcLFY* transcript was detected in differentiating bracts or needles.

Complementation of *Arabidopsis lfy* mutants by the expression of *PcLFY*

The coding region of the *PcLFY* cDNA was fused downstream to the *Arabidopsis LFY* promoter and used to transform *Arabidopsis* plants that were crossed to the strong-phenotype *lfy-26 Arabidopsi*s mutant. Upon identification of homozygous transgenic mutant plants (verified by CAPs genotyping, data not shown, Konieczny and Ausubel, 1993), their phenotype was analyzed. Complete restoration of the wild-type phenotype was observed (Figure 6). Early-arising (basal) flowers were replaced by bracts with secondary inflorescence shoots in the *Arabidopsis lfy-26* mutants, whereas later arising flowers were replaced by small bracts, in whose axils abnormal flowers developed (Figure 6B and C; Weigel *et al*., 1992). These abnormal flowers contained sepals and carpels but

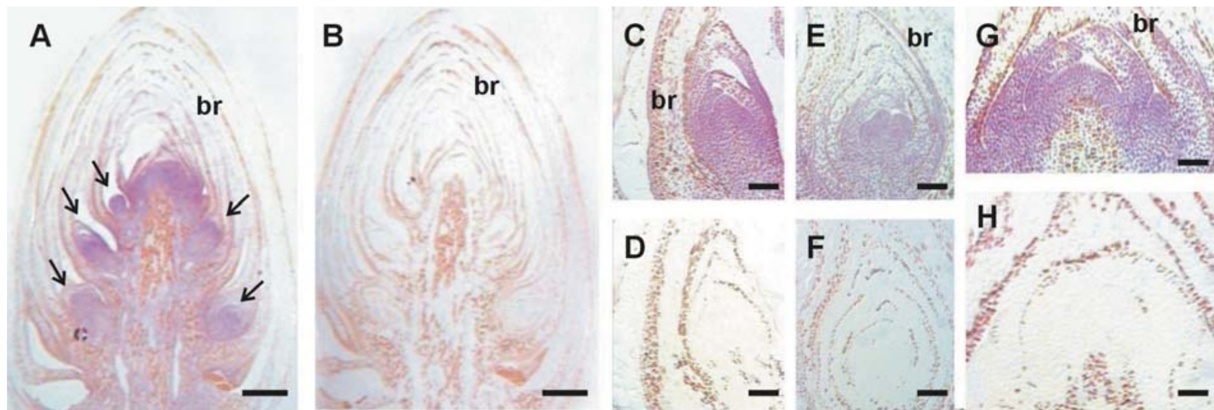

Figure 5 - *In situ* localization of *PcFY* transcripts. All sections are longitudinal. The pink-purple color indicates the hybridization signal. Hybridizations were performed with an antisense probe in A, C, E and G. In B, D, F and H a sense probe was used. In this case, no hybridization signal was observed above background level. A and B: Long-branch apex during the initiation of axillary meristems (arrows) on the side of apical meristem; C and D: developing short-branch female cone with initiating bract primordia (br). E and F: developing short-branch male cone with initiating bract primordia (br). G and H: Detail of an apical meristem of an adult (15 years-old) plant. Bars: A and B: 600 μm; C-F: 250 μm; G and H: 100 μm.

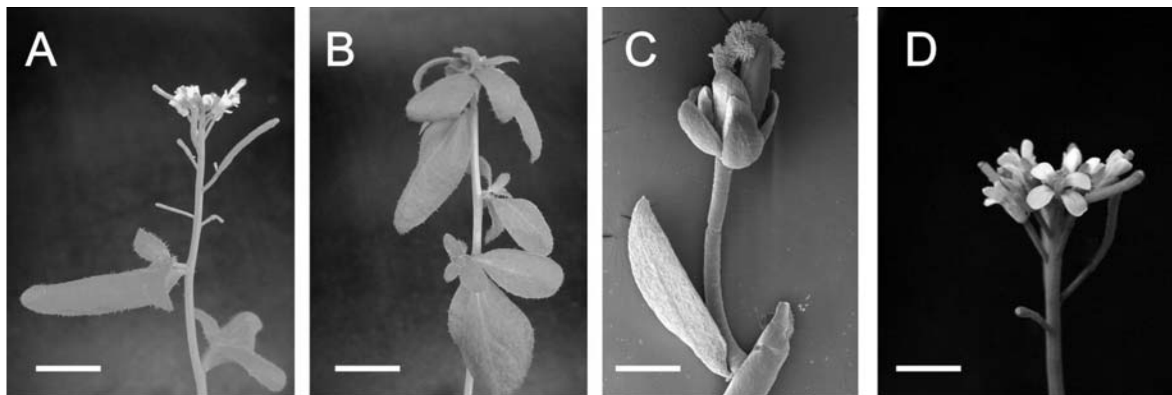

Figure 6 - The *PcLFY* expression driven by the *LFY* promoter can complement an *Arabidopsis* strong *lfy* mutation. A: wild-type inflorescence (Columbia ecotype) showing flower buds at different developmental stages. B: main inflorescence axis of an *lfy-26* mutant. Solitary flowers are replaced by a cauline leaf (bract) adjacent to a lateral inflorescence axis or an abnormal flower. C: SEM image of an abnormal flower of the *lfy-26* mutant. Note the cauline leaf and the homeotic conversion of petals and stamens in sepal-like organs and carpel-like organs, respectively. D: A homozygous transgenic (LFY::*PcLFY*) *lfy-26* mutant showing a wild-type phenotype, indicating the complementation of the *lfy* mutation by the expression of *PcLFY*. Bars: A and B: 6 mm; C: 400 μm; D: 3 mm.

no petals or stamens, these later being usually homeotically substituted by more sepals and carpels, respectively (Figure 6C; Weigel *et al*., 1992). In contrast, wild-type flowers typically contain four sepals, four petals, six stamens and two carpels. The *lfy*-26 floral phenotype was largely complemented by the *LFY*::*PcLFY* transgene. The main shoot of these plants developed flowers in both basal and apical positions, and most of these contained all four floral organ types (Figure 6D).

Discussion

The LFY homologs have been consistently reported to be involved in flower formation in angiosperms (Lohmann and Weigel, 2002). The existence of its homologs in pre-angiosperm plant species that do not form flowers was confirmed by cloning of cDNAs from some gymnosperm species (Mouradov *et al*., 1998; Mellerowicz *et al*., 1998; Frohlich and Parker, 2000). Phylogenetic analysis (Figure 3) has demonstrated that gymnosperm sequences form a separate clade from angiosperms and that gymnosperms have, in contrast to angiosperms (Kelly *et al*., 1995), two paralogous genes resembling *FLO/LFY* (see also Frohlich and Parker, 2000). These two genes, represented by *PcLFY* and *NLY*, had rather divergent sequences, yet they shared some features that distinguished them from their angiosperm counterparts. The existence of two divergent *LFY*-related gene lineages in gymnosperms and the presumptive loss of one of these lineages in angiosperms may be one of the causative forces of the origin of bisexual flowers in angiosperms (Frohlich, 2003). Proline-rich and acidic domains in LFY and FLO (Coen *et al*., 1990; Weigel *et al*., 1992), whose presence indicates that these proteins

are transcription factors, were not evident in conifers (Figure 2). In contrast, the C-terminal part of the protein, whose function has not been elucidated so far, was highly conserved between angiosperms and conifers. The significance of differences between angiosperms and conifers will become clear when the LFY protein is functionally analyzed by deletion and domain-swamping experiments.

As in angiosperm species, *PcLFY* was preferentially expressed in reproductive tissues and was not detected in fully differentiated vegetative tissues (Figures 4B and 5; Kelly *et al*., 1995; Anthony *et al*., 1993) Mouradov *et al*. (1998) have shown that *NLY* was highly expressed only in the very early developing female reproductive structures, before any specifically female attributes have arisen. In contrast, Mellerowicz *et al*. (1998) reported the expression of *PRFLL* in vegetative buds and in the early stages of the male reproductive cone. Thus, the *PcLFY* expression pattern may be considered intermediary between the *NLY* and *PRFLL* expression patterns, as it is expressed in the early stages of the female reproductive cone as well as in the vegetative buds (Figures 4B and 5). The published data on ontogenetic changes in *LFY* expression in vegetative meristems of *Arabidopsis* are contradictory (Blázquez *et al*., 1997; Hempel *et al*., 1997), indicating that factors other than maturation affect the level of expression more directly.

The biological role of the expression of *PcLFY* or angiosperm *LFY* orthologs in vegetative buds is still unclear. Whereas neither lesions in LFY or FLO (Coen *et al*., 1990; Huala and Sussex, 1992; Weigel *et al*., 1992) nor ectopic over-expression of *LFY* (Weigel and Nilsson, 1995; Nilsson and Weigel, 1997) affected vegetative development in several angiosperms, it might not be the case in more primitive species. If the flower is homologous to an entire shoot of a cordaite-like progenitor (Hickey and Taylor, 1996), LFY function might have evolved from a gene that controls some aspects of shoot development, for example cell determinacy (Kelly *et al.*, 1995; Hofer *et al.*, 1997). Regardless of the ground level of *LFY* expression, floral stimulus, be it photoperiod, far-red light treatments, or gibberellin application, was reported to rapidly up-regulate the expression of this gene in diverse angiosperms (Coen *et al*., 1990; Weigel *et al*., 1992; Carpenter *et al*., 1995; Anthony *et al*., 1993; Blázquez *et al*., 1997; Hempel *et al*., 1997). The up-regulation preceded the attainment of developmental commitment to flower (Hempel *et al*., 1997), and the level of LFY activity determined how rapidly plants started producing flowers (Blázquez *et al*., 1997). These data are consistent with LFY being a necessary and quantitative, but not always sufficient, factor of floral determination. Our observations of high *PcLFY* expression in buds with undifferentiated, and therefore possibly not determined, female cone primordia is consistent with PcLFY playing an analogous role in the determination of the female cone primordium identity. In contrast, PcLFY does complement the defects observed in male reproductive development in *Arabidopsis lfy*-26 mutants (Figure 6). It is thus tempting to speculate that two LFY-like conifer paralogs represented by PcLFY and NLY have separate roles in the reproductive determination of separate male and female reproductive organs. Unlike unisexual angiosperm flowers that have a perfect flower ancestry, conifer cones evolved as truly unisexual organs. It is conceivable that separate meristem identity genes were involved in the determination of male and female organs, and that the function of female organ determination became redundant when the perfect flower evolved. Future comparative *in situ*-expression studies, analysis of transgenic plants, and studies of a broad range of pre-angiosperm species are likely to resolve these questions.

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