EgLFY, the *Eucalyptus grandis* homolog of the *Arabidopsis* gene *LEAFY* is expressed in reproductive and vegetative tissues

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The *EgLFY* gene cloned from *Eucalyptus grandi*s has sequence homology to the floral meristem identity gene *LEAFY (LFY)* from *Arabidopsis* and *FLORICAULA (FLO)* from *Antirrhinum*. *EgLFY* is preferentially expressed in the developing eucalypt floral organs in a pattern similar to that described previously for the *Arabidopsis LFY*. *In situ* hybridization experiments have shown that *EgLFY* is strongly expressed in the early floral meristem and then successively in the primordia of sepals, petals, stamens and carpels. It is also expressed in the leaf primordia of adult trees. The expression of the *EgLFY* coding region under control of the *Arabidopsis LFY* promoter could complement strong *lfy* mutations in transgenic *Arabidopsis* plants. These data suggest that *EgLFY* plays a similar role to *LFY* in flower development and that the basic mechanisms involved in flower initiation and development in *Eucalyptus* may be similar to those occurring in *Arabidopsi*s.
Key words: *Eucalyptus*, flowering, *LEAFY*, reproductive development.

***EgLFY*: O homólogo em *Eucalyptus grandis* do gene *LEAFY* de *Arabidopsis* é expresso em tecidos vegetativos e reprodutivos:** O gene *EgLFY*, clonado de *Eucalyptus grandis*, possui homologia de seqüência com o gene de identidade meristemática *LEAFY* (*LFY*) de *Arabidopsis* e *FLORICAULA* (*FLO*) de *Antirrhinum*. *EgLFY* é, preferencialmente, expresso nos órgãos florais em desenvolvimento de eucalipto, obedecendo a um padrão similar ao descrito para o gene *LFY* de *Arabidopsis*. Experimentos de hibridização *in situ* mostraram que o gene *EgLFY* é fortemente expresso em meristemas florais no início de seu desenvolvimento e, então, sucessivamente, durante a formação dos primórdios de sépalas, pétalas, estames e carpelos. Há expressão também nos primórdios foliares de árvores adultas. A expressão da região codificadora de *EgLFY* sob o controle do promotor do *LFY* de *Arabidopsis* pôde complementar mutantes *lfy* nulos em plantas de *Arabidopsis* transgênicas. Essas observações sugerem que o gene *EgLFY* possui um papel similar ao de *LFY* no desenvolvimento floral e que os mecanismos básicos envolvidos na iniciação e no desenvolvimento floral em *Eucalyptus* podem ser semelhantes aos de *Arabidopsis*.
Palavras-chave: eucalipto, desenvolvimento reprodutivo, *LEAFY*, florescimento.

INTRODUCTION

In the model species *Antirrhinum* and *Arabidopsi*s, the apical meristem switches from vegetative to floral development as plants enter the reproductive phase (Coen and Meyerowitz, 1991; Hempel et al., 1994).

In *Antirrhinum* and *Arabidopsi*s, the shoot apical meristem (SAM) initiates lateral primordia that develop into either shoots or flowers. The development of flowers instead of shoots is mediated by the action of floral meristem identity genes which include *LEAFY (LFY) in Arabidopsis* (Weigel et al., 1992) and its homologue *FLORICAULA (FLO)* in *Antirrhinum* (Coen et al., 1990). Inactivation of the *FLO* gene in *Antirrhinum* causes formation of indeterminate shoots in place of flowers and in *Arabidopsis lfy* mutants the structures that would normally develop into flowers develop into structures intermediate between shoots and flowers. FLO and LFY share 70% amino acid identity and each has a proline rich region and an acidic domain, which indicates their possible role as transcriptional activators (Coen et al., 1990). In *Arabidopsi*s, *LFY* has been found to activate homeotic genes, which regulate floral organogenesis (Weigel and Meyerowitz, 1993). Both *LFY* and *FLO* are expressed in the

floral meristem prior to initiation of floral organ primordia while expression at later stages of floral development in both species is less conserved (Coen et al., 1990; Weigel et al., 1992). In *Antirrhinum*, *FLO* expression is also observed in the leaf-like bracts which subtend the flower (Coen et al., 1990). *LFY* might act in suppressing bract formation in wild-type *Arabidopsis* since in *lfy* mutants lack of functional *LFY* RNA leads to ectopic bract formation (Weigel et al., 1992).

In contrast to what is observed for *Arabidopsis*, the apical meristem in eucalypts (*Eucalyptus* spp, Myrtaceae) generally remains vegetative. Lateral meristems, formed in the axils of the leaves, may give rise to a leafy shoot or to an inflorescence in response to inductive environmental conditions, such as day-length and temperature, if the tree is sufficiently mature (Drinnan and Ladiges, 1991). The *E. grandis* inflorescence is determinate and converts directly to a floral meristem(s). Both the inflorescence and flower meristems are completely enveloped by a pair of bracts which protect the primordia. While eucalypt flower buds and flowers are obviously structurally different from those of *Arabidopsis* and *Antirrhinu*m, the pattern and timing of organ development is similar in the three species (see figure 1). Within the bracts enclosing the eucalypt inflorescence (figure 1C), the flower is initiated on the sides of the floral meristem as four protusions, corresponding to sepals, which enlarge, elongate and rapidly fuse, forming the outer layer of the protective structure known as the calicine operculum (Pryor and Knox, 1971; Drinnan & Ladiges, 1991; Steane et al., 1999). The four primordia from the second whorl, which normally give rise to petals in *Arabidopsi*s, arise similarly in *Eucalyptus*, forming the inner (coroline) operculum (figure 1D). Stamen primordia, often in the number of several hundreds, arise in tightly packed whorls surrounding the central gynoecium and correspond to the third whorl of *Arabidopsis* and other plants. The gynoecium generally consists of four to five carpels in the innermost whorl (figure 1D). Early during reproductive development the bracts covering the flowers are shed. Depending on the *Eucalyptus* species, the calicine operculum also dehisce during early floral development (Steane et al., 1999; Drinnan & Ladiges, 1991). At anthesis, the coroline operculum is shed and the prominent stamens surrounding the single style are clearly visible (figure 1E).

As some common developmental features exist between the flower ontogenesis in eucalypts and model species such as *Arabidopsis* and *Antirrhinu*m, it may be suggested that the key floral regulatory genes, described for these model species, would be conserved in eucalypts (Southerton et al., 1998a,b). Nevertheless, it is expected that these genes would display some altered patterns of expression consistent with the unique structural features of the eucalypt flower.

Orthologs of FLO/LFY have been cloned and characterized in several woody perennial species such as Monterey pine *(Pinus radiat*a; Mellerowicz et al., 1998; Mouradov et al., 1998), *Populus trichocarpa* (Rottmann et al., 2000) kiwifruit *(Actinidia deliciosa*; Walton et al., 2001) and grape vine (*Vitis vinifera*; Carmona et al., 2002). Additionally, Southerton et al. (1998) described the cloning of a *LFY* homolog from *Eucalyptus globulus* and suggested that the biological function of *LFY* may be conserved in woody species. However, its specific role in the characteristic features of tree reproductive development has not yet been elucidated. Furthermore, partial or total *FLO/LFY*-like sequences have been reported from other basal angiosperms and gymnosperms (Frohlich and Meyerowitz, 1997; Frohlich and Parker, 2000), although in these cases functional information is not available.

We are currently studying genes involved in the early stages of floral development in woody tropical angiosperm trees. In this paper we describe the cloning of the *Eucalyptus grandis LFY/FLO* putative homolog (named *EgLFY*). We also describe and analyze its expression pattern during eucalypt reproductive and vegetative development. The *EgLFY* gene appears to be the functional homolog of *LFY* as deduced from data on its expression patterns during eucalypt reproductive development and from complementation experiments with *Arabidopsis lfy* mutants.

MATERIAL AND METHODS

Plant Material: Samples of vegetative and reproductive tissues of *Eucalyptus grandis* (var. Coffes Harbour) were collected in the fields of the Escola Superior de Agricultura Luiz de Queiroz, at the University of São Paulo (Piracicaba, SP, Brazil). Young expanding leaves were also used for isolation of genomic DNA. RNA-blot and *in situ* hybridization and SEM analyses were performed on plant tissues collected and fixed in different developmental stages during two growing seasons.

Cloning of EgLFY: Genomic DNA for PCR amplification, Southern analysis and construction of genomic libraries was isolated by the tradit ional CTAB-based method (Sambrook et al., 1989). Total RNA samples for cDNA library construction and Northern Blot were isolated from eucalypt leaves, vegetative apices and from a mix of inflorescences at different developmental stages using the Rneasy plant minikit (QIAGEN) following the supplier's instructions.

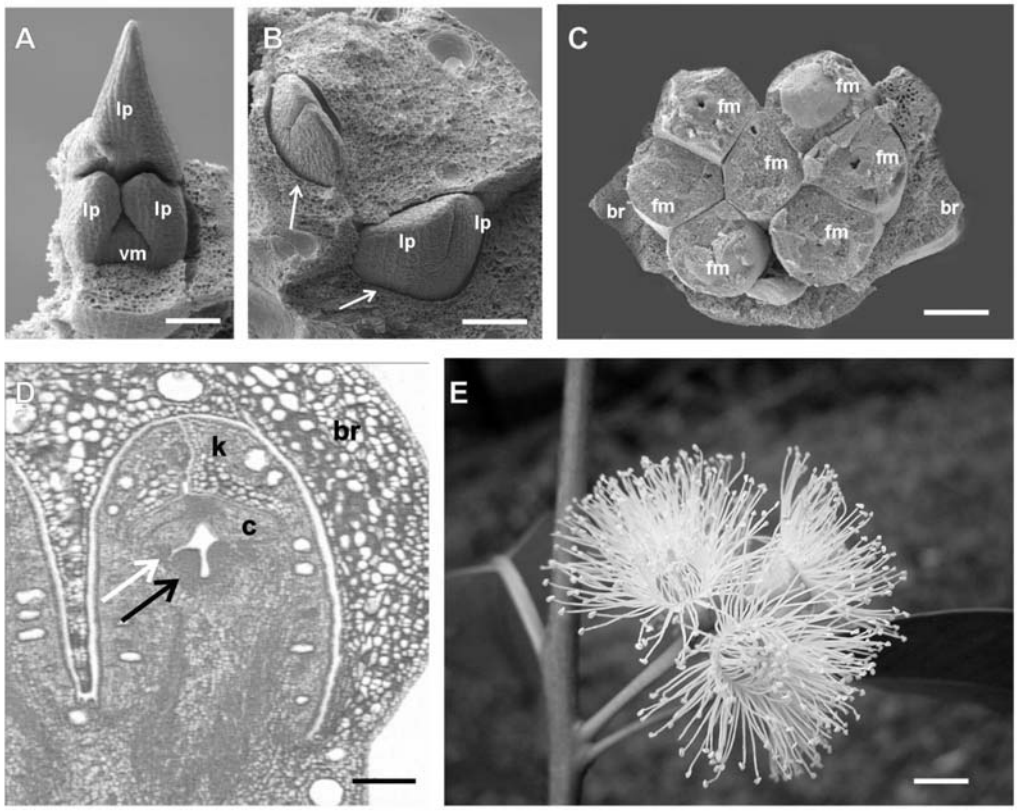

Figure 1. Development of *Eucalyptus grandis* shoots and floral buds. **A-C**: Scanning electron micrographs (SEM). **D**: longitudinal section in light microscopy. **A**: A terminal shoot with some of the leaf primordia (lp) removed to reveal the vegetative meristem (vm). The opposite and decussate phyllotactic pattern can be seen at the apex where two leaf primordia are forming opposite to each other and at right angles to the previous pair of leaves that have been removed. **B**: Young axillary vegetative buds (arrows) are forming opposite leaf primordia (lp). **C**: A developing axilary inflorescence, containing 6 flower meristems (fm) at roughly the same developmental stage. Bract primordia (br) that protect the inflorescence were removed. **D**: A longitudinal section though a developing flower. **k**: calicine operculum (fused sepals); **c**: coroline operculum (fused petals). The white arrow points to the site of stamen primordia development and the black arrow points to developing carpel primordia. **E**: Mature *E. grandis* inflorescence showing flowers at anthesis. Bars: A, B and F: 50 µm; C: 500 µm; D: 100 µm; E: 1cm.

The genomic clones of *EgLFY* were isolated by screening 165,000 plaques from an *E. grandis* genomic library (22 x 10^{-6} pfu) constructed with partially *Sau*3A-digested genomic DNA, using the Packagene Lambda Packing Systems (Promega). For this screening, we have used a biotin-labeled probe (North2South chemiluminescent system, Pierce) using the entire *Arabidopsis LFY* cDNA from plasmid pDW124 (Weigel et al., 1992) as a template. Two adjacent BamHI fragments (E28B with 2Kbp and E6B with 6Kbp) spanning the entire genomic *EgLFY* sequence were subcloned into pBluescriptKS (Clontech). Subclones were prepared by nested deletions (Zhu and Clark, 1995) and sequenced on an ABI Prism 377 (Perkin-Elmer/Applied Biosystems) automated sequencer using the DYEnamic ET terminator Cycle Sequencing Kit (Amersham/Pharmacia Biotec, USA) coupled with M13 reverse and forward primers following the manufacturer instructions.

A cDNA library was constructed using total RNA from a mix of *E. grandis* inflorescences at different developmental stages. The poly-A fraction of RNA was isolated 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′-CGGAYATIAAYAARCCIAARATGMGICAYTA-3′ and L4: 5′-CGGATCCGTGICKIARIYKIGTIG-GIACRTA-3′

(Frohlich and Meyerowitz, 1997). The insert size of the positive clones was determined by PCR using the M13 forward and reverse primers and the three longest clones were sequenced on both strands. The sequences of both genomic and cDNA versions of the *EgLFY* gene were deposited at GenBank databases with the accession numbers AY640313 and AY640314, respectively.

Southern and Northern Hybridization: Southern blotting was performed as described in Sambrook et al. (1989) using genomic DNA digested with XhoI and PstI and blotted on Hybond-N Plus membrane (Amersham). Northern experiments were performed using ten micrograms of total RNA extracted from leaves, vegetative apices and from a mix of inflorescences at different developmental stages, separated in a denaturing agarose gel (Sambrook et al., 1989) and hybridized to an *EgLFY* probe.

The *EgLFY* probe used in both Southern and Northern experiments was a 235bp PCR product obtained from the 3′ transcribed region of the gene, using primers E13:5'-TGGCGGAGCTTGGTGGGGACA-3' and E25:5'-CTTCCTCCTCCAAGTCCAATC-3', and an'*EgLFY* cDNA as a template. PCR reactions were performed in a final volume of 25 ïL with an initial 3 min denaturation at 96ºC, followed by 40 cycles of 96ºC for 40 sec; 45ºC for 30 sec and 72ºC for 2 min. The PCR product was purified using the Concert Kit (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 a cDNA for an *Arabidopsis* ubiquitin (Gen Bank accession AB5432) as a template.

*In situ hybridization:*Digoxigenin labeling of RNA probes, tissue preparation and hybridization conditions were performed as described before (Dornelas et al., 1999, 2000). The template for the *EgLFY* digoxigenin-labeled riboprobes was the 1,400bp fragment, containing the complete coding region, cloned in pGEM-T easy vector. The hybridized sections were viewed immediately and photographed under a Zeiss Axiovert 35 microscope.

Scanning electron microscopy (SEM) and light microscopy: The collected plant material was immediately fixed in 4% paraformaldehyde under vacuum for 24 h and dehydrated with absolute ethanol, where they were stored at 4ºC until needed. For light microscopy the dehydrated samples were embedded in Historesin (Leica, hydroxyetilmethacrilate). The 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 dried under CO_2 in a Balzer´s critical point 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 10-20kV using a ZEISS DSM 940 A or a LEO 435 VP scanning electron microscope, at the University of Sao Paulo (ESALQ-NAP/MEPA).

Sequence comparisons: The trimmed partial *EgLFY* genomic and cDNA sequences obtained were aligned using Clustal W (Thompson et al., 1994), before being checked for similarity with sequences already deposited in public databases using BLASTX (Altshul et al., 1997). Nucleotide and protein sequences of different *LFY* homologs were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and aligned with Clustal W (Thompson et al., 1994). Distance matrixes were obtained from the alignments and comparative trees were built using TreeView (Page, 2000).

Complementation of the Arabidopsis lfy-26 mutant: The *XbaI–Sma*I *EgLFY* fragment, carrying the coding region of *EgLFY*, with its endogenous start and stop codons, was obtained from plasmid pEGLFY and blunt-ended using DNA polymerase I (Klenow fragment). An intermediate pDW132E vector was prepared by cloning the polished fragment described above into the SmaI site of pDW132, containing the *Arabidopsis LFY* promoter (Weigel et al., 1992). The correct orientation of the cloning process was checked by endonuclease digestion. The *Pst*I-*Spe*I fragment from the resultant pDW132E (*LFY::EgLFY*) vector was blunt-ended with Klenow and cloned into the plant transformation vector pSKI015 (a gift from D. Weigel, Salk Insitute, LA Jolla CA, USA), which contains the BAR gene, allowing selection with the herbicide Basta (Sylvet), constituting the pSKI015E vector. *Arabidopsis* plants (Columbia ecotype) transgenic for pSKI015E T-DNA were obtained by using *Agrobacterium tumefacien*s-mediated *in planta* transformation, as described by Bechtold et al. (1998). Putatively transformed seeds were selected upon germination on sand wetted with a Basta (Sylvet) solution at 500 µL.ml^{-1}. Homozygous (Basta-resistant) lines were obtained by selfing the primary transformants. The

segregation ratio of resistant:sensitive was used to estimate the number of transgene insertions. T2 lines, homozygous for the *LFY*::*EgLFY* T-DNA locus, 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 lights: long day (LD) conditions (16 h of light / 8 h of dark) or short day (SD) conditions (8 h of light / 16 h of dark). Finally, *LFY::EgLFY* 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, 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.edu/LABS/pbio-w/caps.html). Transgenic and non-transgenic *Arabidopsis* flowers and inflorescences at different developmental stages were photographed under a stereomicroscope or analyzed by SEM.

RESULTS

The EgLFY *gene is an expressed Eucalyptus grandis homolog of* LFY: The *EgLFY* gene contains two introns (figure 2) and encodes a putative protein with high sequence similarity to FLO/LFY-like proteins (figure 3). The deduced protein sequence of *EgLFY* is 95.2% identical to the previously published *ELF1* gene, the *LFY* homolog in *E. globulus* (Southerton et al., 1998b). The *EgLFY* gene encodes a putative protein of 359 amino acids, which is 67% identical to *Arabidopsis* LFY and 71% identical to the FLO protein (figure 3). These three protein sequences are most similar in their C-

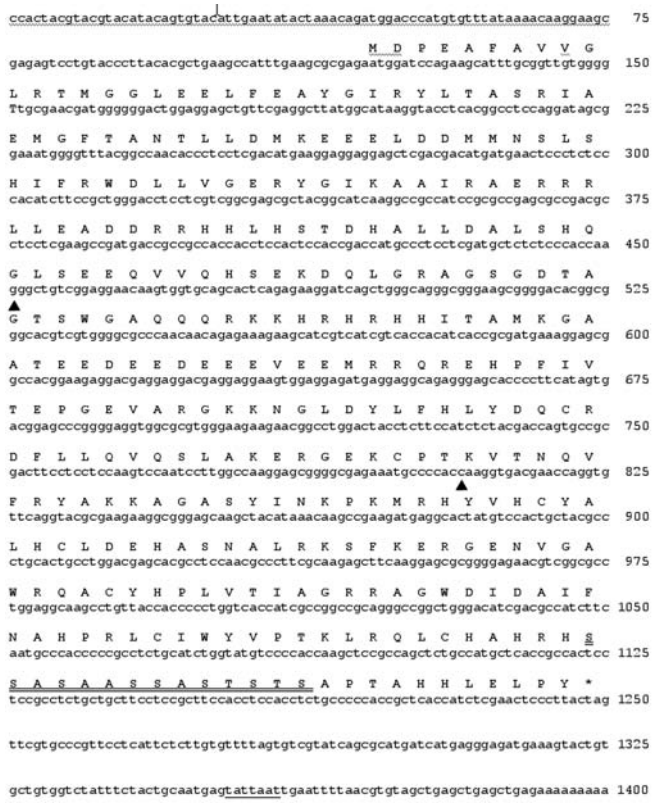

Figure 2. Nucleotide and deduced amino acid (single-letter code) sequence and structure of the *EgLFY* gene, an *E. grandis* homolog of *LFY*. The asterisk indicates the position of the stop codon. The arrowheads indicate the positions of the introns. The putative poly-adenylation site is underlined. The Ser-rich region at the C-terminal portion of the protein is double-underlined.

terminal regions. Beyond Arg-177, EgLFY is 80% identical to LFY and 84% identical to FLO. In this region, a stretch of 30 amino acids is identical in all three proteins, and a total of 156 amino acids in which virtually all changes are conservative replacements. N-terminal of Arg-177, the EgLFY protein is 55% identical to LFY and 58% identical to FLO. The EgLFY protein sequence contains a highly acidic region between glutamates 163 and 174, a short leucine zipper of leucines 45, 52 and 59, and a basic region between Arg-145 and His-153, all features observed in similar positions in the LFY and FLO sequences. EgLFY differs from LFY and FLO in that it lacks the proline rich region at its N-terminus and contains a serine and alanine rich region between Ser-335 and Ala-349.

The number of loci that hybridize with an *EgLFY* probe was investigated by Southern hybridization. This experiment was performed due to the report by Southerton et al. (1998) that *E. globulus* has a second *LFY*-like homolog that appears to be a pseudogene. Figure 4A shows a Southern blot of *Xho*I- and *Pst*I-digested genomic *E. grandis* DNA, probed with the *EgLFY* probe. Two hybridizing bands were detected at low-medium stringencies (washes in 2xSSC at 40ºC). Nevertheless, these additional bands could not be detected in Southern blot experiments when higher stringencies were used (0,1xSSC at 65ºC; data not shown). Thus, the presence of a second *LFY*-like gene in the *E. globulus* genome can not be ruled out. The Northern blot experiments (figure 4B) were always performed at high stringency and the cross-detection of transcripts of *LFY*-like loci other than *EgLFY* was unlikely. The Northern blot results (figure 4B) indicate that the expression of *EgLFY* is restricted to adult plants and that *EgLFY* is preferentially expressed in reproductive tissues.

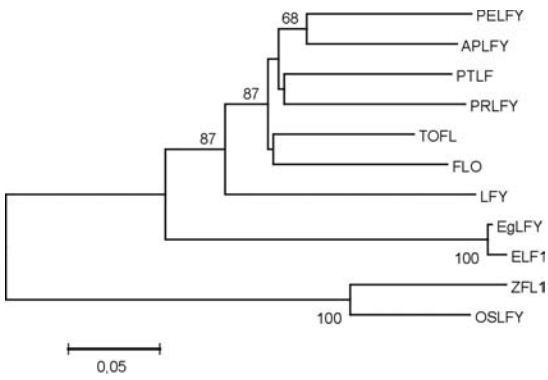

Figure 3. Phylogenetic relationships of EgLFY with other LFY/FLO homologs. The deduced amino acid sequence of EgLFY was compared with (accession nos. in parentheses): PTLF from *Populus balsamifera* (U93196); PRLFY from *Platanus racemosa* (AF106842), TOFL from tomato (AF197934), APLFY from apple (BAB83097); PEgLFY from pea (AAC49782), NTLFY from tobacco (U16172), ZFL1 from maize (AY179883), OSLFY from rice (AB005620), FLO from snapdragon (M55525), LFY from *Arabidopsis* and ELF1 from *E. globulus* (AF34806). Bootstrap support values (for 1000 replicates) are indicated when over 50.

EgLFY *is expressed in the tip of leaf primordia of adult trees and during floral organ development:* The expression pattern of *EgLFY* in vegetative and reproductive tissues was determined more precisely by *in situ* hybridization of longitudinal sections of vegetative and reproductive meristems of *E. grandis* (figure 5). No hybridization signal was detected in the shoot apical meristems of juvenile (6 months-old) plants (figure 5A), agreeing with the Northern blot results. In both apical and lateral vegetative meristems of adult (6 years-old) plants, the *EgLFY* transcripts were detected at the tip of the leaf primordia. No signal was detected in the shoot apical meristem itself (figures 5B and 5C). During reproductive development *EgLFY* expression was detected only in young floral buds, similar to the expression of the *FLO* and *LFY* genes in *Antirrhinum* and *Arabidopsis*, respectively. Eucalypt tissues tended to stain light brown during fixation, noticeably in oil glands and epidermal cells. However, the characteristic purple color generated from alkaline phosphatase substrates observed during the detection of the digoxigenin-labelled antisense probes was easily distinguished from the non-specific staining. No labeling other than background was observed in serial sections probed with sense probes (figure 5G). The patterns of *EgLFY* expression

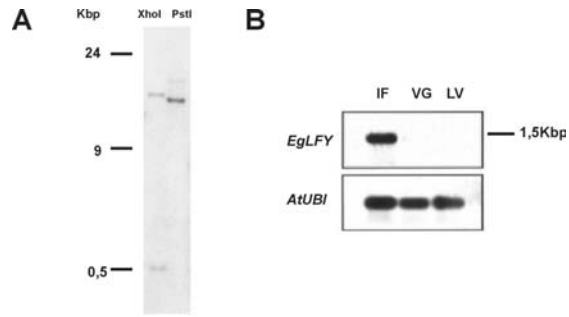

Figure 4. A. Southern blot of genomic DNA from *Eucalyptus grandis* probed with *EgLFY*. Lane 1, digested with *Xho*I; lane 2, digested with *Pst*I. **B**. Northern blot made with total RNA extracted from a mix of inflorescences in different developmental stages (IF), vegetative apices of juvenile plants (VG), young leaves of adult plants (LV) and probed with *EgLFY*. The same blot was re-probed with a heterologous *Arabidopsis* ubiquitin sequence (AtUBI) to show uniform loading and transfer of all lane contents.

in the floral buds of *E. grandis*, were similar to those described for *ELF1* expression in *E. globulus* and *E. macandra* (Southerton et al., 1998b) and a selection of the patterns observed at different floral stages are shown in figure 5. In developing flowers of *E. grandis EgLFY* was first detected uniformly in early floral meristems, before the onset of the floral organ primordia, (figure 5D). Later, the *EgLFY* hybridization signal was preferentially detected in areas corresponding to the developing floral primordia (figures 5E, 5H and 5I). Expression was briefly observed in sepal primordia and then in petal primordia (figures 5E and 5F). *EgLFY* expression declined in the sepals as they enlarged and fused, and was then observed in the petal primordia. As the petal primordia enlarged, expression became restricted to the center of the floral meristem, where the carpels form, and in the stamen primordia (figures 5H and 5I). Afterwards, expression declined in the petals and no hybridization signal was detected anymore in the operculum tissues. Expression was maintained during stamen development and in the region of the developing gynoecium, particularly in the developing ovules (data not shown). *EgLFY* expression was not detected in fully developed floral buds, but these tissues were extremely difficult to section and contained high levels of phenolic compounds and oils that interfered with proper *in situ* hybridization.

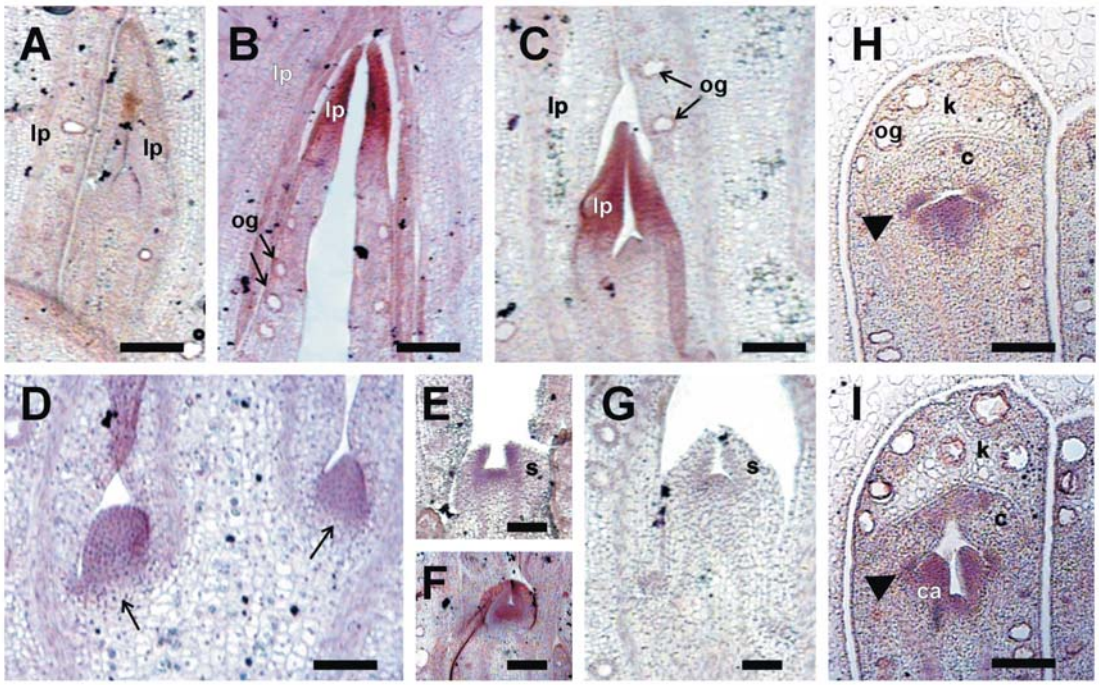

Figure 5. *In situ* localization of *EgLFY* transcripts during vegetative and reproductive growth of *E. grandis*. All sections are longitudinal. All hybridizations were done with the antisense probe, except when mentioned otherwise. The hybridization signal with the *EgLFY* probe was observed as a purple precipitate. **A**: vegetative meristem of a juvenile (6 months-old) plant. No signal was detected above background. **B**: *EgLFY* transcripts were detected at the tip of leaf primordia (lp) of the apical vegetative meristem of an adult (6 years-old) plant. **C**: Lateral vegetative meristem of an adult (6 years-old) plant showing no hybridization signal in the meristem and *EgLFY* transcript accumulation in the young leaf primordia. **D**: The early inflorescence meristems (arrows) expressed *EgLFY.* **E**: The *EgLFY* expression was detected in flower meristems and sepal primordia (s). **F**: Flower meristem hybridized with the *EgLFY* probe at a slightly later stage than that shown in **E**. **G**: Flower meristem at the same stage as that shown in **F** hybridized with an *EgLFY* sense probe. No hybridization signal was detected above background. **H**: Flower meristem at a late developmental stage, showing the fused calicine (k) and the coroline (c) opercula (**K**). This section is slightly oblique, so that the region of petal primordia fusion is not seen. The *EgLFY* expression was restricted to the site of stamen development (arrowhead) and at the center of the floral meristem. **I**: Flower meristem at a later developmental stage than that shown in **H**, the hybridization signal was more intense at the site of stamen formation (arrowhead) and in the carpel primordia (ca). **og**: oil gland. Bars: **A, B, C**: 50 μm; **D**: 25 μm; **E** and **G**: 20 μm; **F**: 15 μm; **H** and **I**: 100 μm.

The EgLFY *coding region can complement transgenic* Arabidopsis lfy *mutants:* When the *EgLFY* coding region was fused downstream to the *Arabidopsis LFY* promoter and introduced into the strong-phenotype *lfy-26 Arabidopsi*s mutant, complete restoration of the wild type development was observed (figure 6). The early arising (basal) flowers in the *Arabidopsis lfy-26* mutants were replaced by bracts adjacent to secondary inflorescence shoots, whereas later arising flowers were replaced by small bracts, in whose axils abnormal flowers developed (figures 6B and 6C; Weigel et al., 1992). These abnormal flowers contained sepals and carpels but no petals or stamens, these later being usually homeotically substituted by more sepals and carpels, respectively (figures 6C and 6D; 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::EgLFY* 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 (figures 6E and 6F).

DISCUSSION

We have isolated an expressed eucalypt *LFY* homolog named *EgLFY*. The *EgLFY* gene contains two introns that occur in identical positions to those found in all the described *LFY/FLO* homologs clones to date (Frohlich and Parker, 2000)

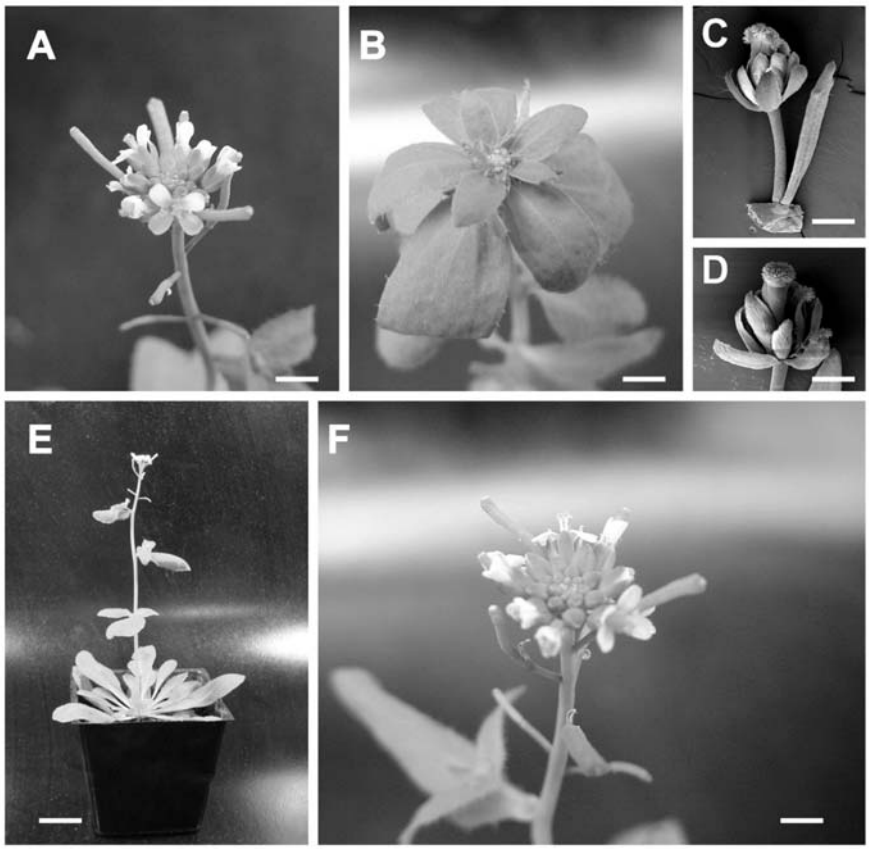

Figure 6: Complementation of an *Arabidopsis* strong *lfy* mutation by *EgLFY* expression driven by the *LFY* promoter. **A**: Wild type inflorescence (Columbia ecotype) showing flower buds at different developmental stages. **B**: main inflorescence axis of a *lfy-26* mutant. Solitary flowers are replaced by a cauline leaf (bract) adjacent to a lateral inflorescence axis or an abnormal flower. **C** and **D**: SEM images of abnormal flowers of the *lfy-26* mutant. Note the cauline leaf in **C** and the homeotic conversion of petals and stamens in sepal-like organs and carpel-like organs, respectively. **E**: A homozygous transgenic (LFY::*EgLFY*) *lfy-26* mutant showing a wild type phenotype, indicating the complementation of the *lfy* mutation by the expression of *EgLFY*. **F**: A higher magnification view of the inflorescence of the same plant shown in **E**. Bars: **A**, **B** and **F**: 3 mm; **C**: 300 μm; **D**: 250 μm; **E**: 3cm.

and its sequence and expression patterns are very similar to those described for most dicot *LFY*/*FLO* homologs in the literature. Expression of *EgLFY* driven by the *Arabidopsis* LFY promoter is able to restore the wild type phenotype of transgenic *Arabidopsis lfy*-26 mutants. These close structural and functional similarities strongly suggest that *EgLFY* is the functional eucalypt homologue of *LFY/FLO*. *LFY/FLO* homologues similar to *EgLFY* have also been isolated from other plants (Frolich and Parker, 2000). Weigel and Nilsson (1998) have reported that transgenic hybrid aspen *(Populus tremula x P. tremuloide*s) constitutively expressing the *Arabidopsis LFY* cDNA flowers precociously and shows similar phenotypes to *Arabidopsis* transformed with the same construct. Similarly, Peña et al. (2001) also reported the early flowering of citrus plants overexpressing a *LFY* homolog. These data add further weight to the hypothesis that floral regulatory mechanisms, and hence regulatory genes, are conserved among the angiosperms. The putative protein encoded by *EgLFY* shares a number of sequence motifs with other characterized LFY/FLO proteins (Frolich and Parker, 2000). The acidic domain is not conserved with respect to sequence and occurs in a region of relatively poor sequence conservation among the *LFY* homologs. The putative EgLFY protein, as well as its *E. globulus* homolog (Southerton et al., 1998) is shorter at the N-terminal end when compared to other LFY/FLO homologs and thus lacks the proline rich region suggesting that this motif may not be functionally significant. None of these protein sequence motifs has yet been demonstrated to be functionally important in any of the floral meristem identity genes. It is of interest to note that eucalypts probably have two *EgLFY*-like genes, although one of these is probably now inactive (Southerton et al., 1998). This duplication is probably a general phenomenon within the genus, and suggests that eucalypts may have experienced ancient genome duplications and many of their genes might be expected to be present in at least two copies (Southerton et al., 1998). In addition to being expressed in floral primordia in a pattern similar to *LFY* and *FL*O, th*eEgLFY* gene is strongly expressed in leaf primordia forming on vegetative meristems of adult plants, but not in the shoot apical meristem itself. The overall pattern of expression of *EgLFY* is, however, similar to other described *LFY/FLO* homologues (Coen et al., 1990; Weigel et al., 1992; Southerton et al., 1998; Peña et al., 2001; Carmona et al., 2002).

Experiments by Hempel et al. (1994) and Blázquez et al. (1997) using *in situ* hybridization and GUS reporter gene expression driven by the *LFY* promoter have now also established vegetative expression of *LFY* in both vegetative apices and young leaves of three different ecotypes of *Arabidopsis* grown under short day conditions. The *Arabidopsis LFY* gene is the earliest of the known floral identity genes to be expressed, and directly activates at least one of the later genes, *APETALA1* (Wagner et al., 1999). Plants carrying fusions of the *LFY* promoter to the *GUS* marker gene were used to demonstrate that *LFY* expression responds both to the long-day flowering pathway and to gibberelic acid (GA). Furthermore, deletion of a putative MYB transcription factor binding site within the *LFY* promoter prevented activation by GA, but not by the long-day pathway (Blázquez and Weigel, 2000). We have failed to identify any putative MYB transcription factor binding site within the *EgLFY* promoter (data not shown). However, exogenous application of paclobutrazol reduced the concentration of endogenous GA in apical tissues of different *Eucalyptus* species and enhanced the reproductive activity of grafted trees (Moncur and Hasan, 1994), suggesting that in *Eucalyptus*, high concentrations of GA inhibits the flowering process, as opposed to what is observed in *Arabidopsis*. It would be interesting to investigate whether paclobutrazol can interfere with *EgLFY* expression.

Although the available information suggests that overexpression of *LFY* is sufficient to promote the conversion of shoots into flowers in woody species such as *Populus* spp. (Weigel and Nilsson, 1995) and *Citrus* spp. (Peña et al., 2001), the role of the endogenous *FLO/LFY* homologs and their function during meristem development are poorly understood. Genetic studies in *Eucalyptus* are difficult because of the long time to flowering of trees and no characterized flowering mutants have been described in this genus. Nevertheless, recent advances in the transformation of *Eucalyptus* species (unpublished data from our own lab) and the large-scale cloning of a number of other floral gene homologues (https://forests.esalq.usp.br) may allow us to use reverse genetic approaches and to define more clearly the role played by *EgLFY* in *Eucalyptus* vegetative and floral tissues.

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