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Eucalyptus has a functional equivalent of the Arabidopsis floral meristem identity gene *LEAFY*

Simon G. Southerton', Steven H. Strauss[2], Mark R. Olive, Rebecca L. Harcourt[3], Veronique Decroocq[4], Xiaomei Zhu, Danny J. Llewellyn, W. James Peacock and Elizabeth S. Dennis*

*CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia (*author f'or correspondence; E-mail: E.Dennis@pi.csiro.au); Present addresses: 'Department of Biochemistry, University of Queensland, St. Lucia 4072, Australia; [2]Department of Forest Science, Oregon State University, Corvallis, OR 97331-7501, USA; [3]CSIR0 Entomology, PO. Box 1700 Canberra City, ACT 2601, Australia; [4]1NRA Centre de Bordeaux, UREFV, BP 81, 33883 Villenave d'Ornon, France*

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Abstract

Two genes cloned from *Eucalyptus globulus, Eucalyptus LeaFY (ELF]* and *ELF2),* have sequence homology to the floral meristem identity genes *LEAFY* from *Arabidopsis* and *FLORICAULA* from *Antirrhinum. ELFI* is expressed in the developing eucalypt floral organs in a pattern similar to *LEAFY* while *ELF2* appears to be a pseudo gene. *ELF] is* expressed strongly in the early floral primordium and then successively in the primordia of sepals, petals, stamens and carpets. It is also expressed in the leaf primordia and young leaves and adult and juvenile trees.

The *ELFI* promoter coupled to a GUS reporter gene directs expression in transgenic *Arabidopsis* in a temporal and tissue-specific pattern similar to an equivalent *Arabidopsis LEAFY* promoter construct. Strong expression is seen in young flower buds and then later in sepals and petals. No expression was seen in rosette leaves or roots of flowering plants or in any non-flowering plants grown under long days. Furthermore, ectopic expression of the *ELF]* gene in transgenic *Arabidopsis* causes the premature conversion of shoots into flowers, as does an equivalent *35S-LFY* construct. These data suggest that *ELF]* plays a similar role to *LFY* in flower development and that the basic mechanisms involved in flower initiation and development in *Eucalyptus* are similar to those in *Arabidopsis.*

Introduction

In the plant species *Antirrhinum* and *Arabidopsis,* the apical shoot meristem 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 [24]* and its homologue *FLORICAULA (FLO)* in *Antirrhinum [6].* 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. *APETALAI (API) is* a second floral meristem identity gene in *Arabidopsis* that acts together with *LFY* to determine meristem fate [15]. When the *LFY* and *API* genes are both disrupted *[24]* there is a complete lack of flower development, and only inflorescence shoots are found.

FLO and LFY share 70% amino acid identity and each has a proline rich region and an acidic domain which indicates they may have a role as transcriptional activators *[6].* In *Arabidopsis, LFY* has been found to activate homeotic genes which regulate floral organogenesis *[26].* Both *LFY* and *FLO* are expressed in

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF034806.

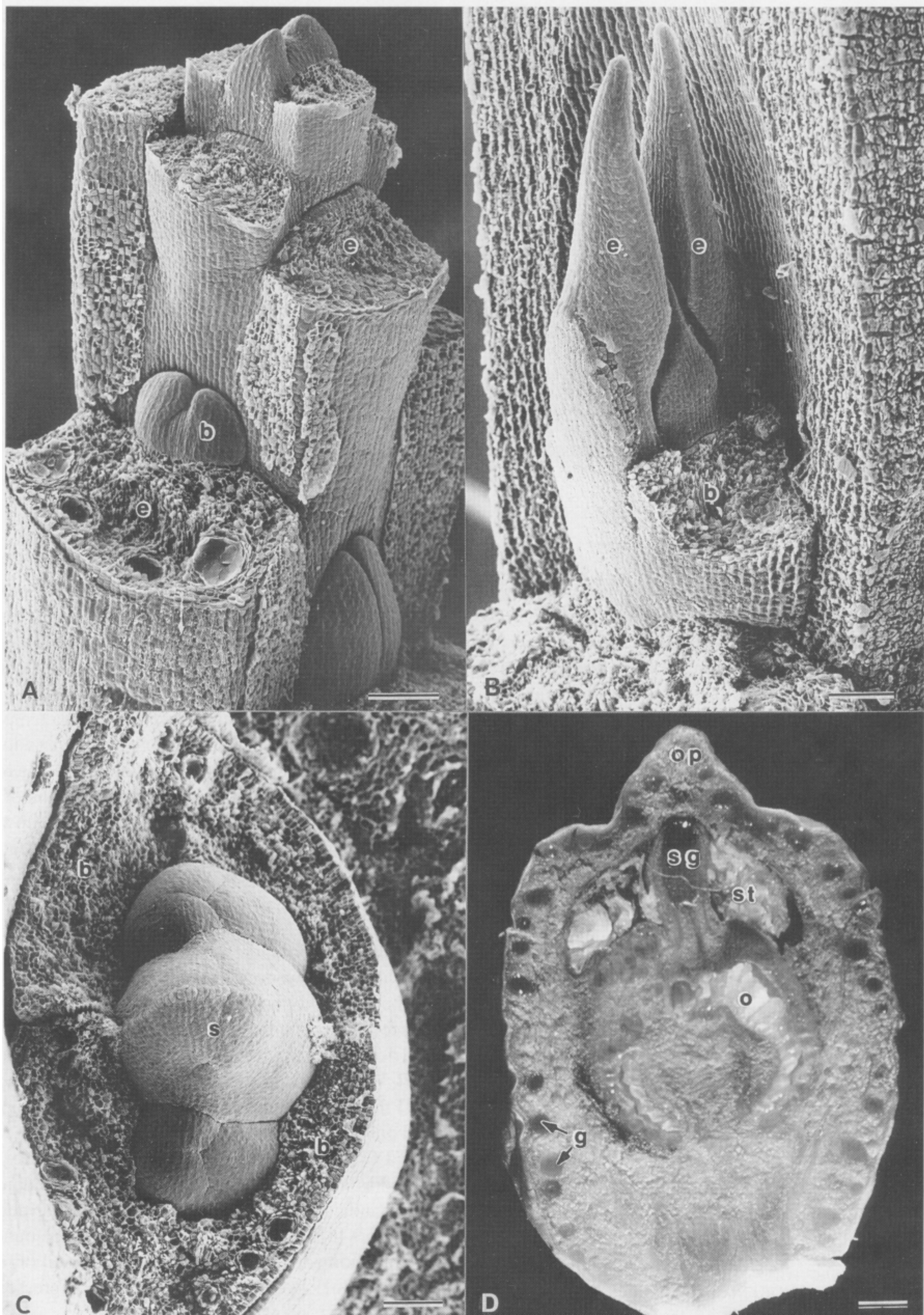
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Figure 1. Development of *Eucalyptus globulus* shoots and floral buds. A. Scanning electron micrograph (SEM) of a terminal shoot with leaves (e) removed to reveal the axillary buds. The opposite and decussate phyllotactic pattern can be seen at the apex where two leaf primordia are forming opposite each other and at right angles to the previous pair of leaves which have been removed. Bract primordia (h) can he seen enlarging and ultimately covering the lateral buds. Bar = 100 μm. B. SEM of a young axillary leafy shoot with the protective bracts (b) removed. The first pair of leaves (e) have formed at right angles to the bracts. Bar = 100 It m. C. SEM of a young axillary inflorescence bud with the bracts (b) removed, revealing three developing floral buds. The smaller buds have formed in the axil between the terminal bud and the bracts and do not develop to maturity *E. glohulu.s ssp. globulus*. Scars on the surface of the buds occur where the sepal primordia (s) have fused forming the outer layer of the operculum. Bar = 100 μm. (D) Photograph of a longitudinally sectioned developing *E.. globu!us* flower. op, operculum; sg, stigma; st, stamens: o, ovules; g, oil glands. Bar = 1 mm.

the floral meristem prior to initiation of floral organ primordia while expression at later stages of floral development in both species is less conserved 16, 241. In *Antirrhinum, FLO* expression is also observed in the leaf-like bracts which subtend the flower [6]. *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 124 1.

We are studying genes involved in the early stages of floral development in woody angiosperm trees of the genus *Eucalyptus*. In *Antirrhinum* and *Arabidop*sis, the apical meristem switches from vegetative to floral development [5. 13] and the plants enter the reproductive phase. In contrast, the apical meristem in eucalypts generally remains vegetative. Lateral meristems, arising in the axils (Figure 1A), may give rise to a leafy shoot (Figure 113) or, to an inflorescence (Figure 1C), in response to inductive environmental conditions, such as daylength and temperature, if the tree is sufficiently mature. Both of these structures are completely enveloped by a pair of bracts which protect the primordia. The eucalypt inflorescence is determinate and converts directly to a floral meristem(s). In *E. globulus,* two more floral meristems may arise in the axils of the first flower and the bracts, as shown in Figure 1 C, however, in *E. globulus ssp. globulus* these often do not develop completely and a single flower is formed. In *E. globulus ssp. bicostata,* used ~for the *in situ* analyses described below, umbels of three flowers are formed. In *E. macrandra,* also used in the in *.situ* studies, up to 15 flowers are formed from successive floral meristems that arise almost simultaneously within the covering bracts.

While eucalypt flower buds and flowers are obviously very different structurally from those of *Arabidopsis* and *Antirrhinum,* the pattern and timing of organ development is similar in the three species. Within the bracts enclosing the eucalypt inflorescence, the flower is initiated on the sides of the floral meristem as four swellings, corresponding to sepals, which enlarge, elongate and rapidly fuse, forming the outer layer of the protective structure known as the operculum (see Figure 1D and [4, 10, 21 1). The four primordia from the second whorl, which normally give rise to petals in *Arabidopsis,* arise similarly, forming the inner layer of the operculum. Stamen primordia, often numbering several hundred, 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 carpets in the innermost whorl. Early during development the bracts covering the flowers are shed and the buds appear with the growth of the developing flower or flower cluster. At anthesis, the operculum is shed and the prominent stamens surrounding the single style are clearly visible. These common developmental features suggest that the key floral regulatory genes, like *LFY* and *FLO,* will all be functioning in eucalypts, but they and other floral regulatory genes might be expected to display some altered patterns of expression consistent with the unique structural features of the eucalypt flower.

In this paper we describe the cloning from *Eucalyptus globulus ssp. globulus* (Tasmanian blue gum) of the genes *Eucalyptus LeaFy (ELFI* and 2) that have sequence homology to the *LFY* and *FLO* genes and analyse their expression in eucalypts. The *ELF]* gene appears to be the functional homolog of *LFY* as deduced from its expression patterns in eucalypt flowers and promoter expression in *Arabidopsis* as a reporter gene fusion. Furthermore, when the coding region of *ELFI* is introduced into *Arabidopsis* under the control of the 35S promoter, phenotypic effects are observed similar to those seen with a *35S-LFY construct. ELF2* appears to be a non-expressed pseudogene.

Materials and methods

Plant material

Tissues from a single adult *Eucalyptus globulus ssp.* globulus tree growing in isolation from other *E. globulus* trees at CSIRO Forestry and Forest Products,

Canberra, Australia, was used as a source of DNA and RNA for library construction, and Southern analysis. Selfed seeds collected from this tree were germinated in the glasshouse and DNA prepared from individual seedlings for inheritance studies. The same parent tree and unrelated young trees grown in a glasshouse were sources of plant tissue used for RT PCR while *in situ* hybridization studies were performed on tissues from a *E. globulus ssp. bicostata* tree growing near our laboratory at CSIRO Plant Industry, Canberra. Inflorescences of *E. macrandra grown* at Central Tilba, NSW were also used for *in situ* hybridization experiments.

Extraction of nucleic acids from eucalypt tissue

Genomic DNA for PCR amplification, Southern analysis and construction of genomic libraries was isolated from all tree species by a CTAB-based method. Typically, 7 g of leaves was frozen then ground to a fine powder in liquid nitrogen in a mortar and pestle. The powder was thoroughly mixed with 25 ml cold extraction buffer (50 mM Tris pH 8.0, 35 mM sorbitol, 5 mM EDTA, 10% w/v PEG 4000, 5% w/v polyvinylpyrrolidone, 2% v/v 2-mercaptoethanol, 0.1 % w/v spermine, 0.1 % w/v spermidine and 0.1 % w/v bovine serum albumen) then passed through two layers of cheesecloth. The filtrate was centrifuged at 3000 x g for 10 min at 4 °C and the pellet resuspended in 5 ml ice-cold wash buffer (50 mM T

pH 8.0, 35 mM sorbitol, 25 mM EDTA and 0.4% v/v 2-mercaptoethanol). This solution was sequentially mixed with 600 μl 10% w/v sarkosyl, 800 p l 5 M NaCl and 650 p 18.6% w/v CTAB, 0.7 M NaCI, then incubated at 65 °C for 20 min. The solution was extracted with 10 ml chloroform isoamyl alcohol (24:1), centrifuged at 3000 x *g* for 10 min and nucleic acids in the aqueous phase precipitated by addition of 2/3 volume isopropanol and swirling on ice. DNA was spooled from the solution and washed several times with 70% ethanol and allowed to air-dry before dissolving in TE (10 mM Tris-HCI pH 8, 1 mM EDTA pH 8). Southern blotting was performed as described in Dennis *et al.* [7] using Hybond N membrane from Amersham.

RNA was isolated from various eucalypt tissues using the method described for DNA extraction to the stage of the precipitation of the nucleic acids with isopropanol. Nucleic acids were then pelleted by centrifugation at 12 000 *x g* for 15 min at 4 °C and the pellet redissolved in 10 ml of 1.5 M CsCI in 100 mM Tris-HCI, pH 8.0. This solution was layered onto a

2 ml cushion of 5.7 M CsCI in 100 mM Tris-HCI, pH 8.0, in a 12 ml polyallomer tube then centrifuged at 33 000 rpm for 16 h at 15 °C in a Beckman SW55 rotor. The supernatant was carefully removed and the pellet rinsed briefly with water. The pellet was dissolved in 100 p l TE, 0.1 % w/v SDS for 4 h at 4 °C and the RNA precipitated by addition of 0.1 volume of 3 M sodium acetate pH 6 and 2.5 volumes of ethanol on ice for 10 min and resuspended in TE.

PCR amplification of LFY/FLO *horrcologues.from eucalypt DNA*

PCR primers were designed based on the amino acid similarity between the *LFY* gene from *Arabidopsis* [24] and the *FLO* gene from *Antirrhinum* [6]. The target amino acid sequences are shown in Figure 3. The sequences of the two primers are shown below. `I' denotes inosine and degenerate bases are in parenthesis. *LFY-309-H*
5'-CGGAATTCATG(AC)GICA(TC)TA(TC)GTICA(TC)T G(TC)TATGC
LFY-351 -J
5'-CGGGATCCGG(CT)TT(AG)TA(AG)CAIGC(CT)TGI C(TG)CCA
PCR was carried out in 12 It] reactions in capillary tubes using a Corbett FTS-1 Thermal Sequencer (Sydney, Australia). To minimize generation of nonspecific products a `touchdown' thermal cycle program was used [81 that began with 5 min denaturation at 94 °C followed by cycles of annealing for 30 s, extension for 30 s at 71 °C and denaturation for 30 s at 94 °C. The annealing temperature was reduced from 60 °C by 2 °C every second cycle until 46 °C, where 8 cycles were carried out, followed by an additional 25 cycles at 48 °C. Apart from annealing temperature, other reaction conditions remained the same as in the initial cycle. Buffer components were 2.9 MM $MgC1_2$, 1.5 μg template DNA, 250 μM dNTPs, 1.0 μM primers, 2.5 units *Taq* polymerase (fetus, Amplitaq), 50 mM KCl, 10 mM Tris pH 8.8, 0.1 % v/v Triton-X100. The PCR product of 141 by was excised from a polyacrylamide gel, digested with BamHI and EcoRl, and cloned into pBluescriptKS(+) (Stratagene).

Library screening

The genomic clones of *ELF]* and *ELF2-I* were isolated from a *E. globulus* partial Sau3A genomic library constructed in *EMBL* 4, by screening 300000 plaques with the 141 by PCR product. *ELF2-1 was* subcloned as a 10 kb BamHI fragment into pBluescriptKS(+).

nni

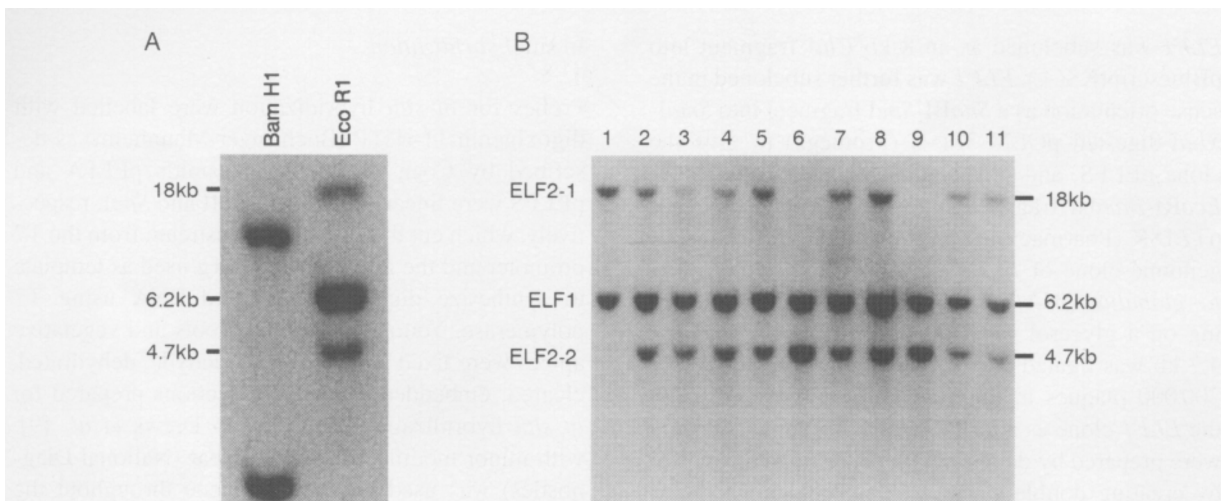

Figure 2. Multiple *ELF* loci in *Eucalyptus*. A. Southern blot of genomic DNA from the parent *Eucalyptus globulus* ssp. *globulus* tree probed with *ELF*. Lane 1, digested with *BamH*I; lane 2, digested with *EcoR*I. B. *ELF2-1* and *ELF2-2* alleles segregate in the progeny of the parent tree. Southern blot of genomic DNAs from 11 progeny plants digested with *EcoR*I and probed with *ELF*.

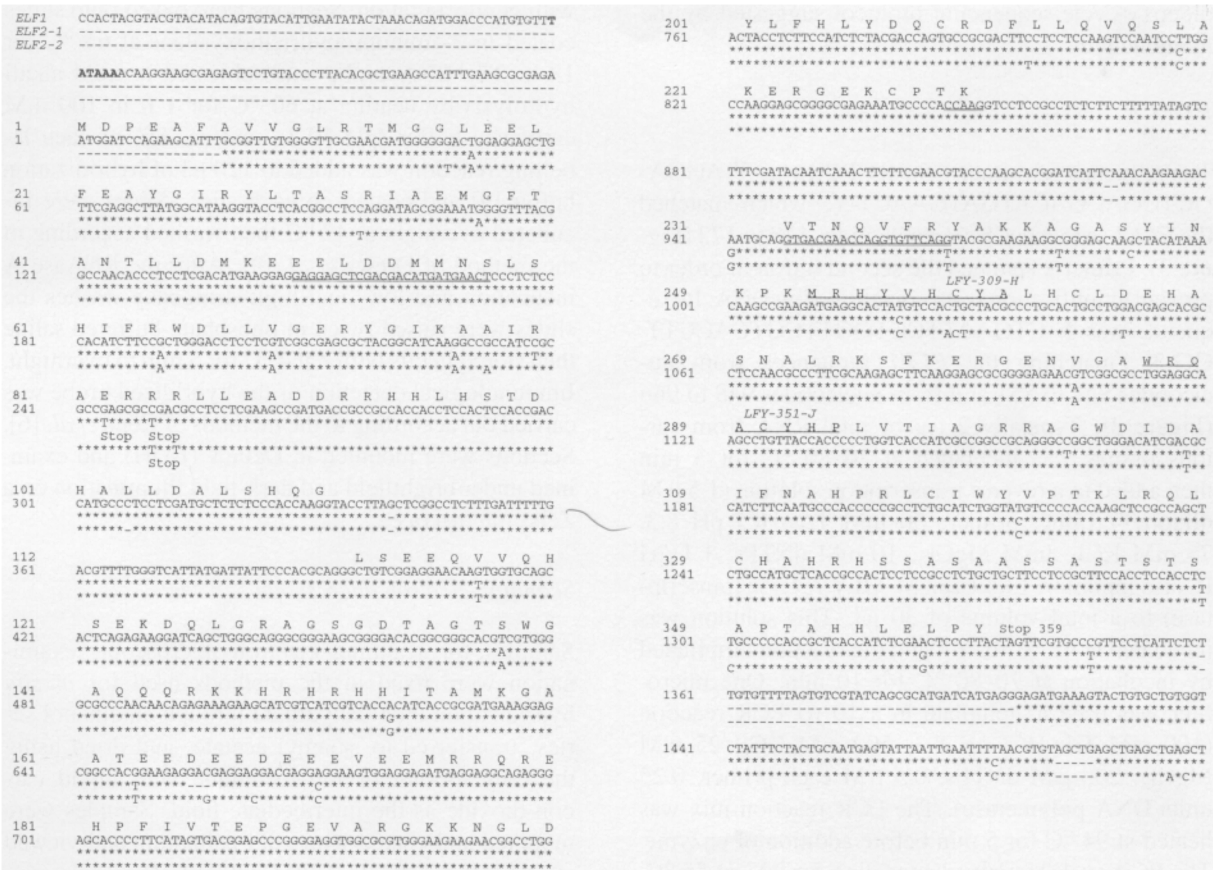

Figure 3. Nucleotide sequences of *ELF1*, *ELF2-1* and *ELF2-2* and deduced amino acid sequence of *ELF1*. The deduced amino acid sequence of *ELF1* is shown above its nucleotide sequence. Below the sequence, nucleotides that are different to *ELF1* in the *ELF2-1* and *ELF2-2* are shown and an asterisk indicates identical nucleotides. 'Stop' indicates the start of the translation stop codon or where a nucleotide substitution has caused the formation of a stop codon. Gaps in the sequences are indicated by dashes. The position of a putative TATA box is indicated in bold type. The positions of the primers based on the *LFY* and *FLO* genes that were used to amplify the genomic fragment of *ELF* are underlined. The double-underlined regions indicate the positions of the primers used in RT-PCR.

ELF1 was subcloned as an 8 kb ClaI fragment into pBluescriptKS(+). *ELF1* was further subcloned in the sense orientation as a ***SnaBI-Spel fragment*** into SmalXbaI-digested pGEM-3Z(-) (Promega) to give the clone pELFS, and in the antisense orientation as an EcoRI-BamHI fragment into EcoRI-BamHI-digested pTZ18R (Pharmacia) to give the clone pELFA. The genomic clone of *ELF2-2* was isolated by digesting E. globulus DNA with EcoRl and size fractionating on a glycerol gradient. The size fraction of ca. 4.7 kb was ligated into ZAP II (Stratagene). About 200000 plaques in this library were screened using the *ELFI clone* as a probe. Subclones for sequencing were prepared by deletion with restriction enzymes or by creating double stranded nested deletions (Pharmacia). Subclones in plasmid vectors pGEM-3Z(-) and pBluescriptKS(+) were sequenced on an Applied Biosystems 373A automated sequencer using the dideoxy-cycle sequencing protocol suggested by the manufacturer.

RT-PCR

The sequence of primer 1 for RT PCR was 5'-AGGAGCTCGACGACATGATGAACT-3', which matched the *ELF1 sequences* from nucleotides 148 to 172 (Figure 3). Primer 2 spanned the second intros in order to prevent amplification from contaminating DNA. Its sequence was 5'-CTGAATTCCTGGTTCGTCACCTTGG-3', matching the *ELF1* sequences from nucleotides 847 to 851 and from nucleotides 948 to 966 (Figure 3). Typically 2 O g of total RNA from various tissues was incubated at 70-80 °C for 3 min then added to a reverse transcription solution (1.5 RM oligo(dT) I Smer primer; 50 mM Tris-HCI pH 8.3, 75 mM KCI, 3mM MgC12, 10 mM dNTPs, 1 U/pl RNase inhibitor, 13.3 U/pl M-MLV reverse transcriptase) to a total volume of 30 R1. This solution was incubated at 42 °C for 1 h and the reaction terminated by incubation at 70-80 °C for 10 min. One microliter was used as template in a 10 p l PCR reaction (100 mM Tris-HCI pH 8.3, 500 mM KCI, 25 mM MgC12, 200 RM dNTPs, 0.5 [LM each primer, 0.25 units DNA polymerase). The PCR reaction mix was heated at 94 °C for 5 min before addition of enzyme. The PCR cycles involved annealing for 20 s at 55 °C, extension for 30 s at 72 °C and denaturation at 94 °C for 20 s for 40 cycles.

In situ *hybridization*

Probes for in ***situ*** hybridization were labelled with digoxigenin-1 1-rUTP (Boehringer-Mannheim) as described by Coen ***et al.*** [6]. Plasmids pELFA and pELFS were linearized with BamHI and ***SpeI,*** respectively, which cut the plasmid downstream from the T7 promoter and the gene, and ca. 1 μg used as template to synthesize digoxigenin-labelled RNA using T7 polymerase. Young flower buds, roots and vegetative apices were fixed in 3.7% formaldehyde, dehydrated, cleared, embedded in wax and sections prepared for in situ hybridization according to Drews ***et*** al. [9], with minor modifications. Histoclear (National Diagnostics) was used instead of xylene throughout the method. Older flower buds were pre-sectioned to about 1 mm thickness to aid infiltration of the tissue. Buds were typically fixed for a period of 2.5 h with gentle agitation. Sections were baked onto slides coated in 3-aminopropyltriethoxysilane at 65 °C for 18 h. The RNA probe was subjected to mild alkali hydrolysis by heating at 60 °C for 1 h in 100 mM carbonate buffer (pH 10.2), and ca. 8% of each labelling reaction was added to 120 01 of hybridization buffer [9] and coated on each slide. Slides were incubated overnight at 42 °C then washed according to the method of Drews ***et*** al. [9]. After the RNAase A incubation and low- and high-stringency washes the slides were rinsed twice in phosphate-buffered saline then stored in this buffer at 4 °C for 5 min to overnight. Immunological detection of the hybridized probe was carried out according to the methods of Coen ***et*** al. [6]. Sections were mounted in DePeX (BDH) and examined under brightfield and dark-field illumination on a Zeiss microscope.

Scanning electron microscopy

Samples for scanning electron microscopic examination were fixed in the methods used for in .situ hybridization then dehydrated through an ethanol series, transferred to isoamyl acetate, and dried using the critical-point drying technique with liquid carbon dioxide as the intermediate fluid. Samples were mounted and coated with gold-palladium and viewed at 15 kV through a Jeol JSM-6400 SEM.

Arabidopsis transformation experiments

A 1.343 kb fragment of the *ELFI* gene from 120 by upstream of the initiator ATG to the stop codon was

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      1                                                           60
 ELF  MDPEAFAVVG L......... .......... .......... .......... ...RTMGGLE
 LFY  ****G*TSGL FRWN...... .PTRALVQAP PPVPPPLQQQ PVTPQTAAFG MR...L****
 FLO  ***D**...L FKWDHRTALP QPNRLLD.AV APPPPPP... ...PQAPSYS MRP*EL****
NFL1  ******SASL FKWDPRGAMP PPTRLLEAAV APPPPPPVLP PPQPLSAAYS IRT*EL****
NFL2  ******SASL FKWDPRGAMP PPTRLLEAAV APPPPPPALP PPQPLSAAYS IKT*EL****

      61                                                         120
 ELF  ELFEAYGIRY LTASRIAEMG FTANTLLDMK EEELDDMMNS LSHIFRWDLL VGERYGIKAA
 LFY  G**GP****F Y**AK***L* ***S**VG** D***EE**** *******E** **********
 FLO  ***Q****** Y**AK***L* **V******R D****E**** *CQ******* **********
NFL1  ***Q****** Y**AK***L* **V******* D********* **Q****E** **********
NFL2  ***Q****** Y**AK***L* **V******* D********* **Q****E** **********

      121                                                        180
 ELF  IRAERRRL.. .LEADDRR.. HHLH.....S TDHALLDALS HQ....GLSE EQVVQHSEKD
 LFY  V*******QE EE*EESS*RR *L*LSAAGD* GT*HA***** QEDDWT**** *P*Q*QDQT*
 FLO  V******I.. .D*EEV**R. *L*L.....G DTTHA***** QE....**** *P*.*Q.**E
NFL1  ********.. .E*EEL**RS *L*S.....D GGTNA***** QE....**** *P*Q*Q.*RE
NFL2  ********.. .E*EEL**RG *L*S.....D GGTNA***** QE....**** *P*Q*Q.*RE

      181                                                        240
 ELF  QLGRAGSG.. ......DTAG TSWGAQQQRK KHRHRHHITA MKGAATEEDE EDEEEVEEMR
 LFY  AA*NN*G*GS GYWDAGQGKM KKQQQ*RR** *PMLTS.... ..VETD*DVN *G*DDDGMDN
 FLO  AM*SG*G*VG GVWEMMGA.* GRKAP*RR** NYKG*SRMAS *EEDDDDD*D *T*GAEDDEN
NFL1  AV*SG*G..G TTWEVVAAV* GGRMK*RR** *VVSTGRERR GRAS*E*DE* TE*GQED*WN
NFL2  AV*SG*G..G TTWEVVAA** GGRMK*RR** *VVAAGREKR GGAS*E*DE* TE*GQEDDWN

      241                                                        300
 ELF  .......... RQREHPFIVT EPGEVARGKK NGLDYLFHLY DQCRDFLLQV QSLAKERGEK
 LFY  GNGGSGLGTE ********** ********** ********** E***E***** *TI**D****
 FLO  I......VSE ********** ********** ********** E********* *TI*******
NFL1  INDAGGGISE ********** ********** ********** E********* *NI*******
NFL2  INDASGGISE ********** ********** ********** E********* *NI*******

      301                                                        360
 ELF  CPTKVTNQVF RYAKKAGASY INKPKMRHYV HCYALHCLDE HASNALRKSF KERGENVGAW
 LFY  ********** *****S**** ********** ********** E******RA* ********S*
 FLO  ********** ********N* ********** ********** A******RA* **********
NFL1  ********** ********** ********** ********** E******RA* **********
NFL2  ********** ********** ********** ********** E******RA* **********

      361                                                        420
 ELF  RQACYHPLVT IAGRRAGWDI DAIFNAHPRL CIWYVPTKLR QLCHAHRHSS ASAAS.....
 LFY  *****K***N **C*H.**** **V******* S********* ****LE*NNA VA**AALVGG
 FLO  *****K***A **A*Q.**** *T******** S********* *****E*S*A *V**T.....
NFL1  *****K***A **A*Q.**** *T******** A******R** ****SE*SN. *A**A.....
NFL2  *****K***A **A*Q.**** *T******** A********* ****SE*SNA *A**A.....

      421                  443
 ELF  .....SASTS TSAPTAHHLE LPY
 LFY  ISCTG*STSG RGGCGGDD*R F..
 FLO  .....*S..I *GGGP*D**P F..
NFL1  .....*S*V* ..GGVGD**P HF.
NFL2  .....*S*V* GGGGGGD**P HF.
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Figure 4. Comparison of predicted protein sequences of ELF, LFY, FLO. NFL- I and NFL-2. The predicted protein sequence of ELF is shown above the sequences of LFY, FLO and *NFL.* Asterisks indicate identi~eal amino acids and gaps in the sequence alignments are indicated by dots. The basic domain in the middle of the ELF sequence and the acidic domain found in all three sequences are double underlined and underlined, respectively. The leucines forming a potential leucine zipper motif are indicated in bold.

inserted in the sense orientation into the transcriptional fusion vector pART7 [11] containing the 35S promoter of cauliflower mosaic virus (CaMV), and the terminator from nopaline synthase. The resulting *35S-ELFI-NOS* fragment was excised with *Notl* and inserted into the binary transformation vector pART27 [11]. This construct was transferred into the *Agrobacterium tumefaciens* strain *AGL1 [19]* by triparental mating and used to transform *Arabidopsis thaliana* C24 plants by standard root transformation methods [23]. 10 mg/1 of kanamycin sulfate was used to select for transformed plants and transformation was also confirmed by PCR analysis using primers designed to amplify a fragment of the *NptII* gene. Seeds were collected from the primary transformants and morphological analyses were performed on the T 1 generation. Seeds of three different terminal flower to mutants (tfl-2, *tfl-3* and *tfl-4),* wild-type C24 and transgenic *Arabidopsis* containing *35S-LFY* [25 J were also grown at the same time under the same conditions for comparison. Seeds were treated at 4 °C overnight before transfer to soil in a growth cabinet set at 21 °C under long-day conditions (16 h light/8 h dark) to promote flowering. The first day at 21 °C was counted as day zero. The LFY-GUS construct was constructed by cloning the *BamHI* promoter fragment from pDW 132, that contains 2.3 kb of upstream promoter sequence and includes the start of translation just upstream of one of the *BamHI* sites, in-frame with the coding region of GUS in the binary vector pBI101.2. The ELF-GUS construct was constructed by substituting the *LFY* promoter with a similar 2.3 kb

fragment from the *ELFI* gene. The two reporter gene constructs were mobilised to *Agrobacterium* strain AGLI as described above. C24 *Arabidopsis* roots were transformed with both the LFY-GUS and ELF-GUS constructs as described and T 1 seed collected *in vitro*. T 1 plants were stained with X-gluc (Progen Industries, Brisbane, Queensland, Australia) as described in Jefferson *et al.* [16] and sectioned and photographed as described in Andersson *et al.* [2].

Results

Eucalyptus globulus *has one expressed* ELF *gene* (ELF 1) *and one pseudogene* (ELF2)

Primers matching two highly conserved regions in the *LFY* and *FLO* genes, were used to amplify a 141 by product from *E. globulus* DNA prepared from tissues from a single tree. This PCR product was used to screen a *E. globulus* genomic library and three genomic segments with different restriction patterns were isolated. When the coding region of one of these clones was used as the probe for Southern analysis (Figure 2A), three *EcoRl* bands (18 kb, 6.2 kb and 4.7 kb) and two *BamHI* bands hybridized. Southern analysis (Figure 213) on selfed progeny from the same tree showed that all seedlings inherited the 6.2 kb EcoRl band *(ELFI), but* two (lanes 6 and 9) lacked the 18 kb band *(ELF2-1)* and one (lane 1) lacked the 4.7 kb band *(ELF2-2)*. This suggests that in *E. glob*ulus there are two *ELF loci (ELFI* and *2),* with two alleles from one of these loci *(ELF2-I* and *ELF2-2)* segregating in this family.

ELF] contains two introns and codes for a putative protein (Figure 3) with high sequence similarity to both FLO and LFY. Neither *ELF2-I* nor *ELF22* can encode a functional protein. The arginine at amino acid 84 in *ELFI* is converted to a stop codon in both *ELF2* sequences; an extra cytosine residue immediately before intron 2 causes a shift in the reading frame. *ELF2-1* contains an additional stop codon at amino acid 82 and *ELF2-2* another frame shift mutation before intron 1. Both alleles lack 18 nucleotides at the 5' end of the coding region, including the initial ATG, and are not similar to *ELFI* further upstream suggesting that they lack a functional promoter. *ELF2*I is 97.0% identical to *ELF]* and *ELF2-2* is 97.6% identical to *ELF7*.

The *ELFI* gene encodes a putative protein of 359 amino acids which is 67% identical to LFY and 71% identical to FLO (Figure 4). The three protein sequences are most similar in their C-terminal regions. Beyond Arg-177, ELF 1 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 there are 156 amino acids in total where virtually all changes are conservative replacements. N-terminal of Arg-177, the ELF1 protein is 55% identical to LFY and 58% identical to FLO. The ELF1 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. ELF1 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 occurrence of *ELF* loci in species related to *E. globulus* was examined by Southern hybridization. Figure 5 shows a Southern blot of EcoRl-digested DNAs, probed with the *ELF/ probe,* from six closely related eucalypt species, three distantly related eucalypt species and species from five related genera within the Myrtaceae. Two or three hybridizing bands were present in all of the eucalypt species and in *Angophora,* which is regarded as the genus most closely related to *Eucalyptus*. In four of the other five genera, either one or two bands hybridized. It appears that *ELF* is ubiquitous amongst the Myrtaceae.

ELF mRNA could not be detected in total RNA or poly(A)+ RNA by northern blotting techniques. *How*ever, using cDNA reverse-transcribed (RT) from RNA extracted from various plant tissues as template, we were able to amplify a fragment of the ELF cDNA from young floral buds. Analysis of RT-PCR products by Southern hybridization using the *ELF1* probe (not shown), indicated that *ELF* was expressed only in young floral buds and not in mature leaves, roots, stems or mature floral buds. The cDNA PCR product derived from the young buds was cloned and found to have the same nucleotide sequence as the coding region of *ELFI*. No products corresponding to *ELF2* were seen suggesting that *ELF2* is not transcribed.

ELF *is expressed strongly in the floral primordium and during floral organ development*

In floral material *ELF* expression was detected only in young buds, similar to the expression of the *FLO* and LFY genes in *Antirrhinum* and *Arabidopsis,* respectively. The pattern of expression of *ELF* in floral tissue

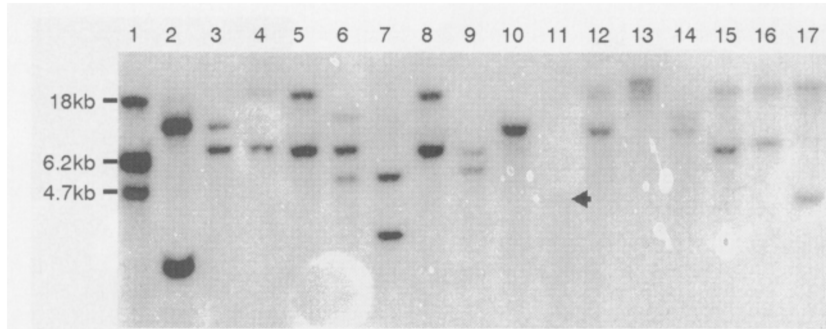

Figure 5. ELF loci in species related to *E. globulus*. Southern blot of genomic DNA from species closely related (lanes I to 6) or distantly related (lanes IS to 17) to *E. globulus ssp. globules* and species in related genera within the Myrtaceae (lanes 9 to 14) digested with ***EcoRl*** (unless otherwise noted) and probed with the *ELF2* genomic clone pELFA. Sizes in kilobases. *1, E. globulus ssp globulus; 2 E. globules ssp. globulus* digested with ***BamH l*** *; 3, E. nitens;* 4, *E. globules ssp. pseudoglobulus: 5, E. globules ssp. bico,stata; 6, E. ciuerea;* 7, *E. vinrirralis; 8, E. globules ssp. rrraidenii; 9, Augophora sp.; 10, Melaleuca sp.; 11, Leptosperruum; 12, Calli.stemou sp.; 13, Lophostemou sp.;* 14, *Svrwarpia sp. 15, E. grandis;* 16, *E. camaldulensis;* 17, *E. incrassata.* The faint band in lane 1 1 is indicated by an arrow.

was determined more precisely by *in situ* hybridization of longitudinal sections of floral buds of *E. globules* and *E. macrandra.* Eucalypt tissues tended to stain light brown during fixation, noticeably in oil glands, epidermal cells and stigmatic cells of more mature flowers. However, the characteristic blue-purple color generated from alkaline phosphatase substrates observed during the detection of the digoxigenin-labelled antisense probes was easily distinguished from the non-specific coloration. No labelling other than background was observed with serial sections probed with sense probes (not shown).

The patterns of *ELF* expression in floral buds of two eucalypt species, *E. globulus* and *E. macrandra,* were similar and a selection of the patterns observed at different floral stages are shown in Figure 6. In *E. macrandra ELF* was first detected at multiple foci corresponding to the developing floral primordia before they had enlarged into distinct floral buds and burst through the covering bracts (Figure 6A). Expression was observed briefly in sepal primordia and then in petal primordia (Figure 6B). In *E. globules,* at the earliest stage of lateral shoot development, it was not always possible to be certain whether a lateral shoot was vegetative or floral, but at a slightly later stage, strong expression was observed in sepal primordia which arose on the sides of the floral meristem (Figure 6C). *ELF* expression declined in the sepals as they enlarged, partially fused and became pointed at the apex, and was then observed in the petal primordia which enlarged, fused and became joined to the sepals (Figure 6C and 6D). As the petal primordia enlarged, expression appeared in the centre of the floral meristem (Figure 6E), where the carpels are formed, and in the stamen primordia adjacent to the base of the petals (Figure 6E). After this time, expression declined in the petals and was not observed again in the operculum tissues. Expression was maintained during stamen development and in the region of the developing gynoecium, particularly in the developing ovules (Figure 6F). *ELF* expression was not detected in fully developed floral buds that lie quiescent over winter and flower in the following spring, but these tissues were extremely difficult to section and contained high levels of phenolics and oils that interfered with proper *in situ* development.

ELF is *expressed strongly in adult leaf primordia*

The expression of *ELF* was also investigated by *in situ* hybridization in vegetative tissues of mature and juvenile eucalypts. *ELF* was not detected in roots of *E. globules* (not shown). In adult shoots of *E. globules* expression was observed in the young leaves and leaf primordia at the sides of the apical meristems (e.g. Figure 6G). *ELF* expression was not detected in the centre of the apical meristem. Expression in the leaf primordia persisted as they enlarged, before it then became restricted to the adaxial side of the leaf near its middle and then subsequently declined and disappeared in older leaves. New expression appeared in bract primordia of lateral shoots as they developed in the leaf axils (Figure 6G). Expression in the bracts declined as they expanded and the leaf primordia began to develop. Weaker *ELF* expression was also detected in young leaves and both leaf and bract primordia forming at the apex of 4-month-old juvenile seedlings which were not yet competent to flower (Figure 6H). A comparison between young vegetative

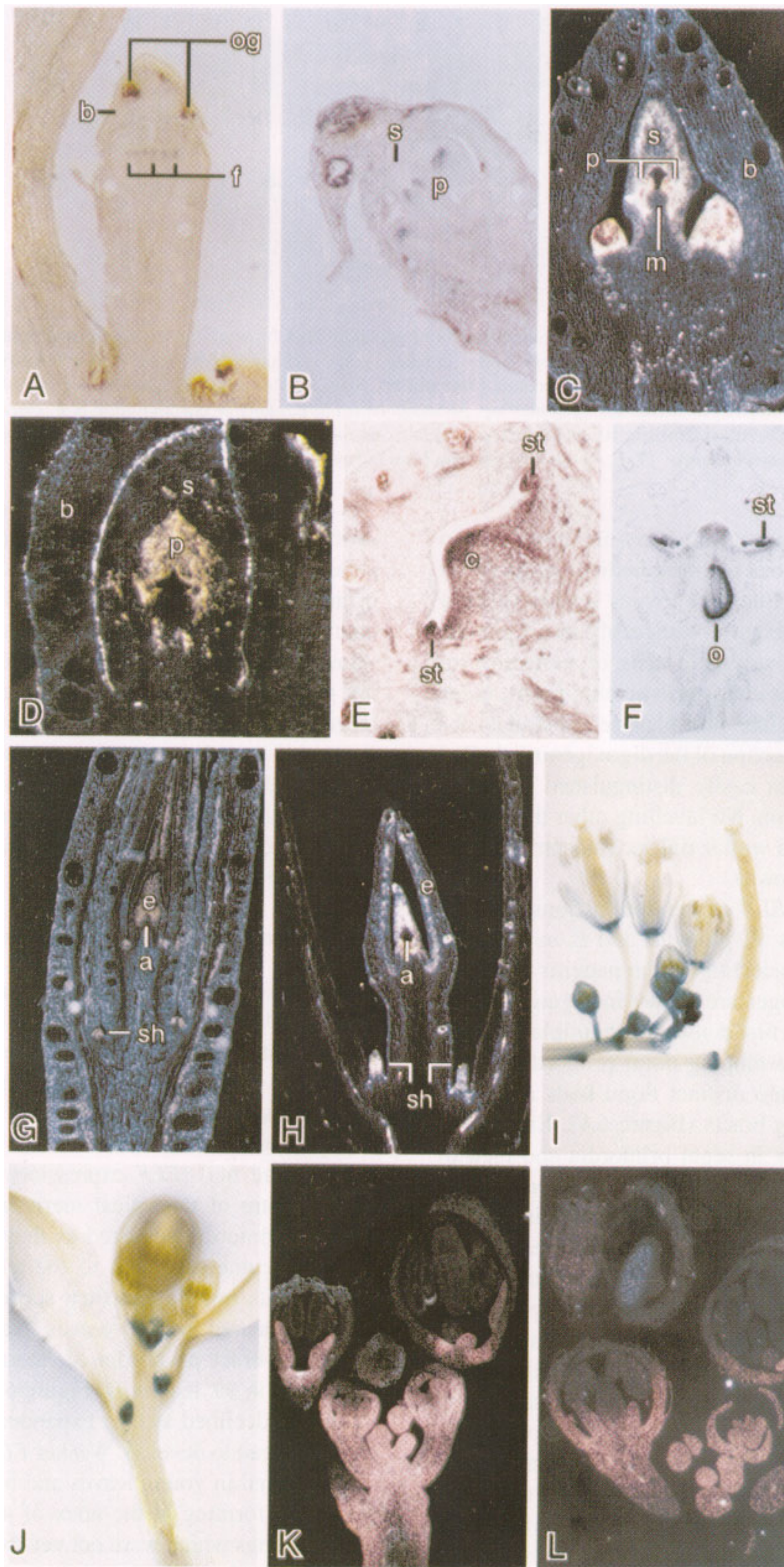
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Figure Expression of *ELF* RNA in longitudinal sections of young Eucalyptus floral and vegetative tissues using digoxigenin-labelled RNA probes and expression of the reporter gene, GUS, driven by the *ELF* and *LEAFY* promoters in transgenic *Arabidopsis*. Sections were photographed under dark-field illumination except for A, B, E and F which were photographed under bright-field illumination. In dark field the hybridization signal is yellow-orange and in bright field it is blue-purple. Micrographs A and B are of *E. macrandra* floral buds and C-H are sections of flower and leaf buds of *F. globulus ssp. bicostnta. A. ELF* expression in the developing floral primordia (f) forming in an l.. *uuwrcmdra* inflorescence (h = bract, og = oil glands). B. *ELF* expression in developing petal (p) primordia after the decline in expression in the sepals (s). C. In *F. globulus.* strong hybridization signal appeared sequentially in the sepal and then the petal primordia as they arose from the sides of the floral meristem, expression declined in the center of the floral meristem (m). D. Hybridization declined in the sepals, but remained in the petals until a later stage when it too declined. E, F. Strong hybridization appeared in the stamen (so primordia and the developing carpels (c) (E) and remained in the mature filaments and developing ovules (o) (F) G. *ELF is* expressed uniformly in leaf primordia (e) as they form on the apical meristem (a). The first detectable expression is on the flanks of the meristem, but not in the meristem itself. As the leaves enlarge expression contracts to the adaxial side in the central region of the leaf and then disappears. *As* expression declines in the leaves it is observed in the lateral shoots (sh) in the leaf axils. H. Weak signals were detected in juvenile vegetative apices in the same pattern as it was observed in the mature apices. I. Inflorescence from a transgenic *Arabidopsis* plant containing the ELF-GUS construct stained for GUS with X-gluc. *J. A* similar inflorescence from a LFY-GUS *Arabidopsis* transformant. K. Thin section of the apical buds from 1 photographed under dark-field illumination. L. Similar thin sections from *J.*

lateral shoots (Figure 6G) and floral buds (Figure 6C) which were collected and treated at the same time suggested *ELF* expression was slightly higher in floral tissue than in vegetative tissues.

The ELF *promoter can direct expression in transgenic* Arabidopsis

When the *ELF* promoter *(-2300* to +1 relative to the start of translation) was fused to the *GUS* reporter gene and introduced into *Arabidopsis,* strong *GUS* expression was seen in very young flower buds of TO and T1 plants (Figure 61 and 6K). As the buds developed, expression generally declined and became restricted to the pedicel and the developing sepals and petals. Low expression was seen in the developing stamens and carpels but this declined and became restricted to the base of the filament. In fully mature flowers, expression was only observed at the base of the petals and sepals. No expression was observed in mature siliques but expression remained strong at the base of the silique at the top of the pedicel. Plants transformed with a *LFY-GUS* construct showed an almost identical pattern (Figure 6.1 and 6L) to the *ELF-GUS* transgenics. Although the levels of expression of the reporter genes were comparable, expression of the *LFY* promoter construct always appeared lobe slightly lower than the *ELF* construct. No expression from either construct was observed in vegetative tissues when the transgenic plants were grown under short days to inhibit flowering (not shown).

Ectopic expression of ELF *in* Arabidopsis *causes similar phenotypes to ectopic expression of* LEAFY

Twenty independent transgenic *Arabidopsis* plants were produced containing the *ELF-coding region* under the control of a *35S* promoter of CaMV, a strong constitutive promoter active in most plant tissues, including flowers. Nine of the primary transformants displayed features similar to *Arabidopsis* transformed with *35S-LFY [25]*. These included early flowering (Figure 7A), decreased height and abnormal terminal flowers subtended by a single cauline leaf (Figure 7B and 7C). The terminal flower phenotype was very similar to that observed with *35S-LFY* transformants (Figure 7D) and with the *terminal flower* mutants, *tfi-2, tfl-3* and *tfl-4,* of *Arabidopsis [1]* grown under the same conditions. Seeds were collected from these nine plants and ten seeds of each of four lines were grown in soil for further analysis. These four families flowered, on average, *5* days earlier than *C24.* The abnormal phenotypes co-segregated with the kanamycin resistance gene used to select the transformants.

Discussion

We are investigating the genetic regulation of flowering in the genus Eucalyptus, a genus that contains many species important in world forestry. Flower development in model plants, such as *Arabidopsis,* has been shown to be regulated by distinct sets of genes, among the earliest acting of which are the floral meristem identity genes, such as *LFY,* that are thought to establish and maintain the floral state, as opposed to the vegetative shoot fate, in apical and lateral meristems. Assuming that basic mechanisms for floral regulation would be at least partially conserved among dicots we have isolated an expressed eucalypt gene, *ELF,* using PCR with degenerate primers to a conserved region of the two floral meristem identity genes *LFY* and *FLO*. The expressed *ELF* gene contains two introns that occur in identical positions to

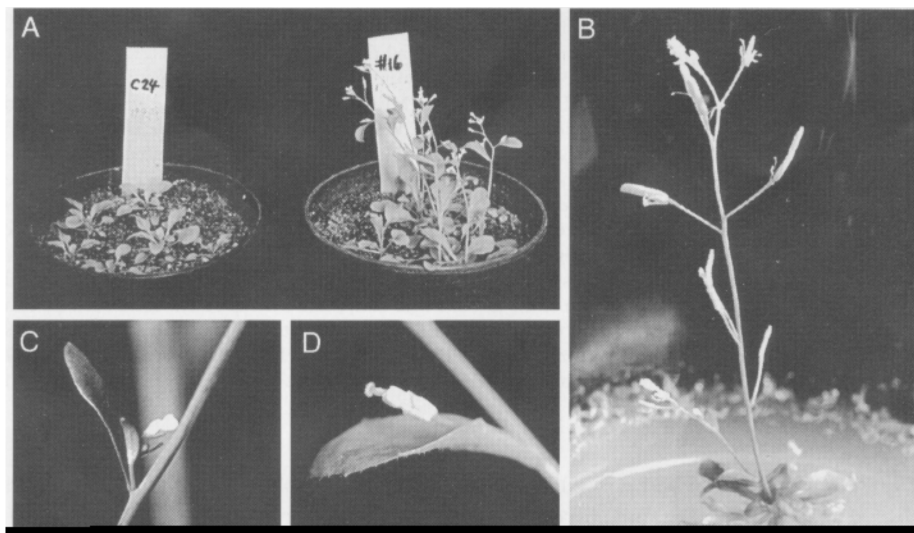

Figure 7. Ectopic expression of *ELF* mimics ectopic expression of *LFY* in transgenic Arabidopsis. A. Early flowering of 35SELF transformants (right) compared with non-transformed C2-l ecotype (left) grown under long day conditions. B. terminal flower phenotype seen with TO plants of 35SELF transformant line 16. Note the single siliques forming from axils where whole inflorescences would normally form. C. Close-up view of the single terminal flowers subtended by a leafy bract formed on T1 progeny of transformant line 16. D. Terminal flower phenotype observed with 35S-LFY transformants grown under the same conditions.

those found in *LFY* and *FLO* and its sequence and expression pattern are very similar to *LFY* and *FLO*. Ectopic expression of *ELF* phenocopies the ectopic expression of *LFY* in transgenic *Arabidopsis* [25] and its promoter directs expression of a reporter gene in *Arabidopsis* in a manner identical to the *LFY* promoter. These close structural and functional similarities lead us to believe that *ELF* is the functional eucalypt homologue of these two floral meristem identity genes. *LFYlFLO* homologues similar to *ELF* have also been isolated from tobacco [17] and poplar [22]. Weigel and Nilsson [25] have reported that transgenic hybrid aspen *(Populus tremula x P. tremuloides)* constitutively expressing the *Arabidopsis LFY* cDNA flowers precociously and has phenotypes similar to *Arabidop*sis transformed with the same construct. *ELF* genes were identified by hybridisation in diverse members of the Myrtaceae (Figure 5) and using similar degenerate primer PCR strategies we have recently isolated eucalypt homologues of the *Arabidopsis* floral organ identity gene *AGAMOUS* [12] and the floral meristem identity gene *APETALA* 1 [18]. These data add further weight to the hypothesis that floral regulatory mechnisms, and hence regulatory genes, are conserved across the angiosperms.

The putative protein encoded by *ELFI* shares a number of sequence motifs with the LFY and FLO proteins which are thought to be characteristic of plant transcription factors. These include in the centre of the protein a highly acidic region of 12 amino acids characteristic of transcriptional activation domains and a region of repeated leucines forming part of a potential leucine zipper that might be involved in protein-protein interactions. Both structural motifs are found in similar positions in the ELF1, LFY and FLO proteins, as well as in NFL, the tobacco homologue of these presumed transcriptional regulators [17]. The acidic domain is not conserved with respect to sequence and occurs in a region of relatively poor sequence conservation among the four floral meristem identity genes. The region immediately N-terminal to the acidic domain is highly basic and like the acidic domain is not highly conserved with respect to sequence in the four proteins. The putative ELF 1 protein is shorter at the N-terminal end than the three other homologs and thus lacks the proline rich region seen in the LFY, FLO and NFL protein sequences 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 have two *ELF* genes, although one of these is now inactive, as we have also shown that they have two *API* homologues [18] and two *AGAMOUS* homologues [12], both copies of which appear to be still functional.

This duplication is probably a general phenomenon within the genus, at least for *ELF* (Figure 5), and suggests that eucalypts may have experienced an ancient genome duplication and many of its genes might be expected to present in at least two copies.

In addition to being expressed in floral primordia in a pattern similar to *LFY* and *FLO,* the *ELF* gene is expressed strongly in leaf primordia forming on vegetative meristems, but not in the shoot apical meristem itself. The detection of *ELF* mRNA in vegetative tissues was surprising as significant expression of *LFY* and *FLO* had not previously been seen in leaf primordia [6, 24]. The pattern of expression of *ELF* is, however, similar to the *LFY/FLO* homologues *NFL1* and *NFL2* isolated from tobacco [17] and the homologues from poplar ([22J, Steve Strauss, pers. comm.). Like *ELF, NFL* is highly expressed in mature vegetative apices with expression localized to the adaxial sides of young leaves, leaf primordia and sites of leaf primordia initiation. The poplar *LFY/FLO* gene is also expressed in vegetative buds but no data is yet available on its precise cellular distribution. A cluster analysis of the amino acid sequences of the various floral meristem identity genes and their presumed homologs (not shown) performed with the Wisconsin Genetic Computer Group program PILEUP does not cluster the *ELF* gene with *NFL1* or *NFL2* (which are more closely related to *FLO)* so strong vegetative expression does not define a distinct sub-class of floral meristem identity genes.

Recent experiments by Blazquez *et al.* [3] and Hempel *et al. [14*] 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* (Columbia, Nossen and Landsberg *erecta)* grown under short day conditions. These authors used a reporter gene construct almost identical to the one described here to detect GUS expression in vegetative tissues using both histochemical and fluorometric assays on excised apices of transgenic *Arabidopsis* plants. We observed GUS activity driven by the *LFY* promoter, or the equivalent *ELF* promoter, in young flower primordia of transgenic *Arabidopsis,* but saw no significant expression with either construct in vegetative apices of the C24 ecotype grown under short-day conditions, so there is presumably some influence of either environmental growth conditions or genetic background on the absolute level of vegetative *LFY* expression in *Arabidopsis.*

Genetic studies in *Eucalyptus* are difficult because of the long time to flowering of trees; and no flowering mutants are known in this genus, but recent advances in the transformation of *Eucalyptus camalduleusis* [20] and the cloning of a number of the other floral gene homologues may allow us to use reverse genetic approaches to more clearly define the role played by *ELF* in vegetative and floral tissues in these important tree species.

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