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Expression of a *FLOWERING LOCUS T* homologue is temporally associated with annual flower bud initiation in *Eucalyptus globulus* subsp. *globulus* (Myrtaceae)

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Abstract. The transition to flowering in plants is the result of the balance of endogenous processes and environmental signals that act through a complex genetic pathway that has been studied extensively in annual plants such as *Arabidopsis*. Perennial trees are characterised by a juvenile non-flowering phase lasting several years followed by an adult phase in which there is repeated cycling between vegetative and reproductive growth. The genetic control of flowering time is potentially more complex in perennials than in annuals and is less understood. Here, we examine the control of flowering in *Eucalyptus globulus* subsp. *globulus*, an important forestry species in temperate parts of the world. The *E. globulus* subsp. *globulus* homologues of two important flowering genes *FLOWERING LOCUS T* (*FT*) and *LEAFY* (*LFY*) were isolated and quantitative RT-PCR was used to measure their expression over a 2-year period. The expression of the homologue of *FT* in *E. globulus* subsp. *globulus* leaves was associated with the annual transition from vegetative to reproductive growth (i.e. flower bud initiation). Expression of the *LFY* homologue was associated with early flower bud development. In a comparison of *FT* and *LFY* expression patterns in two clones each of an early and late anthesis genotype, no association between the expression of these genes and the timing of anthesis was shown. Taken together, this indicates that *FT* and *LFY* could form part of the flower initiation pathway in *Eucalyptus* but do not regulate the observed differences in anthesis time.

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Introduction

The transition to flowering in plants is influenced by external signals such as light and temperature, and internal signals, which interact through a complex genetic pathway mainly studied in the annual plant, *Arabidopsis* (reviewed in Amasino 2010). In perennial trees, the regulation of flowering time is potentially different to that in *Arabidopsis* and other annual species since trees are characterised by a juvenile, non-flowering phase lasting years, followed by an adult phase characterised by repeated cycling between vegetative and reproductive growth. As it was the first tree genome to be sequenced, tree flowering studies have mainly focused on poplar, a deciduous tree, while non-deciduous trees such as *Eucalyptus* have received less attention. *Eucalyptus globulus* subsp. *globulus* (hereafter referred to as *E. globulus*) is an important dominant tree of south-east Australian lowland forests, and it is also the main hardwood species grown in pulp-wood plantations in temperate regions of the world (Eldridge *et al*. 1993). An understanding of the genetic control of flowering time in this species is thus of ecological and economic significance.

In *Arabidopsis*, the photoperiod, vernalisation and endogenous pathways converge at a common set of genes to enable the integration of these different signals into a coordinated flowering response (Amasino 2010). *FLOWERING LOCUS T* (*FT*) is expressed in the leaf and the FT protein moves to the apex (Corbesier *et al*. 2007), where it interacts with the transcription factor FLOWERING D (FD, Abe *et al*. 2005; Wigge *et al*. 2005) to activate inflorescence identity genes including *APETALA1* (*AP1*) and *LEAFY* (*LFY*), which trigger flowering (Putterill *et al*. 2004). There are different gene families which are involved in the *Arabidopsis* flowering pathway. One of these families is the *FT*/*TERMINAL FLOWER1* (*TFL1*) family, which consists of six genes in *Arabidopsis*, including the mobile flowering stimulus *FT* and the flowering repressor *TFL1*. Other genes in this family are not as well studied, but may also be involved in flowering.

Variation among plant species in their flowering response to environmental signals is important for adaptation to a wide variety of environmental conditions, as it ensures that flowering can occur at the most favourable time for reproduction and seed dispersal. Although much has been learned from *Arabidopsis*, in order to understand the diversity of flowering responses, there is a need to study related flowering pathways in other plant species. Homologues of some of the genes in the *Arabidopsis* flowering

pathway have been identified in many tree species, and in many cases their function has been supported by transgenic complementation of *Arabidopsis* mutants. The presence of these genes in a wide range of plants suggests that the general framework of the pathway to reproductive initiation may be evolutionarily conserved to some extent. Transgenic expression of flowering genes in trees have shown that some genes, at least those later in the flowering pathway, are functionally conserved, and can be used to promote early flowering in trees. For example, *Arabidopsis LFY* induced early flowering when overexpressed in transgenic poplar and citrus trees (Weigel and Nilsson 1995; Rottmann *et al*. 2000; Peña *et al*. 2001), though in poplar the flowers were abnormal. Transgenic expression of *Arabidopsis AP1* also induced flowering in citrus (Peña *et al*. 2001). Transformed apple plants expressing the antisense apple homologue of *TFL1* flowered at an early age (Kotoda *et al*. 2006) and juvenile *Populus* stems transformed with *Populus* homologues of *FT* initiated normal inflorescences within weeks to months of transformation, the first reports of juvenile transgenic trees producing inflorescences (Bohlenius *et al*. 2006; Hsu *et al*. 2006).

In poplar, flowering studies have focused on *FT*, a key integrator of the flowering pathway (Zhang *et al*. 2010). *FT* homologues are involved in regulating the age of first flowering in poplar, with trees exhibiting a gradual increase in levels of *FT* until the time of first flowering (at age 5–6 years, Bohlenius *et al*. 2006; or at age 11 years, Hsu *et al*. 2006). The authors postulated that epigenetic mechanisms may play a role in the regulation of *FT* in trees, and that each annual cycle of growth in juvenile trees leads to a gradual release of the chromatin-based repression. Modification of chromatin structure plays a key role in repressing floral development during embryo and vegetative development in *Arabidopsis* and similar processes may maintain the juvenile phase in trees (Brunner and Nilsson 2004). Expression experiments in *Populus* have suggested that *FT* is also associated with the seasonal initiation of flowers (Hsu *et al*. 2006) and, surprisingly, the photoperiodic control of vegetative bud set (Bohlenius *et al*. 2006). Similarly, a homologue of *FT* is proposed to play a role in the control of growth rhythm in Norway spruce; *FT* upregulation is correlated with the induction of growth cessation and bud set under short days in autumn, and a decline in *FT* expression was associated with bud burst as temperatures increased in spring (Gyllenstrand *et al*. 2007). This alternative role of *FT* in a non-flowering plant could indicate that the ancestral function of *FT*-like genes was in the control of growth (Gyllenstrand *et al*. 2007).

Like most forest trees, *E. globulus* has a long juvenile, non-flowering phase (1–5 years; Jordan *et al*. 1999) followed by an adult phase characterised by repeated cycling between vegetative and reproductive growth. It is evergreen, but over the winter months in south-eastern Australia, vegetative growth is limited. Following the annual spring flush of vegetative growth, floral buds appear in the axils of new leaves. These buds clearly must initiate at the microscopic level several weeks before they are visible to the naked eye (6–8 weeks in *E. nitens*, Moncur *et al*. 1994) and even earlier at the molecular level. The inflorescences are usually single-flowered but occasionally have three flowers per umbel. During the early stages of flower development, the sepal primordia fuse to each other to form an outer (sepaline) operculum and the petal primordia fuse to form an inner (petaline) operculum. The entire umbel is enclosed by a pair of bracts which are shed early in flower development as the bud elongates, followed by the shedding of the sepaline operculum. The shedding of the petaline operculum to expose the stamens and style occurs once all parts of a flower are fully developed, from late autumn to early summer in *E. globulus* (Jones *et al*. 2011) and is the process commonly referred to by eucalypt researchers as 'flowering'. Here, the shedding of the petaline operculum will instead be termed 'anthesis' to avoid confusion with the flower initiation stage, as the latter corresponds to the stage generally referred to as 'flowering' in the model annual plant *Arabidopsis*.

Several homologues of *Arabidopsis* flowering genes have already been identified in *Eucalyptus*. Two homologues of *AP1* (*EAP1* and *EAP2*) and a homologue of *LEAFY* (*ELF1*) were identified in *E. globulus* subsp. *bicostata* and *E. globulus* subsp. *globulus*, respectively, and transgenic expression of these genes in *Arabidopsis* showed that they are functionally equivalent to their *Arabidopsis* homologues (Kyozuka *et al*. 1997; Southerton *et al*. 1998*b*). *EgrSVP*, the *E. grandis* homologue of *SVP*, has been identified; its ectopic expression in *Arabidopsis* delayed the transition to flowering and altered the flowering phenotype in a variety of ways, including the homeotic conversion of the perianth into leaves, indeterminate flower development, and altered plant architecture (Brill and Watson 2004). Southerton *et al*. (1998*a*) identified three MADS-box genes in *E. grandis* (*EgM1*, *EgM2* and *EgM3*) that had strong homology to floral organ identity genes. *EgM2* was part of the *PISTILLATA* clade, genes of which function as 'class B' genes in the ABC model of flower development (Bowman *et al*. 1991; Coen and Meyerowitz 1991). *EgM1* and *EgM3* had strongest homology to the *AGL2* (*SEPALLATA*) class E genes. The expression of these genes in sepals, petals, stamens and carpels of developing flowers was congruent with the gene homology, despite the modified floral structure of eucalypts. Dornelas and Rodriguez (2005) identified many *Eucalyptus* homologues of *Arabidopsis* flowering time genes. These EST (from the Brazilian FORESTS database) are not yet publicly available. Jaya *et al*. (2010) isolated the *E. occidentalis* homologues of *TFL1*, *LFY* and *AP1* (*EOTFL1*, *EOLFY* and *EOAP1*) and showed that the peak in the expression of *EOAP1* was associated with peak flower bud initiation.

While it has been shown that the *Eucalyptus* homologues of the *Arabidopsis* flowering pathway genes are expressed in specific tissue types, to date there have been few studies of how the expression of these genes may be regulated during the processes of flower bud initiation, flower development and anthesis. This study focuses on the annual transition from vegetative to reproductive growth, flower bud development and anthesis in *E. globulus*, the last of which is under strong genetic control (Gore and Potts 1995; Jones *et al*. 2011). To identify whether *FT* and *LFY* are associated with seasonal flower bud initiation and anthesis in *E. globulus*, the homologues of these genes were isolated from *E. globulus* and cloned. Their expression patterns were monitored in leaves, apices and flower

buds of 'early' (late autumn) and 'late' (summer) anthesis genotypes, over a 2-year period.

Materials and methods

Plant material

Tissue samples were harvested every 2 weeks from two ramets (i.e. two separate trees of the same genotype cloned by grafting) each of an 'Early' (late autumn) and 'Late' (summer) anthesis genotype from the Southern Tree Breeding Association breeding population of *E. globulus*. The two ramets of each genotype were named EarlyA, EarlyB, LateA and LateB. The 'Early' and 'Late' genotypes were from the Furneaux and Strzelecki Ranges races, respectively, and were chosen for this study as these races represent two of the extremes in time of anthesis. The Furneaux and Strzelecki Ranges races are well differentiated on many quantitative traits in addition to anthesis time (Dutkowski and Potts 1999) and these particular genotypes differ in their anthesis time, but vary only slightly in the timing of when flower buds are first observed, indicating that flower bud initiation could be a photoperiod-mediated process (Jones *et al*. 2011). Early anthesis genotypes therefore appear to have a shorter flower bud development time than later anthesis genotypes, which is caused by a period of near dormancy during flower bud development in late anthesis genotypes (Jones *et al*. 2011). These genotypes had been grafted onto *E. globulus* seedlings before planting at the seedEnergy Cambridge Orchard between September 2001 and May 2002, and later treated with paclobutrazol, a GA inhibitor that reduces internode length and increases the intensity of, but does not affect the timing of, flower initiation in eucalypts (Hetherington *et al*. 1992; Hasan and Reid 1995). *E. globulus* is a forest tree reaching heights of ~90 m in nature, but in the orchard, with grafting and treatment with paclobutrazol, trees were kept under 5 m in height.

Shoot apices, inflorescences and leaves at different developmental stages (Fig. 1) were harvested and frozen in liquid nitrogen and stored at –80°C until RNA extraction. Samples of the same type and developmental stage were pooled into one sample for RNA extraction. For example, a single sample of 'Leaf 1' in year one included three to four pooled leaves that had an inflorescence in the axil ('Bud 1') and originated from the same ramet and usually the same branch. Over the next year, 'Bud 1' continued to elongate and the sepaline and petaline operculae were shed, so that by year two, 'Leaf 1' had a developing fruit ('Bud 1') in its axis and 'Leaf 2' and 'Bud 2' tissues had initiated during the annual spring flush of vegetative and floral growth (Fig. 1). By year three, 'Leaf 2' had a developing fruit ('Bud 2') in its axis and 'Leaf 3' and 'Bud 3' tissues had initiated during the annual spring flush of vegetative and floral growth (Fig. 1). Samples were harvested every 2 weeks for 46 time points in total, providing replication across two flowering years from southern hemisphere winter 2006 to summer 2008. Tissues were sampled from the time of macroscopic appearance of flower buds or leaves until the end of the study (week 90) where possible.

Initially, two ramets of each genotype were harvested (EarlyA; EarlyB; LateA; LateB), but as no flower buds were observed on the EarlyB ramet in spring 2006, flower buds from this ramet could not be harvested. All ramets were genotyped using nine microsatellite markers (Jones 2009) to confirm that they were clonal (data not shown).

Where possible, tissues were harvested at ~1300 hours, but on occasion, some samples were taken later in the day. To test whether the abundance of key flowering and reference gene mRNA fluctuated during the potential sampling times, the leaves (Leaf 2) and apices of one ramet each of three different genotypes (LateA from this study plus an additional Early genotype from Furneaux and a genotype from the south-eastern Tasmania race) were harvested every 2 h from 1100 to 1700 hours, during vegetative flush and macroscopic appearance of flower buds in year two (spring 2007).

Isolation of nucleic acids

Tissues were crushed in liquid nitrogen in a mortar and pestle before nucleic acid extraction. Flower bud and apex sample RNA were isolated using the RNeasy mini kit (Qiagen, Doncaster, Vic., Australia), with 1% PVP (MW 40 000) and

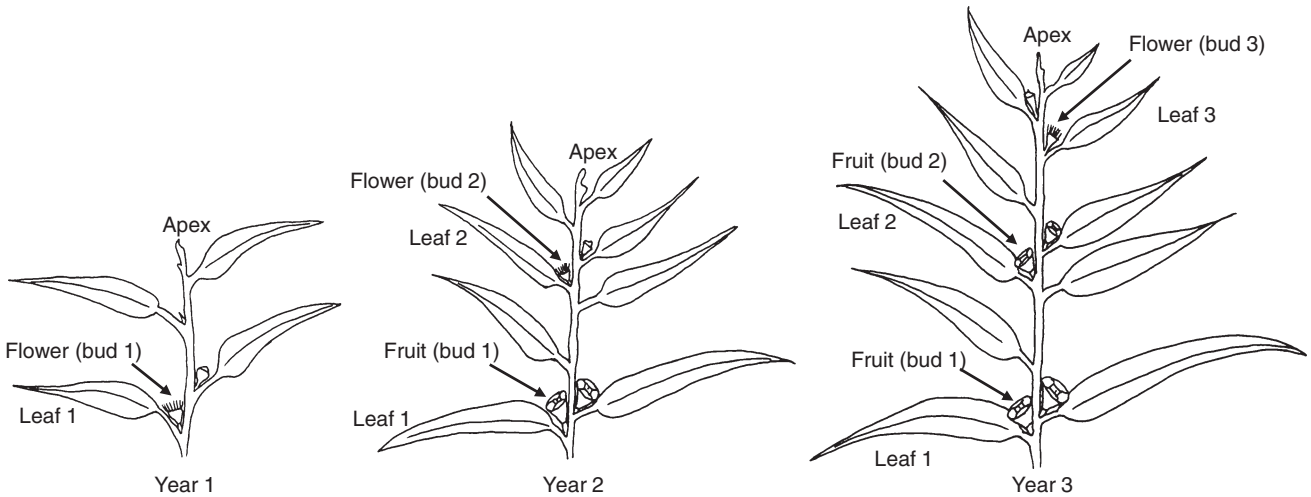

Fig. 1. Tissue types (bud, apex, leaf) harvested for studies of gene expression during *Eucalyptus globulus* flower development. Tissues were sampled from the time of macroscopic appearance of flower buds or leaves until the end of the study (week 90) where possible.

1% PEG (MW 20 000) added to the RLT extraction buffer before use. Leaf RNA was isolated using the SV Total RNA Isolation System (Promega, Sydney, NSW, Australia), with 1% PEG (MW 20 000) added to the lysis buffer. In both protocols, the DNase incubation was increased to 30 min. Genomic DNA (gDNA) for PCR and Southern analysis was extracted using Plant DNA Isolation Kit (Mobio, Carlsbad, CA, USA) with no modifications to the protocol. Nucleic acids were checked for purity on agarose gels and quantified using the Picofluor Handheld Fluorometer (Turner BioSystems, Sunnyvale, CA, USA). The PicoGreen and RiboGreen dyes (Invitrogen by Life Technologies, Grand Island, NY, USA) were used for DNA and RNA quantification, respectively, compared with a λDNA and 18S RNA standard, respectively, according to manufacturer's instructions.

First strand cDNA was synthesised using the Improm II RT kit (Promega), according to the protocol of the supplier, using 500 ng RNA in a total volume of 20 µL. A negative control (i.e. omitting reverse transcriptase enzyme, RT-) was included for each RNA sample in order to check for genomic DNA contamination of RNA samples. The cDNA was diluted five times in sterile distilled water and 5 µL was used in gene-specific PCR for gene isolation while 2 µL was used in quantitative real-time PCR (qRT-PCR).

Isolation of genes

To isolate the *E. globulus* homologues of *FT* and *LFY* and constitutively expressed reference genes, published *Eucalyptus* sequences were used for primer design with Primer 3 version 0.4.0 (Rozen and Skaletsky 2000), or several plant sequences were aligned to design degenerate primers using Block Maker and CODEHOP (Rose *et al*. 1998), or primers from other qRT-PCR studies were tested (Table 1). In order to include the *E. globulus* homologue of *TFL1* in the *FT*/*TFL1* family phylogenetic tree, *EglTFL1* was isolated using degenerate primers and nested PCR as detailed by Sreekantan *et al*. (2004).

Each PCR reaction mixture (25 µL final volume) contained 1 × *Taq* polymerase reaction buffer (Fisher Biotec, Wembley, WA, Australia; 67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL gelatin); 100 µg/mL of BSA; 2 mM $MgCl_2$; 160 µM each of dATP, dCTP, dGTP and dTTP; 7.5 pmol of each primer; ~25 ng cDNA or gDNA and 2.2 units of *Taq* DNA polymerase. PCR amplification was performed in a PTC-100 Programmable Thermal Cycler or Tetrad Thermal Cycler (MJ Research, Inc., Waltham, MA, USA), using the following conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, optimum annealing temperature for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min. The optimum annealing temperature was 60°C for all genes except *EglFT* and *EglACT11* for which annealing temperatures of 55 and 50°C were used, respectively.

PCR products were cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega), quantified using the Picofluor Handheld Fluorometer, and ligated into the pGEM-T Easy Vector (Promega) according to manufacturer's instructions. Gene-specific PCR or pGEM primers (see below) were used to identify positive inserts and these were sequenced at the Australian Genome Research Facility (Brisbane, Qld, Australia) using ABI Prism BigDye Terminator Cycle

Table 1. Sequences used to isolate *Eucalyptus globulus* flowering genes

Primer sequences given are those designed in this study. Observed gDNA and cDNA sequence lengths (in base pairs) and the NCBI accession numbers for the genes isolated in this study are given. For *EglFT1* and *EglTFL1*, the primers used to produce a probe for Southern blotting are also given

Gene (homologue in *Arabidopsis*)	Species (accession for primer design)	Primer sequence/reference	Size (gDNA/ cDNA)	Accession (this study)
EglFT1 (*FT*) (gene isolation)	*Arabidopsis thaliana* (NM_105222) *Populus tremula* (DQ387859) *Glycine max* (TC219541)	F: GMGAYGAYCTSCGGAMNTTYTAYAC R: CCGCCRSAGCCGSWYTCNCKYTG	1007/343	HQ453991 HQ453992
EglFT1 (*FT*) (Southern probe)		F: GGCAGGAGATCGTGTGCTAC R: CCCCAGGTTGTAGAGCTCAG	158/158	
EglTFL1 (*TFL1*) (gene isolation)	*Metrosideros excelsa* (AY170872)[A]	See Sreekantan *et al.* (2004)	627/161	HQ385322
EglTFL1 (*TFL1*) (Southern probe)		F: CAGACCCAGATGTTCCTGGT R: CGAACCTGTGGATACCAATG	619/154	
ELF1 (*LFY*)	*Eucalyptus globulus* (AF034806)	F: GAGGAGCTGTTCGAGGCTTA R: AAGGGAGTTCGAGATGGTGA	Not sequenced/ 1023	HQ453993
EglH4 (*H4*)	*Eucalyptus globulus* (AY263810)	F: GCGGCAAGGGAGGCAAGG R: CGGATCACGTTCTCCAGGAA	193/193	HQ456540 HQ456545
EglTUBA1 (*TUA6*)	*Eucalyptus globulus* (U37794)	F: AGCGCCTGTCTGTGGATTAT R: AGAACGCCTGCAGATTTCAT	185/185	HQ456541
Egl18S (*18S*)	*Pisum sativum* (U43011)	See Ozga *et al.* (2003)	62/62	HQ456544
EglUBQ (*UBQ*)	*Pisum sativum*	See Albrecht *et al.* (1998)	Multi-banded	Not sequenced[B]
EglEF1α (*EF1α*)	*Pisum sativum*	See Foucher *et al.* (2003)	213/~300	HQ456542
EglACT11 (*ACT11*)	*Populus* (CA824001)	See Brunner *et al.* (2004)	228/228	HQ456543

[A] A *Eucalyptus* homologue of *TFL1* was published (*EOTFL1*, Jaya *et al.* 2010) after this study commenced.
[B] *EglUBQ* had a multi-banded product.

Sequencing Ready Reaction Kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) with plasmid DNA as the template and primers specific to the pGEM-T Easy Vector. Phylogenetic analyses were undertaken to confirm the identity of the flowering genes that were isolated. The amino acid sequences were aligned with ClustalX (Larkin *et al*. 2007). A neighbour-joining tree was constructed in PAUP version 4.0b10 (Swofford 2003) with 1000 bootstrap replicates.

Southern blotting was performed following the protocol of Sambrook *et al*. (1989). Genomic DNA was digested by *Bam*HI, *Eco*RI, *Hind*III and *Xba*I. The conserved coding regions of *EglFT1* and *EglTFL1* were used as probes for the Southern analysis at low stringency, isolated using the primers listed in Table 1.

Quantitative real-time PCR

Primers for qRT-PCR (Table 2) were designed based on the sequences of the cloned fragments. When both gDNA and cDNA sequences were available, primers were designed to bind to different exons to allow identification of false positives from gDNA contamination in qRT-PCR (Bustin 2000). Where the sequenced PCR product length was less than 200 bp, the same primers were used (Table 2). Reactions contained 1 × SensiMix (Bioline, London, UK), 1 × SYBR green solution, 0.3 µM each primer and 2 µL each cDNA sample (~10 ng) in a total volume of 10 µL. Real-time PCR was performed on the Rotor-Gene 3000 (Qiagen, Melbourne, Vic., Australia) using the following conditions: 95°C for 10 min; 50 cycles of 95°C for 5 s and 60°C for 40 s; 65°C for 45 s then a ramp from 65 to 95°C in 1-degree increments, with 5 s at each step. Each reaction was performed in duplicate and no-template controls were included with each qRT-PCR run. In addition, seven samples of plasmid DNA with an insert of the gene of interest, in a 10-fold dilution series from 1 ng/µL to 1×10^{-6} ng/µL were included in each qRT-PCR run. Results were excluded if one of the duplicate reactions failed, and if the equivalent reaction using the template produced with reverse transcriptase omitted (RT-) had a quantification cycle (C_q, Pfaffl 2001) of less than 23 (i.e. gDNA contamination) or for a *Egl18S* C_q >20.0 (i.e. low cDNA template). Duplicate C_q were averaged.

To determine which reference gene was most appropriate, the abundance of *EglH4*, *EglTUBA1*, *Egl18S*, *EglUBQ*, *EglEF1α* and *EglACT11* across nearly a year of sampling was measured and a regression line fitted using GraphPad Prism version 5.0. All of the genes were tested in leaf and apex tissue of each genotype, and *Egl18S* was tested in bud tissue of the early anthesis genotype. *Egl18S* was chosen as the most suitable reference gene (see Results) and so it was used to normalise data in subsequent experiments, using the mathematical model in Pfaffl (2001).

Results

Gene isolation and qRT-PCR primer design

To examine the genetic control of flower bud initiation and opening in *E. globulus*, we isolated some key *Arabidopsis* flowering gene homologues. The flowering genes *EglFT1*, *EglTFL1* and *ELF1* were isolated successfully from *E. globulus* cDNA from various tissue types (Table 1). All PCR fragments were cloned and sequenced, and these sequences were used to design primers for qRT-PCR (Table 2).

EglFT1 was isolated from gDNA and leaf cDNA using degenerate primers designed using *Arabidopsis thaliana*, *Populus tremula* and *Glycine max FT* sequences (Table 1). *EglFT1* (partial gene) had four introns in positions conserved across *FT* genes from other species. The annotated sequence is available on NCBI, accession numbers HQ453991 (‘Early’ Furneaux genotype) and HQ453992 (‘Late’ Strzelecki Ranges genotype). *EglTFL1* was isolated from gDNA and apex cDNA using degenerate primers and nested PCR, as detailed by Sreekantan *et al*. (2004). *EglTFL1* (partial gene) had two introns in positions conserved across *TFL1* genes from other species. The annotated sequence is available on NCBI, accession number HQ385322. The amino acid sequences of *EglFT1* and *EglTFL1* are also shown in the alignment in Fig. 1 of the Accessory Publication (available online). A Southern blot analysis revealed that *EglTFL1* was single copy, but there were at least three bands for *EglFT* (data not shown). However, a BLASTN search of the *Eucalyptus* genome on Phytozome (*Eucalyptus grandis* Genome Project 2010, available at http://www.phytozome.org/eucalyptus.php, accessed 28 November

Table 2. Primers, expected fragment sizes (in base pairs) and reaction conditions for qRT-PCR of the *Eucalyptus globulus* homologues of *FT* and *LFY* and the reference genes

The primers used for reference gene isolation (Table 1) were also used for qRT-PCR

Gene	Primer sequences	Size (gDNA/cDNA)	T_m range	Mean reaction efficiency
EglFT1	F: TGATATTCCAGCTACGACAGGA R: CCCCAGGTTGTAGAGCTCAG	331/190	86.5–88.3	1.73
ELF1	F: AACGGCCTGGACTACCTCTT R: GCAGTGGACATAGTGCCTCA	~280/187	86.0–87.3	1.74
EglH4	F: GCGGCAAGGGAGGCAAGG R: CGGATCACGTTCTCCAGGAA	193/193	91.2–91.8	1.76
EglTUBA1	F: AGCGCCTGTCTGTGGATTAT R: AGAACGCCTGCAGATTTCAT	185/185	84.7–85.3	1.75
Egl18S	See Ozga *et al.* (2003)	62/62	81.5–87.7	1.70
EglUBQ	See Albrecht *et al.* (1998)	Multi-banded	85.8–88.2	1.72
EglEF1α	See Foucher *et al.* (2003)	213/~300	83.7–85.0	1.76

2011), which includes the initial 8× mapped *E. grandis* genome assembly and annotation, indicated that only one *FT*-like gene is present in the database so far (scaffold_2:23,918,064..23,919,083), which corresponds to the *FT*-like gene isolated in this study. The *E. grandis* and *E. globulus* gDNA sequences were 96.1% similar, the main differences being two insertions in *E. grandis*, both of which were in intron 2. Protein sequences were 100% similar. There was one *TFL1*-like gene present in the database (scaffold_10:5,248,545..5,249,173; 96.4% similarity). *EglFT1* clustered within the *FT* clade of the *FT*/*TFL1* gene family while *EglTFL1* had close affinities to other plant *TFL1* genes (Fig. 2). Additional non-degenerate primers were designed for qRT-PCR of *EglFT1* (Table 2) and were successful at an annealing temperature of 60°C with a high reaction efficiency and a single product (i.e. a narrow melting temperature (T_m) range) (Table 2).

ELF1 was isolated from *E. globulus* apex cDNA with primers designed using the *E. globulus ELF1* sequence (Southerton *et al*. 1998*b*) (Table 1). The sequence obtained had strong homology with the *ELF1* sequence from Southerton *et al*. (1998*b*). A BLASTN search of the *E. grandis* genome sequence revealed there were two *LFY*-like genes present: scaffold_11:29,183,351..29,184,527 and scaffold_11:35,886,649..35,887,809 but the second sequence contained stop codons. Additional primers were designed for qRT-PCR of *ELF1* (Table 2) and were successful at an annealing temperature of 60°C with a high reaction efficiency and a single product (i.e. a narrow T_m range) (Table 2).

The candidate reference genes *EglH4*, *EglTUBA1*, *Egl18S*, *EglUBQ*, *EglEF1α* and *EglACT11* were all isolated successfully from *E. globulus* apex cDNA using the primers listed in Table 1. The same primers were used for qRT-PCR and were successful at an annealing temperature of 60°C with a high reaction efficiency and a single product (i.e. a narrow T_m range), except for the *EglACT11* primers that did not successfully amplify a product. *EglUBQ*, however, had a wide T_m range and multi-banded product. This is not necessarily a problem, since these *UBQ* primers are used widely in studies of gene expression in pea, as all bands are amplified quantitatively (Albrecht *et al*. 1998).

Expression patterns of reference genes

To determine which reference gene was most appropriate, the abundance of *EglH4*, *EglTUBA1*, *Egl18S*, *EglUBQ*, *EglEF1α* and *EglACT11* transcripts across nearly a year of harvests was measured and a regression line fitted. In leaf tissue, *Egl18S* was the most abundant (i.e. lowest C_q) reference gene and *EglTUBA1* was the least abundant (Fig. 3*a*, *c*). All reference genes showed a more or less consistent expression among samples of leaf tissue, with a low slope and close fit to the regression line (Fig. 3*a*, *c*). *Egl18S* was also expressed at high levels in apex tissue, but *EglUBQ* and *EglTUBA1* were the least abundant transcript in this tissue type (Fig. 3*b*, *d*). All of the reference genes were less stably expressed in apex tissue than they were in leaves (Fig. 3) but the pattern was consistent across genes for a given harvest date. The expression profile of *Egl18S* in bud tissue was also consistent (Fig. 4) and as this gene also had a stable expression pattern in leaves and apices (Fig. 3), it was chosen to normalise data in subsequent experiments.

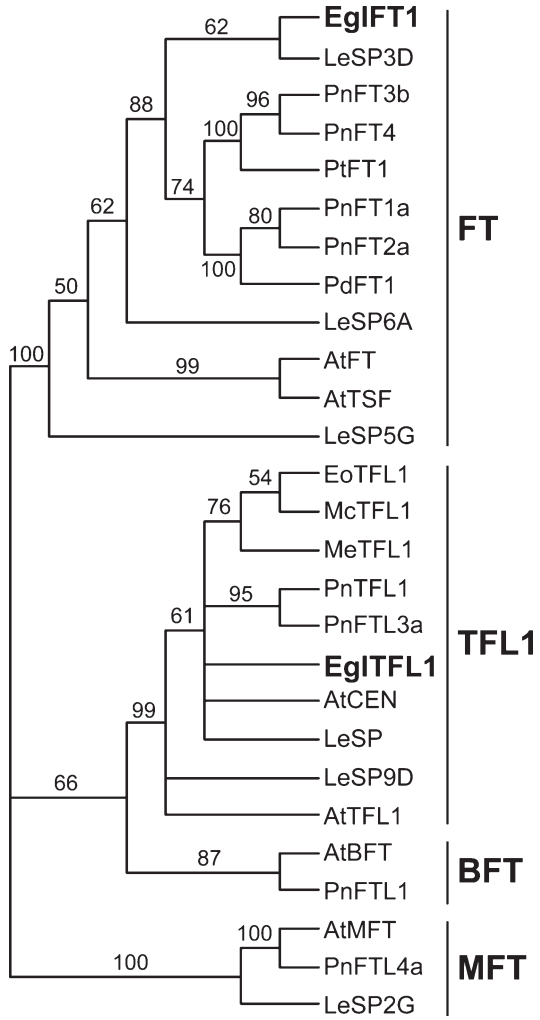

Fig. 2. Neighbour-joining tree of several plant *FT*/*TFL1*-like genes and the *Eucalyptus globulus* putative homologues of *FT* and *TFL1* (*EglFT1* and *EglTFL1*), generated with 1000 bootstrap replicates. NCBI/TAIR accession numbers of sequences are given in the Accessory Publication Table 1, available online. Neighbour-joining tree for the amino acid sequence aligned with ClustalX. Bootstrap values are indicated as a percentage above each branch. Genes isolated in this study (*EglFT1* and *EglTFL1*) are in bold. At, *Arabidopsis thaliana*; Ci, *Citrus unshiu*; Cm, *Cucurbita maxima*; Cr, *Chenopodium rubrum*; Egl, *Eucalyptus globulus*; Eo, *E. occidentalis*; In, *Ipomoea nil*; Le, *Lycopersicon esculentum*; Mc, *Metrosideros collina*; Me, *M. excelsa*; Md, *Malus* × domestica; Mt, *Medicago truncatula*; Pd, *Populus deltoides*; Pn, *P. nigra*; Pt, *P. tremula*; P, *P. trichocarpa*; Ps, *Pisum sativum*; Vv, *Vitis vinifera*.

Expression patterns of flowering genes

To test whether key *Arabidopsis* flowering gene homologues were associated with the seasonal initiation of flower buds and anthesis in *E. globulus*, the abundance of the flowering gene transcripts were compared in early (EarlyA, EarlyB) and late (LateA, LateB) anthesis genotypes. The flowering genes studied represented pathway integrator (*FT*) and inflorescence identity

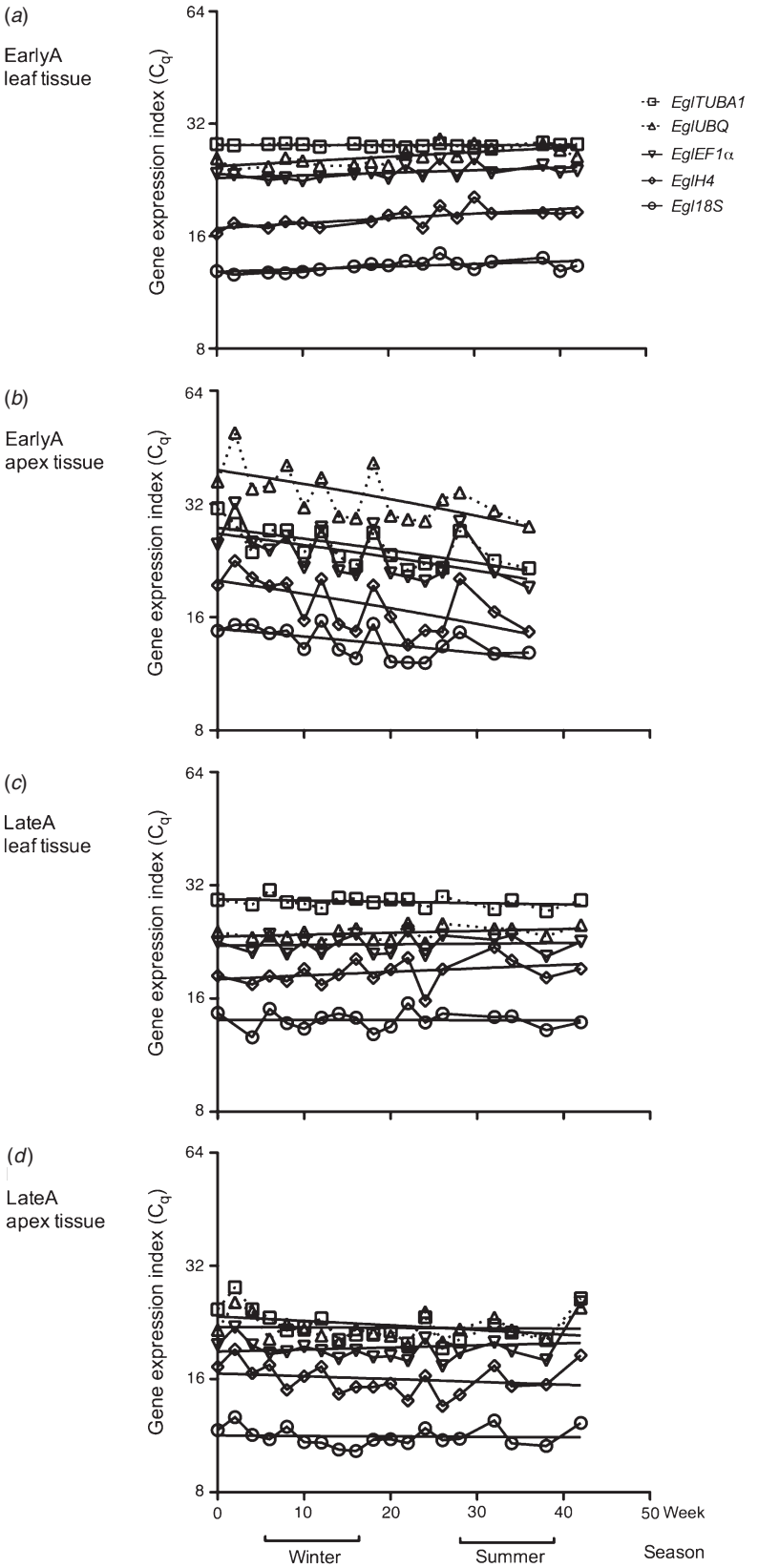

Fig. 3. Variation in expression of reference genes over 46 weeks, showing regression lines for leaf and apex tissue in two genotypes of *Eucalyptus globulus*. (*a*) EarlyA leaf tissue; (*b*) EarlyA apex tissue; (*c*) LateA leaf tissue; (*d*) LateA apex tissue.

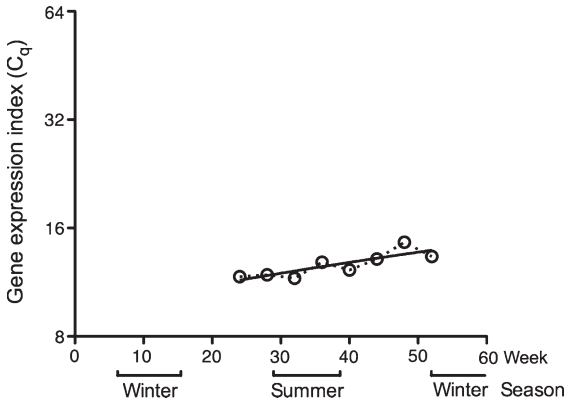

Fig. 4. Variation in expression of the reference gene *Egl18S* over 14 weeks in buds of a single genotype (EarlyA) of *Eucalyptus globulus*. Regression line is shown by a dashed line.

(*LFY*) roles in the *Arabidopsis* flowering pathway (Amasino 2010).

Expression of *EglFT1* in the young leaves of each year was low in late summer through autumn to early winter and then gradually increased during the transition to flower bud initiation in spring of each year. This pattern was most obvious in the late anthesis genotype (LateA, Fig. 5*c*; LateB, Fig. 5*d*) for which the timing of anthesis and the macroscopic appearance of the new flower buds coincided.

Comparison of the early and late anthesis genotypes showed no association between the expression of *EglFT1* and the timing of anthesis, as the timing of *EglFT1* expression in Early was similar to that of the Late ramets (Fig. 5) despite a 6-month difference in the timing of anthesis between the two genotypes. *EglFT1* expression was therefore associated with flower bud initiation but not anthesis time.

EarlyB had a brief peak in *EglFT1* expression in the first year leaves, but this was not ongoing as in the other ramets (Fig. 5). There was, however, an ongoing upregulation of *EglFT1* in these leaves in the second year despite the absence of anthesis (Fig. 5*b*). There were no flower buds initiated in this ramet in the first year and therefore it did not have an anthesis period in the second year, but buds were initiated on this ramet in the second year. *EglFT1* expression was very low in the EarlyA apex samples across nearly a full year of harvests (Fig. 5*a*), and was therefore not measured in the apex tissue of the other three ramets.

In the second year (spring 2006 onwards), the *ELF1* transcript was very abundant in flower buds when they were first visible and first harvested. However, *ELF1* expression in buds was low by late summer in ramets of both genotypes (Fig. 6), despite the difference in the rate of flower bud growth and timing of anthesis between the two genotypes.

To show that the abundance of key flowering and reference gene mRNA did not fluctuate significantly throughout the time of day that tissues were sampled, *Egl18S*, *EglFT1* and *ELF1* transcript levels were measured every 2 h from 1100 to 1700 hours in three genotypes (LateA from this study plus an additional Early genotype from Furneaux and a genotype from the south-eastern Tasmania race). Expression of the reference gene *Egl18S* was relatively constant from 1100 to 1700 hours in leaf and apex tissue (Accessory Publication Fig. 2, available online). The *EglFT1* and *ELF1* transcript levels varied throughout this sampling period, but with no clear pattern that was consistent among genotypes (Accessory Publication Fig. 2, available online). The disparity in the time of day of harvesting among sampling intervals was unlikely to have lead to a biased gene expression profile in this study, as (1) there was little variation in the expression of the reference gene *Egl18S* over the 1100 to 1700 hour period, (2) the patterns of variation in *EglFT1* and *ELF1* transcript levels over this time were inconsistent among genotypes, and (3) the seasonal variation in *EglFT1* transcripts was much greater than the fluctuation shown in the period from 1100 to 1700 hours. The 1100 to 1700 hours expression experiment was conducted for the period during which seasonal expression of *EglFT1* was greatest, and any of the *EglFT1* expression levels in the 1100 to 1700 hour time series would have represented a peak in the seasonal time series.

Discussion

Cloning of the flowering genes EglFT1 *and* ELF1

The flowering genes *EglFT1* and *ELF1* were isolated successfully from *E. globulus* cDNA. This is the first report of a *FT* homologue isolated from *Eucalyptus* cDNA or gDNA. In *Arabidopsis* and most other plants, *LFY* is a single copy gene, while *FT* is part of a gene family (Kardailsky *et al*. 1999; Kobayashi *et al*. 1999). In *Eucalyptus*, however, there are two *LFY*-like genes present, but one is a pseudogene (Southerton *et al*. 1998*b*; Thumma *et al*. 2010). A BLASTN search of the *E. grandis* genome revealed there were two *LFY*-like genes present, one containing stop codons. The Southern blot analysis suggested that there are at least three *FT*-like genes in *E. globulus*, but a BLASTN search of the *Eucalyptus* genome indicated that only one *FT*-like gene is present in the database so far. Given that the phylogenetic analysis clearly confirmed the identity of *EglFT1* as belonging to the *FT* clade of the *FT*/*TFL1* gene family, it appears that the *FT* gene family is relatively small in *Eucalyptus*. Studies in other forest tree species, however, have isolated more than one *FT* homologue per species. For example, at least three *FT*-like genes have been isolated in citrus (Nishikawa *et al*. 2007), four in Norway spruce (Gyllenstrand *et al*. 2007) and five in *Populus* (Bohlenius *et al*. 2006; Hsu *et al*. 2006; Igasaki *et al*. 2008). In poplar, different members of the *FT* clade appear to have different roles (see below).

Selection of reference genes

Reference genes are typically chosen from genes essential for cellular survival, as they are presumably constitutively expressed. However, some studies have shown that, on occasion, these genes can vary in their expression due to regulation, so a reference gene must be validated to show that it is suitable for the tissue types and developmental stages to be sampled. Reference genes such as ubiquitins, histones and *18S* have been used in previous studies in *Eucalyptus* tree species (Brill and Watson 2004; Dornelas *et al*. 2004; Watson and Brill

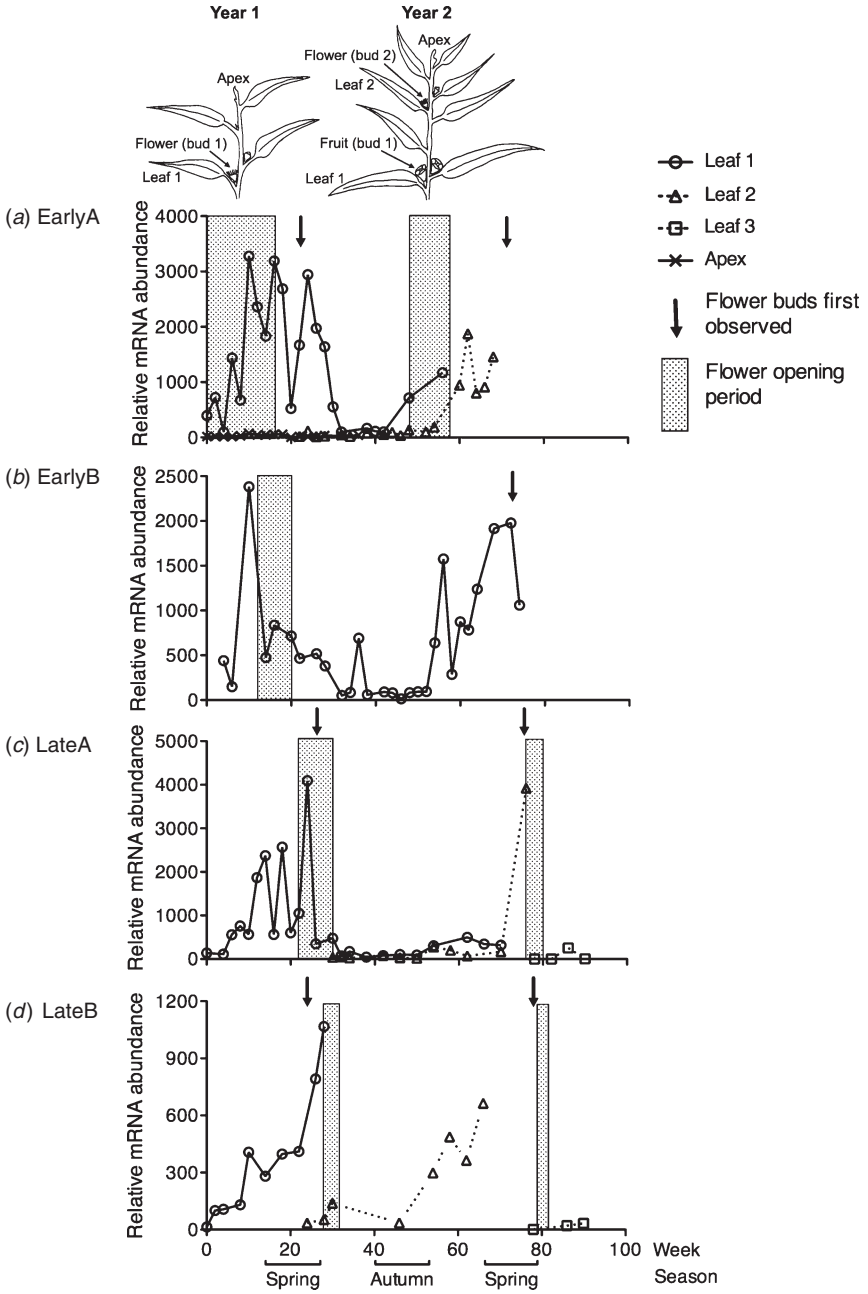

Fig. 5. Relative *EglFT1* mRNA abundance in leaves (Leaf 1, Leaf 2, Leaf 3) of two ramets each of an early and late anthesis genotype of *Eucalyptus globulus* over 90 weeks: (*a*) EarlyA; (*b*) EarlyB; (*c*) LateA; (*d*) LateB. Relative *EglFT1* mRNA abundance in apices is also shown for EarlyA. Relative mRNA levels were determined using qRT-PCR on pooled tissue samples normalised using the housekeeping gene *Egl18S*, and values are the mean of two technical replicates. Timing of flower bud initiation and opening for each ramet is indicated, and representative shoot architecture of EarlyA at the beginning of each year is also shown.

2004; El Kayal *et al*. 2006; Navarro *et al*. 2009; Jaya *et al*. 2010). In many of these studies, however, the stability of the reference gene was assessed using RNA gel blots, or the expression levels were not monitored over different seasons. In quantitative studies, reference genes need to be validated more rigorously, and tested over different organs, developmental stages and seasons. Brunner *et al*. (2004) recommended carefully testing several reference genes for studies that compare gene expression among different tissue types and developmental stages. Their linear regression analysis of reference gene qRT-PCR expression levels, implemented here among samples harvested at different times of the year, provides a quantitative method for choosing an appropriate reference gene for expression studies which is more sensitive than RNA gel blots. While the *EglTUBA1* transcript was the most stable in leaf, in apices the expression of this gene was highly variable, as were *EglH4*,

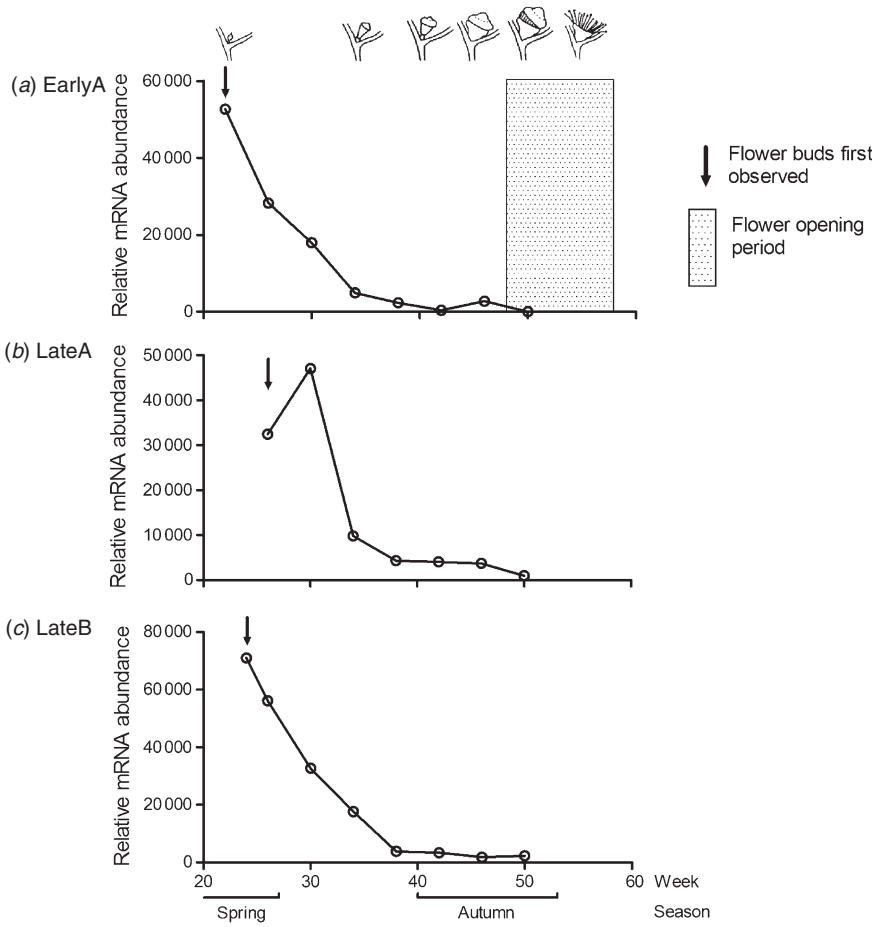

Fig. 6. Relative *ELF1* mRNA abundance in flower bud 2 of two ramets each of an early anthesis and late anthesis genotype of *Eucalyptus globulus* over 90 weeks: (*a*) EarlyA; (*b*) LateA; (*c*) LateB. Relative mRNA levels were determined using qRT-PCR on pooled tissue samples normalised using the housekeeping gene *Egl18S*, and values are the mean of two technical replicates. Timing of flower bud initiation and opening for each ramet is indicated (LateA and LateB flowers did not open during this period), and representative bud morphology of EarlyA at several sampling intervals is also shown.

EglUBQ and *EglEF1*α. In studies such as ours, use of the same reference gene to normalise target gene expression profiles for all tissue types will enable the comparison of gene expression patterns across different tissue types. The *Egl18S* transcript was the most consistent in abundance among the different tissue types and harvest dates, as evidenced by the low slope of, and close fit to, the regression line in leaf, apex and bud tissue. The validation of reference genes presented here, using qRT-PCR rather than RNA gel blots, provides a valuable point of reference for other studies that plan to compare gene expression among different organs and developmental stages in *Eucalyptus*.

When selecting suitable reference genes, it is important to consider not only the stability of expression, but also the abundance of the transcript. It is usually more appropriate to choose a reference gene that has an expression level comparable to that of the genes being analysed (Bustin 2000). In leaf tissue, *Egl18S* was the most abundant reference gene and *EglTUBA1* was the least abundant. *Egl18S* was also expressed at high levels in apex tissue, but *EglUBQ* and *EglTUBA1* were the least abundant transcripts in this tissue type. High expression levels of *Egl18S* could make it an unsuitable reference gene for studying weakly expressed genes, and in certain cases the use of *EglTUBA1* may be more appropriate.

All reference genes showed a more or less consistent expression among samples of leaf tissue, with a low slope and close fit to the regression line, but they were less stably expressed across different samples of apex tissue. However, the pattern was consistent across genes for a given apex harvest date, indicating that this was caused by variation in RNA quality or quantity in apex tissue, rather than instability of the internal control gene. During spring, the apex samples consisted of newly expanding shoots and it is likely that these included more leaf tissue than apex samples taken during winter which included more stem than young leaf tissue. This could account for some of the variation in RNA quality or quantity. Another source of variation could be the variation in the developmental stage of the apex over the course of the year; the apex was dormant during autumn to winter but undergoing active growth in spring. This issue did not pose a problem in a study of the circadian clock in chestnut, where the internal control rRNA levels were similar across winter bud, leaf and stem samples (Ramos *et al*. 2005).

Seasonal expression patterns of flowering genes

The expression of *EglFT1* in the leaf of both early and late anthesis genotypes was associated with annual flower bud initiation, and the pattern was repeatable over a 2-year period. Comparison of early and late anthesis genotypes showed no association between the expression of *EglFT1* and the timing of anthesis, as the expression profiles were similar despite a 6-month difference in the timing of anthesis between genotypes. Together, this indicates that the levels of *EglFT1* alone are unlikely to regulate anthesis time in *E. globulus*, but *EglFT1* could form part of the flower initiation pathway, consistent with the role proposed in other species. A similar pattern has been detected in apple, where *FT* expression peaked during the flower induction period but with only a small peak during the flower blooming (i.e. anthesis) period (Hattasch *et al*. 2008), though interestingly the tissue used in the apple study was apical meristem tissue rather than leaves. Similarly, a study of seasonal flowering gene expression patterns in Satsuma mandarin (*Citrus unshiu*) showed a seasonal increase in *CiFT* expression in the stem and leaves during floral induction (Nishikawa *et al*. 2007), however, the experiment was not extended to include leaves harvested at anthesis time. The abundance of transcripts of poplar homologues of *FT* in leaves was also correlated with seasonal flower bud initiation in adult trees of *Populus deltoides* (Hsu *et al*. 2006, 2011) and *P. nigra* var. *italica* (Igasaki *et al*. 2008).

While the *EglFT1* expression profile showed an overall pattern of upregulation in the lead-up to appearance of flower buds each year, the expression profile was noisy, especially in EarlyA and LateA. In *E. globulus*, not all shoots initiate flowers each year, and the shoot from which some leaves were taken may have been destined to be vegetative rather than reproductive. These factors could explain the troughs of *EglFT1* expression during the period of overall upregulation. There is also some difficulty in harvesting developmentally equivalent leaves across harvesting intervals in the case of adult forest trees, and this could also account for some of the noise in the expression profile.

The *E. globulus* expression data suggest that an ongoing upregulation or critical level of *EglFT1* expression may be required in order to initiate flower buds, as EarlyB had a brief peak of *EglFT1* expression in first year leaves but did not initiate flower buds in the first year. This is in contrast to the ongoing upregulation of *EglFT1* in first year leaves of the other trees, and also in contrast to the ongoing upregulation of *EglFT1* in the second year leaves of EarlyB, after which flower buds were initiated. Alternatively, it may be that other factors that were absent in EarlyB in the first year but were not measured in this study play a role, such as the genes involved in the spring flush of vegetative growth.

Recent studies have shown that homologues of *FT* may have broader roles in plant development than flower initiation alone, and this could also be the case in *E. globulus*. In Norway spruce (*Picea abies*), *PaFT4* expression is correlated with seasonal cycles of growth (Gyllenstrand *et al*. 2007). In *Populus nigra* var. *italica*, *PnFT1* and *PnFT2* may be associated with flower initiation while *PnFT3*/*4* was associated with leaf senescence (Igasaki *et al*. 2008). *Populus trichocarpa PtFT1* appeared to be involved in reproductive onset and the regulation of short-day induced growth cessation (Bohlenius *et al*. 2006). However, Hsu *et al*. (2011) argued that poplar *FT1* and *FT2* have diverged in function and regulation, with *FT1* induced by winter temperatures to mediate the floral transition, and *FT2* promoting vegetative growth and inhibiting bud set in warm temperatures and long days. In this study, *EglFT1* had a broad peak of expression through winter as well as spring, compared with the expression of poplar *FT1* in winter and *FT2* in the spring growing season (Hsu *et al*. 2011). It is unlikely that we have amplified a second *EglFT* as our qPCR primers amplified a single product with a narrow T_m range. The possibility that the expression of *EglFT1* is associated with annual growth flush rather than annual flower initiation in *E. globulus* cannot be discounted, as the timing of the two processes is correlated (Jones *et al*. 2011). EarlyB did not initiate flower buds in the first year of harvesting, but nor did it undergo a vegetative flush in that year. To resolve this, it will be necessary to analyse *EglFT1* expression profiles of adult trees that underwent a vegetative flush but did not initiate flower buds, or to analyse *EglFT1* expression in juvenile trees around spring growth flush.

The expression of *ELF1* was high early in bud development, declining by late summer in both genotypes. By late summer, all four flower whorls were fully developed in both genotypes, but the flower buds on the Late ramets began a period of near dormancy during winter whereas buds on the Early ramets continued to grow until anthesis in late April (Jones *et al*. 2011). The similar pattern of *ELF1* expression in both genotypes, despite the difference in flower bud development and opening time, implies that *ELF1* has a role in early flower bud development but not in regulating later stages of development including anthesis time.

Potential genes associated with anthesis

While the seasonal peaks in *EglFT1* expression are associated with *E. globulus* flower bud initiation, the genes associated with anthesis (shedding of the inner operculum to expose the stamens and style) are still unknown. As anthesis time is a trait of economic and ecological importance in *E. globulus*, the genes that are potentially associated with this process are worth pursuing. Anthesis occurs once all parts of the flower are fully developed, and as the inner operculum is formed from fused petals, the inner operculum abscission zone may be developmentally equivalent to the petal abscission zone in other species. Timing of abscission of flowers and flower parts is controlled by the hormone ethylene in many plant species (Dugardeyn and Van der Straeten 2008) and ethylene inhibitors are commonly used to delay petal abscission and extend vase life of cut flowers (e.g. in roses, Sane *et al*. 2007). Thus, differences in anthesis timing could relate to differences in genes involved in ethylene pathway regulation. There have been no studies examining the effect of ethylene inhibitors on anthesis in *Eucalyptus*, but ethylene inhibitors have been used to prevent leaf senescence and extend the vase life of cut foliage of eucalypts (Ferrante *et al*. 2002). The variation in anthesis time among genotypes of *E. globulus* might also be attributable to differences in the signalling pathway that controls abscission downstream of ethylene or in development of the operculum abscission zone. Several genes have been implicated in abscission

signalling and abscission zone development in plants (reviewed in Taylor and Whitelaw 2001; Cho *et al*. 2008) and it may therefore be worth investigating the eucalypt homologues of these genes in relation to differences we observe in anthesis timing.

Future directions in eucalypt flowering studies

The *E. grandis* genome sequence, recently released into the public domain, will be a valuable resource for genomic and biotechnology research in *Eucalyptus*. It will be relatively easy to identify *Eucalyptus* homologues of *Arabidopsis* genes for expression analyses, and also identify cases where there is no *Eucalyptus* counterpart. Integrating quantitative trait loci (QTL) for flowering traits and the *E. grandis* genome sequence will also assist in finding the genes responsible for natural variation in flower initiation and anthesis. QTL analysis of a large F_2 family with grandparents that differed in time to first flowering and vegetative phase change are currently being undertaken in our laboratory, and may identify QTL associated with these traits. If the confidence intervals around these QTL are not too large and the molecular markers can be found in the *E. grandis* genome sequence, positional candidate genes may be identified. A precocious flowering mutant of *E. grandis* has been identified (Missiaggia *et al*. 2005) and precocious flowering can be induced in *E. occidentalis* (Southerton 2007), and these may also be useful tools for advancing molecular research into flowering of eucalypts.

Supplementary material

Supplementary data are available on the Journal's website and consist of the following: Fig. 1: Alignment of several plant *FT*/*TFL1*-like genes and the *E. globulus* putative homologues of *FT* and *TFL1* (*EglFT1* and *EglTFL1*). Figure 2. Variation in expression of reference and flowering genes in three genotypes of *E. globulus* harvested from 1100 to 1700 hours during flower bud initiation in spring 2007. Table 1. Genes used for phylogenetic analyses of the *FT*/*TFL1* gene family.

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