

# FT overexpression induces precocious flowering and normal reproductive development in *Eucalyptus*

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## Summary

*Eucalyptus* trees are among the most important species for industrial forestry worldwide. However, as with most forest trees, flowering does not begin for one to several years after planting which can limit the rate of conventional and molecular breeding. To speed flowering, we transformed a *Eucalyptus grandis* × *urophylla* hybrid (SP7) with a variety of constructs that enable overexpression of *FLOWERING LOCUS T* (*FT*). We found that *FT* expression led to very early flowering, with events showing floral buds within 1–5 months of transplanting to the glasshouse. The most rapid flowering was observed when the cauliflower mosaic virus 35S promoter was used to drive the *Arabidopsis thaliana* *FT* gene (*AtFT*). Early flowering was also observed with *AtFT* overexpression from a 409S ubiquitin promoter and under heat induction conditions with *Populus trichocarpa* *FT1* (*PtFT1*) under control of a heat-shock promoter. Early flowering trees grew robustly, but exhibited a highly branched phenotype compared to the strong apical dominance of nonflowering transgenic and control trees. *AtFT*-induced flowers were morphologically normal and produced viable pollen grains and viable self- and cross-pollinated seeds. Many self-seedlings inherited *AtFT* and flowered early. *FT* overexpression-induced flowering in *Eucalyptus* may be a valuable means for accelerating breeding and genetic studies as the transgene can be easily segregated away in progeny, restoring normal growth and form.

## Introduction

*Eucalyptus* is an economically important hardwood tree that is widely planted for pulp, energy and timber in many countries. There are several active breeding programmes for *Eucalyptus* focused on improving specific traits, such as biomass yield, wood density, fibre quality and stress tolerance (Grattapaglia and Kirst, 2008). The wealth of genetic diversity in the genus presents a vast resource for modification to suit particular environments and commercial products. The recent sequencing of the *Eucalyptus grandis* genome (Myburg *et al.*, 2014), the expansion of functional genomics data and the continued development of molecular markers will greatly expand the options available to breeders, including marker-assisted breeding, genomic selection and transgenic technology.

While sequencing and phenotyping technologies are advancing at a rapid pace, the physical process of crossing plants to concentrate desired alleles is often limited by the onset of reproduction. While a number of genotypes of *Eucalyptus grandis*, *Eucalyptus urophylla* and *Eucalyptus camaldulensis* can flower in 2–3 years, other commercially important species such as *Eucalyptus globulus*, *Eucalyptus dunnii* and *Eucalyptus nitens* take 10 or more years to flower (CAB, 2000; Eldridge *et al.*, 1993; Missiaggia *et al.*, 2005; Sein and Mitlohner, 2011). The long generation time of many eucalypts is a limiting factor for tree improvement (Grattapaglia *et al.*, 2011). This drawback is especially true for genomic selection, where after development of marker-trait associations, it is usually possible to continue employing them for multiple generations (Resende *et al.*, 2012). Because genomic markers can be readily determined on newly germinated plants or even seeds, the rate

of sexual cycling can become the rate-limiting step for tree improvement.

Although there are eucalypt genotypes and species that flower rapidly (Bolotin, 1975; Missiaggia *et al.*, 2005), introgressing them into breeding programmes takes time and early-flowering varieties may lack traits of interest. It is also possible to accelerate floral induction by physiological methods, such as paclobutrazol application (Griffin *et al.*, 1993; Williams *et al.*, 2003). However, the results for eucalypts are highly variable, environment and genotype specific; and flowering generally does not occur for one to several years after treatment.

Overexpressing *FLOWERING LOCUS T* (*FT*) is known to overcome juvenility in angiosperm trees including poplar, apple and citrus (Bohlenius *et al.*, 2006; Endo *et al.*, 2005; Hsu *et al.*, 2011; Kotoda *et al.*, 2010; Yamagishi *et al.*, 2011). *FT* – often considered ‘florigen’ – is a key regulator of floral transition (reviewed in Wigge, 2011) and can induce early flowering in a variety of woody and herbaceous species (Endo *et al.*, 2005; Lee *et al.*, 2013; Lifschitz and Eshed, 2006; Matsuda *et al.*, 2009). Analysis of the *E. grandis* genome and floral transcriptome revealed the presence of seven expressed *FT*-like genes, indicating that an *FT*-based floral induction pathway is likely to be present (Vining *et al.*, 2014). Other floral regulators can be employed for accelerating flowering in some species, such as overexpressing *MADS4* from silver birch to stimulate flowering in apple (Flachowsky *et al.*, 2007). However, overexpression of many known floral regulators is ineffective or gives poor performance in trees. Overexpressing *LEAFY* (*LFY*), *APETALA1* (*AP1*) or *CONSTANS* (*CO*) led to little to no early flowering in poplar (Rottmann *et al.*, 2000; Strauss *et al.*, 2004). Here, we demonstrate that overexpressed *FT* is a potent stimulator of precocious flowering in

*Eucalyptus* and that the resulting flowers are fertile, with viable pollen and seeds.

## Results

### Precocious floral bud formation

Overexpression of the *FT* gene from *Arabidopsis thaliana* (*A. thaliana*, *AtFT*) is known to promote early flowering in a variety of plant species (McGarry *et al.*, 2013; Yamagishi *et al.*, 2011; Zhang *et al.*, 2010). We generated three constitutive *AtFT* expression constructs with different constitutive promoters and selectable markers along with one heat-inducible *P. trichocarpa FT1* (*PtFT1*) construct (Figure 1). The two constitutive promoters used were the 35S promoter of cauliflower mosaic virus, and the 409S ubiquitin promoter of *Solanum bulbocastanum*, hereafter 409S. We tested 12–28 independent transformation events (events) for each construct, 1–17 ramets (genetically identical trees vegetatively propagated from a single progenitor) per event (Tables S1–S4).

We found that all four constructs successfully led to very early flower bud formation. None of the 39 nontransformed control trees produced floral buds, indicating that SP7 trees do not flower under our glasshouse conditions (Tables S1–S4). The percentage of flowering events varied widely among constructs (Table 1). Events were counted as flowering if at least one tree produced floral buds. Of the 12 pCAM:409S:*AtFT* [409S:*AtFT* (H)] events, trees from five events flowered (41.7% of events) and all 16 plants within flowering events produced flowers (100% of ramets, Table S1). Of the 28 pART27:409S:*AtFT* [409S:*AtFT* (K)] events, only one event had trees with flowers (3.6% of events, Table S2). That single event had 13 trees, 12 of which flowered (92.3% of ramets). Of the 17 35S:*AtFT* events, 11 flowered (64.7% of events) and all plants within those events flowered (100% of ramets, Table S3). Of the 27 HSP:*PtFT1* events, trees from two events flowered before the onset of heat induction (7.4% of events) and trees from 14 events flowered during heat induction (51.9% of events, Table S4). Trees with flower buds prior to the onset of heating failed to produce additional flower

**Table 1** Comparison of floral induction by construct

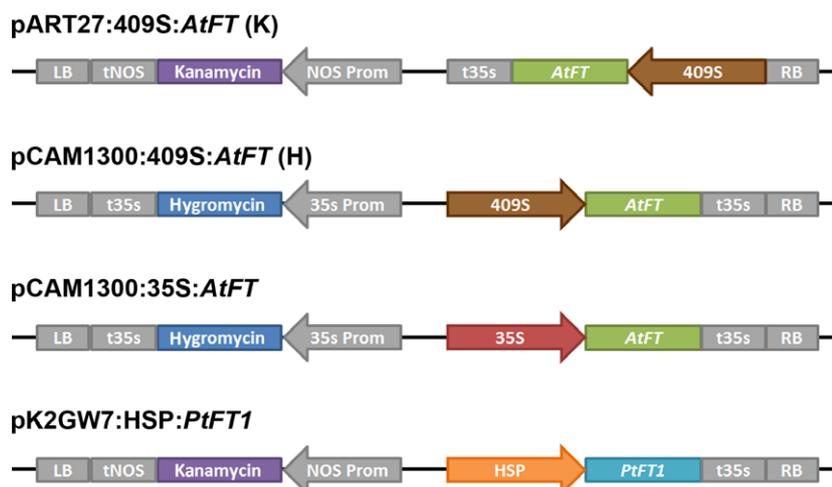
Construct	Total no. of ramets	Total no. of events	Bud formation	
			No. of events	% of events
409S: <i>AtFT</i> (H)	50	12	5	41.7
409S: <i>AtFT</i> (K)	242	28	1	3.6
35S: <i>AtFT</i>	104	17	12	70.6
HSP: <i>PtFT1</i> (preheat)	108	27	2	7.4
HSP: <i>PtFT1</i> (postheat)	108	27	14	51.9

The total number (no.) of ramets and total no. of events columns detail how many ramets (trees) and events (insertion events represented by one or more ramets) were analysed for each construct.

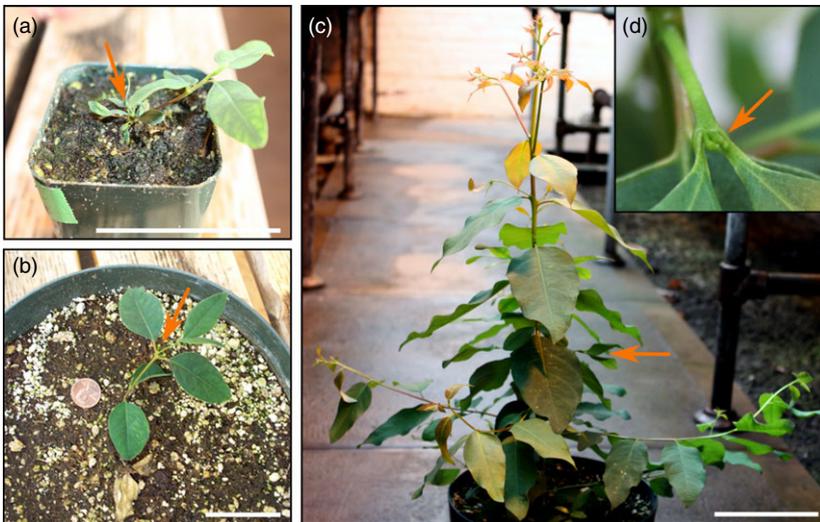
Bud formation, floral buds observed; H, hygromycin selection; K, kanamycin selection.

buds during heat induction. Flowering within HSP:*PtFT1* events ranged from one to four ramets (25%–100% of ramets).

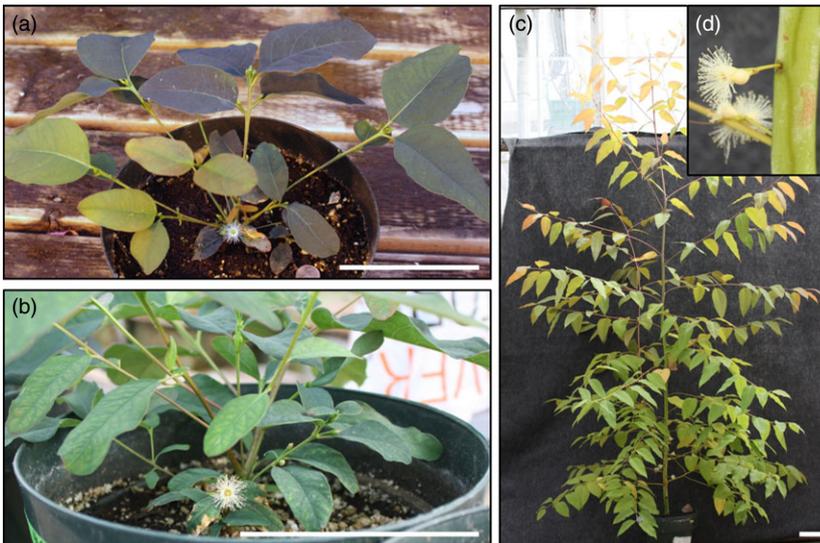
Although all four constructs led to very early flower formation, there were construct-to-construct differences in the rate of formation of initial floral buds. Flower buds were first observed after 1 month in soil for 35S:*AtFT* events, 2–3 months for 409S:*AtFT* (H) events, and 5 months for the 409S:*AtFT* (K) event (Figure 2). This rate is much faster than the 12–14 months needed for SP7 to flower in optimal field conditions (Miron Abramson, Futuragene, personal communication). These initial flowers started to open 2–3 months after floral buds were first observed (Figure 3), when trees were <1 m in height. Some 35S:*AtFT* events showed early floral bud opening coupled with severely reduced vegetative growth, resulting in flowering of trees <5 cm in height (Figure S1). On average, heat induction of HSP:*PtFT1* trees led to flower formation after 9 weeks, and a small number buds opened from five events after 6 months of heat induction.



**Figure 1** *AtFT* and *PtFT1* constructs used for *Eucalyptus* transformation. RB, right border; LB, left border; tNOS, nopaline synthase transcriptional terminator; t35s, 35S transcriptional terminator; 409S, constitutive ubiquitin promoter from *Solanum bulbocastanum*; 35S, cauliflower mosaic virus 35S promoter; HSP, heat-inducible promoter from soybean; K, kanamycin resistance in plants; H, hygromycin resistance in plants, and arrows show direction of transcription.



**Figure 2** *AtFT* overexpression caused precocious flower bud formation. (a) Flower buds were first observed after just 1 month for 35S:*AtFT* plants, (b) 2–3 months for 409S:*AtFT* (H) plants and (c and d) after 5 months in soil for 409S:*AtFT* (K) plants. Arrows indicate locations of developing floral buds. Bar = 5 cm.



**Figure 3** Flower buds opened when trees were <1 m in height. Representative images of (a) 35S:*AtFT*, (b) 409S:*AtFT* (H), and (c and d) 409S:*AtFT* (K) are shown. Note the flowers originating off of the main stem of the 409S:*AtFT* (K) tree (c and d). The initial flowers were all singles or small clusters. Bar = 10 cm.

### Floral production and floral phenotypes

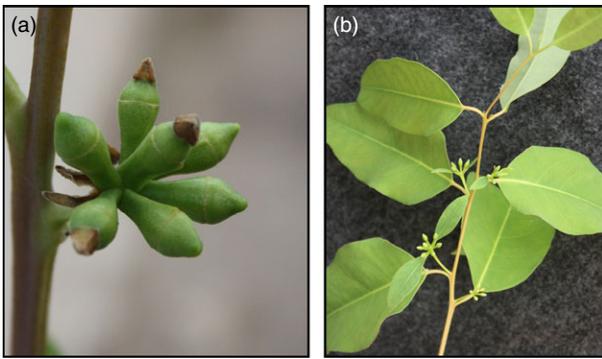
The first flowers formed for all constructs were either single flowers, or small clusters of up to three floral buds. Unusually, the initial 409S:*AtFT* (K) and the preheat HSP:*PtFT1* flowers formed as trios of buds flanked by two leaves and were located on short pedicels initiating from the main stem (Figures 2, 3 and S2). Some of the 409S:*AtFT* (H) flowers also grew directly from larger stems. As the plants matured, flowers were formed in well-developed umbels (Figure 4) and were mostly located on branches. This floral arrangement and location was similar to how flower buds form on SP7 trees in field conditions (Miron Abramson, Futuragene, personal communication). The 409S:*AtFT* (H) and 35S:*AtFT* plants flowered continuously, including during winter months under supplemental lighting in the glasshouse. In contrast, trees from the only flowering 409S:*AtFT* (K) event produced an initial set of floral buds, then did not form new floral buds for another 6 months (Figure S1b). All flower buds formed on HSP:*PtFT1* trees prior to the onset of heat induction died without opening. After heat induction, two trees from different events continued to produce new floral buds. The remaining trees lost an average of 87% of their total buds, with events showing 58%–100% bud

loss. This poor rate of floral bud survival was not observed for the other constructs.

*AtFT*-induced flowers contained all four of the expected whorls of floral organs, with the outer operculum, inner operculum, numerous stamens and single stigma present (Figure 5). Both male and female gametes were also present; floral sectioning revealed the presence of numerous ovules, and anthers contained triangular pollen grains. All *AtFT* constructs led to similar floral morphologies. Flowers located at branch tips were sometimes partially fused with leaves (Figure 5 panel b) and did not open properly. No other obvious abnormalities were observed for *AtFT* constructs. Of the five HSP:*PtFT1* events that had flowers open, three had fairly normal flowers and two had flowers with fused organs and poor opening (Figure S2). However, no pollen was observed in any HSP:*PtFT1* flowers.

### Floral fertility

*FT*-induced flowering is reliable but does not usually lead to production of viable pollen or seed in some genera such as *Populus* (Zhang *et al.*, 2010). To see whether *FT*-induced flowers produced functional seed and pollen in hybrid *Eucalyptus*, we



**Figure 4** Later floral buds developed as umbels. (a) Later buds developed by 409S:*AtFT* (H) and 409S:*AtFT* (K) plants formed as umbels complete with bracts, and (b) buds were often located on branches instead of the main stem.

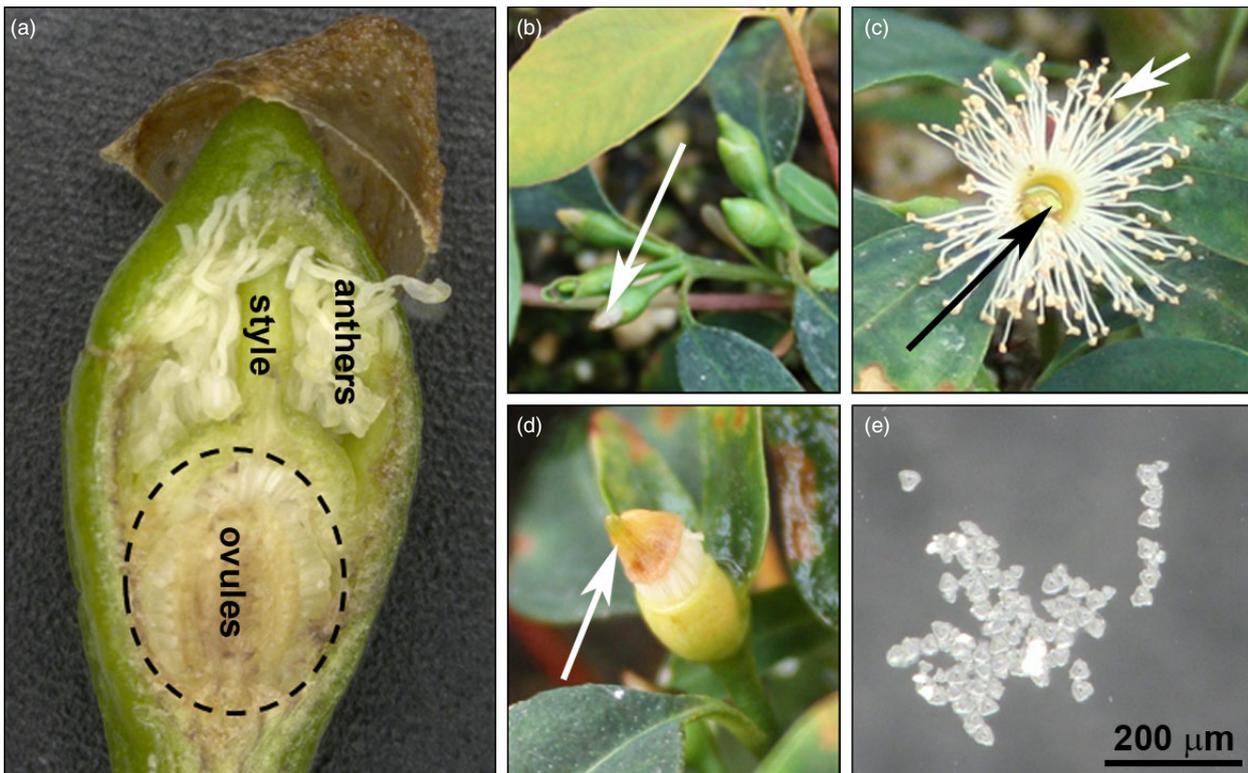
examined pollen and seed viability. Alexander staining revealed that pollen grains of plants from all *AtFT* constructs were viable (Figure 6). We used two types of pollinations to assess the fertility of trees from our 409S:*AtFT* (H) and 409S:*AtFT* (K) flowering events. Trees were allowed to open-pollinate in the glasshouse (houses did not contain any other flowering eucalypt trees), leading to the formation of open-pollinated self-seeds. In addition, flowers were hand-pollinated with *E. grandis* pollen to obtain outcrossed offspring. These cross-pollinated flowers were

emasculated and tented with plastic wrap to reduce the likelihood of self-pollination.

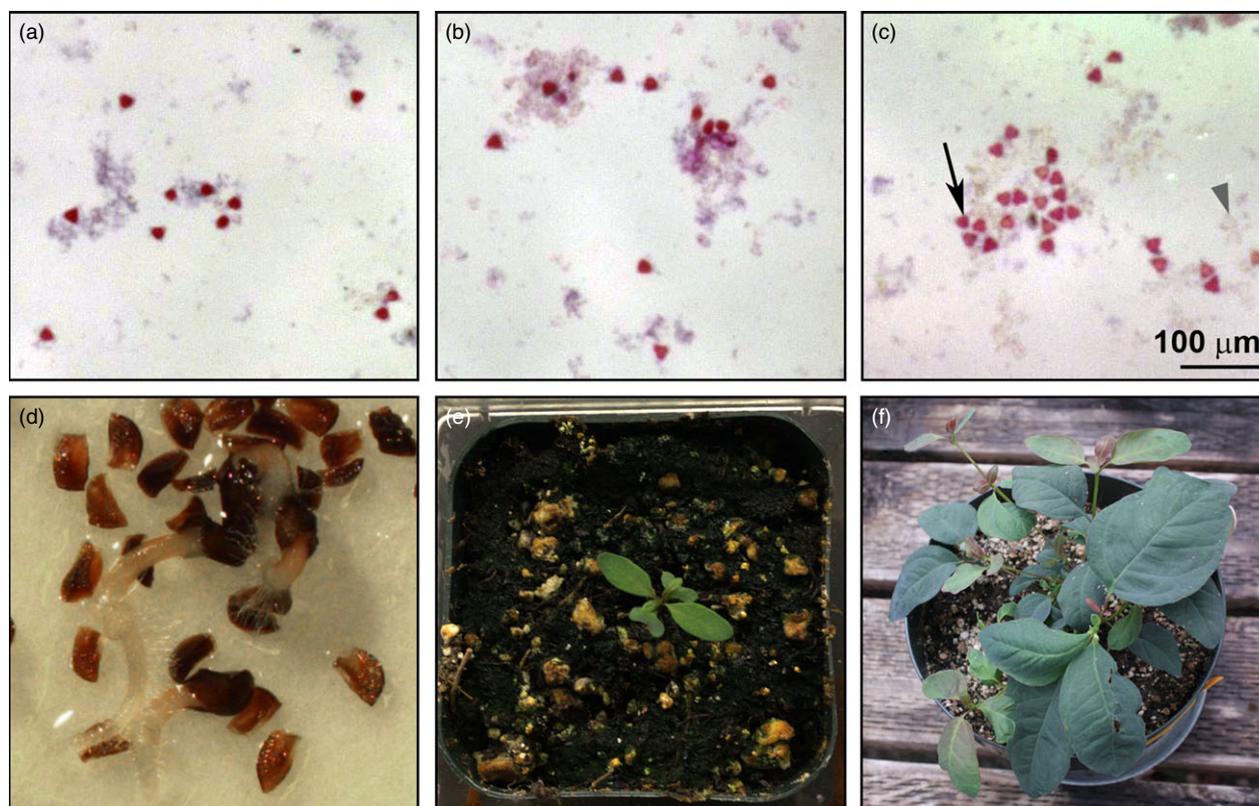
Viable seeds were obtained from *AtFT*-induced flowers (Figure 6), indicating that these flowers had functional female gametes. Germination testing of self- and cross-pollinated flowers showed that cross-pollination led to a higher percentage of germinated seeds than self-pollination (Table 2). Overall, cross-pollination of 409S:*AtFT* (H) trees resulted in a much higher number of seeds pods formed than did the 409S:*AtFT* (K) event (Table 3). However, the average number of germinated open-pollinated seeds obtained from these trees was still low. Therefore, we hand-self-pollinated a select set of 409S:*AtFT* (H) flowers to better determine whether this low viable seed yield was due to poor pollen performance or from the lack of pollinators in the glasshouse. The average number of viable seeds per pod from hand-self-pollinated flowers was higher than from open-pollinated flowers (Table 3). While the number of germinated seeds per seed pod was variable [ranging from 0 to 48 for cross-pollinated 409S:*AtFT* (H)], the number of total seeds obtained from hand-self-pollinated and cross-pollinated flowers was similar and averaged 75 and 76 seeds per pod, respectively (Table S5). However, seeds from open-pollinated 409S:*AtFT* (H) events had a low germination rate (0.18 seeds germinated per pod, Table 3).

**Expression of *AtFT***

We used quantitative real-time PCR (QPCR) to measure relative *AtFT* expression in leaves of flowering, nonflowering and control



**Figure 5** *AtFT*-induced flowers had normal morphology. *AtFT*-induced flowers buds contained normal interior morphology, including (a) ovules. All four expected whorls of floral organs, (b) outer operculum, (c) anthers and stigma and (d) inner operculum were normal in appearance. Both female (ovules, a) and male (pollen grains, e) gametes were also present. Images from 409S:*AtFT* (H) flowers are shown, other constitutive *AtFT* constructs gave similar results. Bar = 200 μm.



**Figure 6** *AtFT*-induced pollen and seeds were viable. Alexander staining of pollen grains from (a) 409S:*AtFT* (H) anthers, (b) 409S:*AtFT* (K) anthers and (c) 35S:*AtFT* anthers showed that pollen grains were viable. Viable grains stain pink/purple (black arrow) while inviolate grains are clear (grey arrowhead). Bar = 100  $\mu$ m. Seeds produced by *AtFT*-induced flowers were viable. (d) Seeds germinated and grew into (e) viable seedlings and (f) robust young trees.

**Table 2** Germinated seed yield by pollination type and construct

Construct	Pollination type	Pods tested	Seeds germinated	Average germinated seeds/pod
409S: <i>AtFT</i> (H)	Open (self)	192	34	0.2
409S: <i>AtFT</i> (H)	Cross	33	518	15.7
409S: <i>AtFT</i> (K)	Open (self)	14	18	1.3
409S: <i>AtFT</i> (K)	Cross	9	39	4.3
409S: <i>AtFT</i> (H)	Open (hand-pollinated)	4	34	8.5

**Table 3** Summary of cross-pollinations and seed pods formed

Construct	Flowers cross-pollinated	Seed pods obtained	% seed pod formation
409S: <i>AtFT</i> (H)	109	81	74
409S: <i>AtFT</i> (K)	24	9	37

H, hygromycin selection; K, kanamycin selection.

events. The only flowering 409S:*AtFT* (K) event had a low rate of flowering and intermittent floral production so it was not used for gene expression analysis. Flowering 409S:*AtFT* (H) events had, in

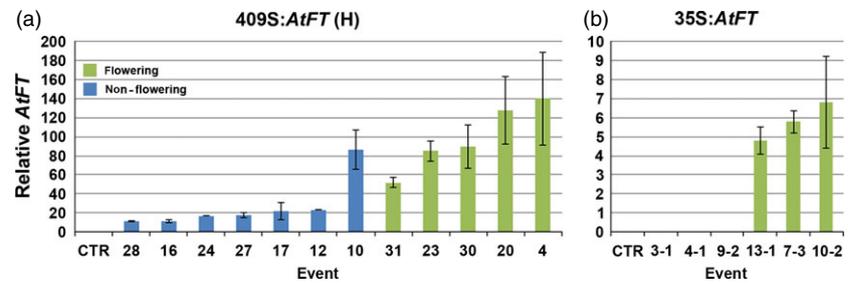
general, fourfold higher expression of *AtFT* than nonflowering events; nonflowering 35S:*AtFT* plants had nearly undetectable levels of *AtFT*, while flowering events had robust expression of *AtFT* (Figure 7).

#### Inheritance of *AtFT* in self- and cross-progeny

We obtained 34 409S:*AtFT* (H) and 18 409S:*AtFT* (K) self-seedlings, 13 and 11 of which survived and grew large enough to test, respectively. Genotyping of these self-seedlings revealed that all of them inherited *AtFT*. We selected seedlings which resulted from cross-pollination of five 409S:*AtFT* flowering events and used PCR to confirm *AtFT* inheritance. We found that *AtFT* was inherited in 54.55%–100% of the progeny, depending on the parental event (Table 4).

#### Floral bud formation in self- and cross-progeny

All of the 409S:*AtFT* (H) open-pollinated self-progeny that inherited *AtFT* had visible floral buds within 2–3 months of transplanting to the glasshouse. The parental trees of these offspring had floral buds within the same length of time. All nine progeny of open-pollinated 409S:*AtFT* (K) trees inherited *AtFT*. Three of these trees had floral buds visible within 2 months, which is much faster than the 5 month time span displayed by the parent trees. The other trees developed flower buds after 3–6 months. Cross-progeny were scored for floral bud formation 3 months after transplanting to soil. Cross-progeny had widely varying rates of flower bud formation, ranging from 42.9% to 95.2% of plants per event (Table 5).



**Figure 7** *AtFT* expression levels were correlated with plant flowering. Relative *AtFT* expression was measured in events from (a) 409S:*AtFT* (H) and (b) 35S:*AtFT* plants. Each bar represents relative expression in 1–4 ramets per event, and error bars show standard error of biological replicates.

**Table 4** Inheritance of *AtFT* in open (self-) and cross-progeny

Construct	Pollination type	No. of seedlings tested	Event(s)	Expected % <i>AtFT</i> progeny	Observed % <i>AtFT</i> progeny	Chi-squared test
409S: <i>AtFT</i> (H)	Open (self)	12	All	75.00	100.00	0.046
409S: <i>AtFT</i> (H)	Cross	20	4	50.00	100.00	$P < 0.001$
409S: <i>AtFT</i> (H)	Cross	20	23	50.00	75.00	0.025
409S: <i>AtFT</i> (H)	Cross	17	30	50.00	64.71	0.225
409S: <i>AtFT</i> (H)	Cross	17	31	50.00	88.14	0.002
409S: <i>AtFT</i> (K)	Open (self)	11	95	75.00	100.00	0.056
409S: <i>AtFT</i> (K)	Cross	14	95	50.00	100.00	$P < 0.001$

**Vegetative phenotypes: leaf coloration**

Young flowering 409S:*AtFT* (H) and 35S:*AtFT* trees had leaves with a noticeably mottled appearance, as well as many dark green leaves (Figure S3). Flowering 409S:*AtFT* (K) trees had leaves with similar coloration to nonflowering and control trees. We found that flowering and nonflowering plants had nearly identical levels of total leaf chlorophyll. By contrast, the dark green leaves of flowering events had a twofold to threefold higher amount of chlorophyll than the average of leaves of control or nonflowering plants ( $P < 0.05$ ).

**Vegetative phenotypes: plant height and diameter**

We measured vegetative phenotypes as a means to assess overall plant growth and development. In general, trees exhibited robust vegetative growth. Quantification of stem heights showed that constitutive *AtFT* flowering plants were, on average, threefold shorter than control and nonflowering plants ( $P < 0.05$ , Figure 8). This difference was more pronounced for 35S:*AtFT* and 409S:*AtFT* (H) events than for 409S:*AtFT* (K) events. Flowering 409S:*AtFT* (H) and 35S:*AtFT* events had greatly reduced height growth to their respective control trees, and nonflowering 409S:*AtFT* (K) events were slightly shorter than the controls. The 409S:

*AtFT* (K) trees were measured at a later age than trees from the 409S:*AtFT* and 35S:*AtFT* constructs, and as such were taller than these trees. Prior to heat induction, HSP:*PtFT1* events, including two events that had preheat induction floral buds, were similar in size (height and stem diameter) to control trees (Figure S4). After heat induction, flowering and nonflowering events were not significantly different in size (Figure S4).

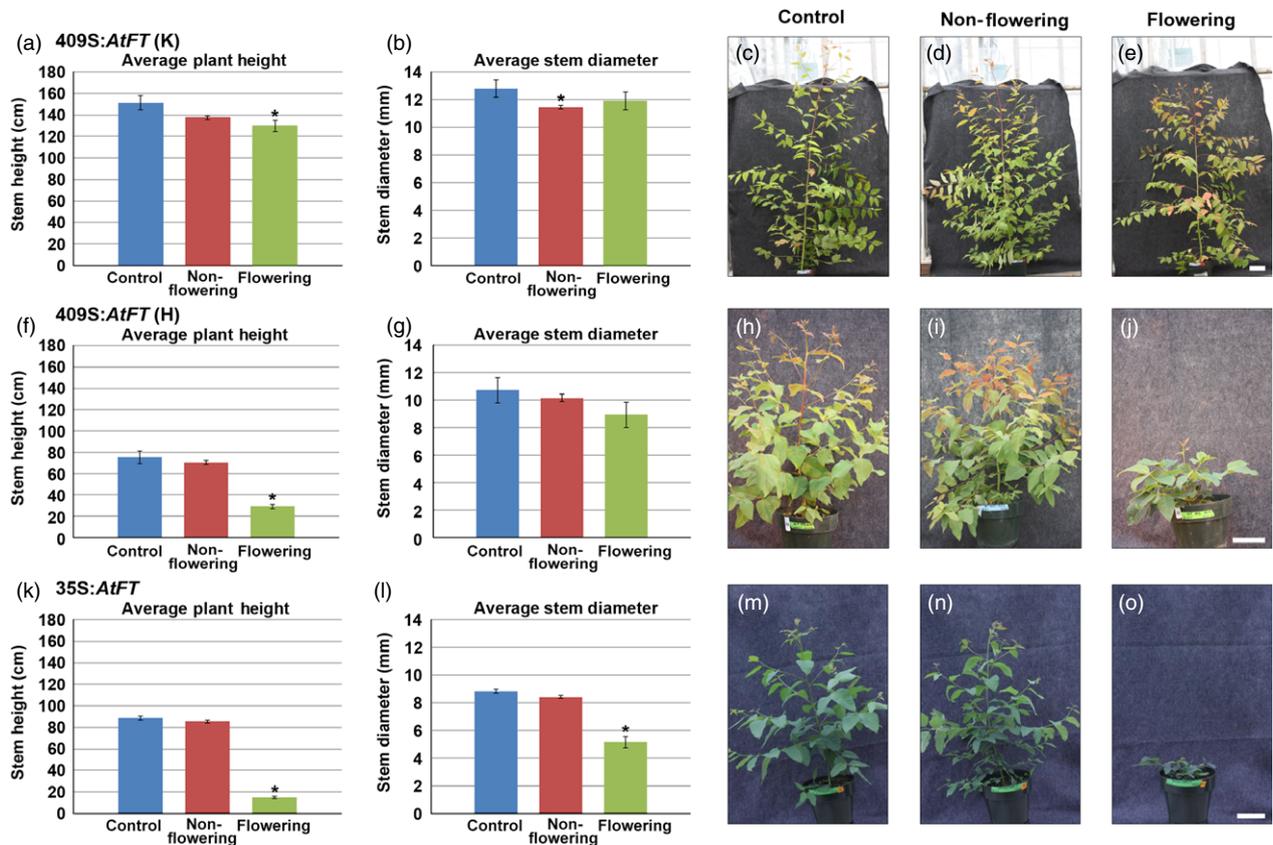
Measurements of stem diameter showed that despite the differences in height, flowering 409S:*AtFT* (H and K) plants had an average stem diameter nearly the same as that of control plants. The flowering 35S:*AtFT* events, however, had twofold smaller stem diameters than the control plants ( $P < 0.05$ ). Nonflowering events from all three constitutive *AtFT* constructs had similar diameters to the control trees.

**Vegetative phenotypes: branch counts and apical dominance**

Flowering trees from 35S:*AtFT* and 409S:*AtFT* (H) constructs had altered plant structure, with reduced apical dominance, numerous branches and an overall bushy appearance. Flowering 409S:*AtFT* (K) and HSP:*PtFT1* plants had a structure similar to that of control trees. Apical dominance was scored for all plants based on the number of large (primary) stem(s) per ramet (Figures S4 and

**Table 5** Floral bud development in open (self-) and cross-progeny

Construct	Pollination type	No. of seedlings tested	Event(s)	Expected % flowering	Observed % flowering	Chi-square test
409S: <i>AtFT</i> (H)	Open (self)	13	All	75.00	100.00	0.037
409S: <i>AtFT</i> (H)	Cross	21	4	50.00	95.24	$P < 0.001$
409S: <i>AtFT</i> (H)	Cross	21	23	50.00	42.86	0.513
409S: <i>AtFT</i> (H)	Cross	26	30	50.00	46.15	0.695
409S: <i>AtFT</i> (H)	Cross	24	31	50.00	79.17	0.004
409S: <i>AtFT</i> (K)	Open (self)	11	95	75.00	78.57	0.758
409S: <i>AtFT</i> (K)	Cross	7	95	50.00	57.14	0.705



**Figure 8** Events with robust flowering were short and bushy. The heights and diameters of all trees were measured for all three constructs. (a–e) The single flowering 409S:AtFT (K) event was, on average, slightly shorter than control or nonflowering events. (f–o) By contrast, flowering 409S:AtFT (H) and 35S:AtFT events were significantly shorter than control or nonflowering events. (l) In addition, flowering 35S:AtFT events had significantly thinner stems. Bars show standard error, and significant differences ( $P < 0.05$ ) are indicated by asterisks. Representative images are shown, bar = 10 cm.

S5). Flowering 35S:AtFT and 409S:AtFT (H) trees often had multiple stems (Figure S5). The bushy phenotype of the 409S:AtFT (H) trees was less pronounced as the plants aged and grew larger, with one stem becoming prominent. Quantification of total branches located within the first twenty cm of the plants showed that 409S:AtFT (H) and 35S:AtFT flowering events had threefold more branches than control trees ( $P < 0.05$ , Figure S6). Flowering 409S:AtFT (K) trees were similar to control trees. Nonflowering trees from all AtFT constructs had similar numbers of total and primary branches as their respective control trees.

## Discussion

*Eucalyptus* trees are grown in plantations worldwide for various purposes. However, unlike most crop species, these trees are not highly domesticated. Their diverse germplasm represents a wealth of genetic potential for creating customized tree varieties, such as those more tolerant of cooler climates, or with improved wood characteristics. However, the process of generating more specialized trees is hampered by the slow breeding cycle of most species. We found that overexpression of AtFT would be an effective means for accelerated breeding of *Eucalyptus*.

We tested three constitutive AtFT constructs, all of which successfully caused early flowering. We also tested a heat-inducible PtFT1 construct, which resulted in early flower bud formation but poor flower bud survival and no viable pollen. AtFT constructs gave differing outcomes in regards to percentage of

floral induction (both between and within events), timing of initial floral bud formation and overall tree form. We observed that our 409S:AtFT (H) and 409S:AtFT (K) constructs resulted in different percentages of floral induction, resulting in 41.7% and 3.6% of events flowering, respectively (Table 1). This difference may be due to the presence of the 35S promoter, a known activator of transgenes (Yoo *et al.*, 2005), in the selectable marker cassette of the 409S:AtFT (H) construct (Figure 1). All constitutive AtFT constructs resulted in the formation of flowers with similar phenotypes (Figure 5). Despite the unusual floral structure of eucalypt flowers, with outer and inner opercula as opposed to sepals and petals, overexpression of AtFT still produced flowers with a normal appearance.

Initial flowers were single in nature, rather than the expected clusters (Figure 3). Later flowers were present in small clusters of buds, perhaps reflecting increased tree maturity (Figure 4). Single flowers have been observed for other tree species with FT overexpression-induced flowering, including poplar and apple (Trankner *et al.*, 2010; Zhang *et al.*, 2010). These early AtFT-induced eucalypt flowers were normal in appearance and, other than their solitary state, were no different from later flowers. By contrast, cotton plants overexpressing FT had early flowers with an abnormal appearance, while later ones were more characteristic (McGarry and Ayre, 2012a). Unusually, some flowers formed on small branches directly off of the stems of 409S:AtFT (K and H) trees (Figure 3). Similar floral placement was also observed for HSP:PtFT1 plants (Figure S2). While cauliflorous flowers are

standard for a few tree species, including, chocolate, ficus and redbud, this placement is not typical of most eucalypts. It has, however, been observed in an early flowering mutant of *E. grandis* (Missiaggia *et al.*, 2005) where flowers occur at locations normally occupied by vegetative branches. It appears that vegetative meristems of flowering SP7 trees, which normally give rise to primary branches, underwent a developmental change to become floral meristems. These early floral buds had vegetative branches located both above and below the flowers, suggesting that trees went through a transient period of *AtFT* responsiveness.

We found that, in general, the greater floral production of the 35S:*AtFT* and 409S:*AtFT* (H) events corresponded to relatively high levels of *AtFT* overexpression (Figure 7). However, some events, such as 409S:*AtFT* (H) event 10, had relatively high levels of *AtFT* expression but did not flower. This difference may be due to the use of leaf tissue, rather than floral tissue, to measure *AtFT* transcript levels. Or, the level of *AtFT* protein may be low in event 10.

We found that two constitutive *AtFT* constructs led to noticeable alterations in the vegetative structure of flowering events, while nonflowering events were similar to control trees (Figures 3 and 8). In general, these flowering trees were very bushy, with more branches and decreased apical dominance as compared to control trees (Figures S6 and S7). Similar phenotypes have been reported in other *FT*-transformed trees, such as plum, citrus and poplars (Endo *et al.*, 2005; Srinivasan *et al.*, 2012; Zhang *et al.*, 2010). Our findings are also consistent with the well-characterized role of *FT* as an important regulator of overall plant architecture, and not for just the timing of floral onset (reviewed in McGarry and Ayre, 2012b). We also observed an increase in total leaf chlorophyll for our flowering 409S:*AtFT* (H) and 35S:*AtFT* events (Figure S3). No alterations in leaf chlorophyll content were reported for citrus, poplar or plum trees overexpressing various *FT* genes (Endo *et al.*, 2005; Hsu *et al.*, 2011; Srinivasan *et al.*, 2012; Zhang *et al.*, 2010). However, this phenotype may be an indirect consequence of the alterations in plant size and growth form. The relatively normal vegetative structure of flowering 409S:*AtFT* (K) and *PtFT1* events could be attributed to a comparatively low amount of *AtFT* or *PtFT1* overexpression, leading to the formation of few flowers. In addition, flowering *PtFT1* trees exhibited poor floral bud survival, with most buds dying without opening. While some trees did form flower buds before heat induction, they failed to produce more buds during heat induction itself. Preheat buds may have been induced by a late summer heatwave that occurred while these plants were growing in a standard glasshouse. For accelerated breeding in a glasshouse, the short stature of *FT*-induced flowering plants may be an advantage, if the dwarfism is not extreme. It will be important to find an expression system which balances early flower production with modest height reduction, profuse flowering and good plant health.

*AtFT*-induced flowers were both morphologically normal and functioned normally. Pollen grains had the expected triangular shape, and Alexander staining revealed that pollen grains induced by all three *AtFT* constructs were viable (Figure 6). Viable pollen grains have been obtained in apple, plum and trifoliolate orange trees transformed with constitutive early-flowering constructs (Endo *et al.*, 2005; Flachowsky *et al.*, 2007; Srinivasan *et al.*, 2012; Yamagishi *et al.*, 2011). We were unable to test the pollen viability of *PtFT1* transformants as the few flower buds which survived to maturity lacked detectable pollen grains. Previous

work with HSP:*AtFT* poplar showed that, under typical heat induction conditions, pollen grains did not fully develop (Zhang *et al.*, 2010). It is possible to alter the timing and duration of heat induction to achieve viable pollen grains (Hoenicka *et al.*, 2014). However, the percentage of plants with pollen grains was low, and grains were obtained through anther dissection.

Seeds from *AtFT*-induced flowers had a normal appearance and germinated in artificial media. We compared total seed yields from self- (open) and cross-pollinated flowers and found that pods formed from self- and cross-pollination contained similar numbers of total seeds (Table S5). However, cross-pollination with *E. grandis* pollen greatly increased the yield of viable seeds (Table 3). The low number of viable seeds obtained from open-pollinated flowers could have been due to lack of pollen transfer, thus we hand-self-pollinated a set of flowers. These pods had a greatly increased average of germinated seeds per pod (Table 3). Overall, we found that *AtFT*-induced flowers could be used for both self- and cross-pollination purposes. Viable seeds were also reported in transgene-stimulated early-flowering plum and apple (Flachowsky *et al.*, 2011; Srinivasan *et al.*, 2012).

*AtFT* was inherited by progeny, but in a complex and variable manner. We found that all self-progeny inherited *AtFT*, rather than the expected 75%, which indicates that some events may have multiple copies of *AtFT* (Table 4). Similarly, analysis of cross-progeny revealed that events 4, 23 and 31 had significantly more *AtFT*-inheriting progeny than would be predicted for single *AtFT* insertions (Table 4). Cross-progeny from event 30 inherited *AtFT* in a manner consistent with a single gene insertion. However, not all seedlings which inherited *AtFT* formed flower buds (Table 5), indicating that perhaps multiple insertions are needed to achieve early flower formation in some events. If so, it would be necessary to genotype nonflowering individuals in order to identify those which do not contain the transgene. An alternate explanation is gene silencing, leading to *AtFT* suppression in some individuals. The presence of multiple insertions could present a complication in the practical usage of *FT* transgenic trees as it would decrease the proportion of null progeny, offspring which do not inherit the transgene. This complication could be reduced by the use of strong promoters, which could make single copies of *FT* transgenes sufficient for obtaining robust flowering. The ability to segregate away the exogenous *FT* gene in each generation would be highly useful as it would allow for the creation of two different sets of progeny. Offspring with the *FT* transgene, and thus potentially early flowering, could be used for accelerated breeding in many successive generations. Analysis of molecular markers in earlier training populations could lead to the selection of trees of interest for cross-pollinations without the need for phenotyping. Offspring without the transgene could be used to test vegetative traits of interest and for commercial production. It is estimated that a reduction in generation time would have great potential benefits to eucalypt tree breeding by genomic selection, leading to the production and testing of elite clones in 9 years instead of 18 (Resende *et al.*, 2012). Currently, early-flowering apple trees are being used, in conjunction with marker-assisted breeding, to accelerate the introgression of genes associated with disease resistance (Flachowsky *et al.*, 2011). In a similar fashion, *FT* from *Arabidopsis* or other species could be transformed into elite eucalypt cultivars and used to accelerate breeding of selected clones. A major challenge to implementing this approach, however, is the recalcitrance of eucalypts to transformation. Eucalypts can be challenging to transform, with poor rates of shoot regeneration, and different genotypes often require

customized transformation protocols (Chauhan *et al.*, 2014; Girijashankar, 2011). Improved transformation efficiency may be needed before the benefits of *FT* can be fully realized.

Other barriers to the practical usage of *FT*-accelerated eucalypts include the uncertainty of how null progeny, those plants which have a transgenic parent but do not inherit the transgene, would be regulated. These are the trees that would be best-suited to actual commercial production. Trees lacking the *FT* transgene would not have inherited the explicit genetic modification but may still be regulated as if they were GMO in some countries. In the United States, it is likely that null segregant progeny would be considered as nonregulated, as is the case with 'FasTrack' plum null segregants (Gregoire, 2011). In Europe and New Zealand, although a scientific workshop has recommended nonregulation for such progeny, and null segregants are recommended comparators to GM plants in regulatory dossiers, it is unclear whether null segregants would be accepted as non-GMO (EFSA, 2011; FSANZ, 2013). One area of scientific concern is that even though null progeny do not inherit the transgene, they may inherit an epigenetic state that is influenced by the effect of the transgene in the parental tree, leading to unintended phenotypic consequences. The plant epigenome can be passed on to progeny which can influence the phenotype of these offspring (reviewed in Hirsch *et al.*, 2012). However, conventional breeding extensively combines and modifies epigenomes, which may affect traits of the progeny (Tsafaris *et al.*, 2008).

We have shown that overexpression of exogenous *FT* genes in eucalypts can be an effective means for acceleration of eucalypt tree breeding. This approach is also valuable as a general research tool for genetic studies, as well as for those focused on genetic modification of flower structure and fertility (Brunner *et al.*, 2007; Vining *et al.*, 2012). Its practical implementation will depend on regulations and market restrictions if null segregants are considered GMOs, and on improvements to transformation efficiency.

## Experimental procedures

### Vector construction and transformation

*AtFT* was PCR-amplified using gene-specific primers and cloned into the pART27 or the pCAM1300 vectors under control of the constitutive 409S ubiquitin promoter. The pCAM35S:*AtFT* construct was generated by replacing the 409S promoter with the constitutive 35S promoter. *PtFT1* was PCR-amplified using gene-specific primers and cloned into pK2GW7 under control of a soybean heat-shock inducible promoter (HSP). The pCAM:409S:*AtFT* (H), pCAM35S:*AtFT* and *HSP:PtFT1* constructs were transformed into hybrid *Eucalyptus* clone SP7 kindly provided by Futuragene (Rehovot, Israel) using *Agrobacterium*-based, organogenic transformation methods (Chauhan *et al.*, 2014). In brief, sterile leaves were wounded and cocultivated with *Agrobacterium* containing the plasmid of interest. Explants were placed on callus-induction medium followed by shoot-induction medium and shoot-elongation medium. All types of media contained selective agents (Kanamycin or Hygromycin). Transformed events were confirmed using PCR with primers specific to *AtFT* (5'-AGCCACTCTCCCTCTGAC-3') and either the 409S (5'-CGGTTTCGCTAGAGTCG-3') or 35S promoter (5'-CATGGAGTCAAAGATTCAAATAGAGG-3'). Shoots from individual confirmed transformed events were propagated, leading to multiple identical ramets (trees) per independent transformation event. Rooted shoots were transplanted to soil and kept in humid conditions before being transplanted to larger pots in a glasshouse.

Plants were grown with 16 h of illumination per day, with average day and night temperatures of 29.4 °C and 21.1 °C (85 °F and 70 °F), respectively. For all constitutive *AtFT* experiments, events from each construct were randomized in one block with nontransgenic controls, SP7 plants which had undergone tissue culture and propagation in the absence of *Agrobacterium*. Prior to heat induction, HSP:*PtFT1* plants were randomized with control plants and grown in standard glasshouse conditions until ramets reached an average height of 44.6 cm. Then, ramets were moved to two heat induction glasshouses and randomized within four blocks, two per glasshouse, with one ramet from each event present in each block, along with nontransgenic control trees. Heat induction consisted of 4 h per day of 40 °C (104 °F).

### Quantification of total leaf chlorophyll

Relative leaf chlorophyll content was measured using a SPAD metre. Readings were taken from all 409S:*AtFT* (H) ramets, and five ramets per event for 409S:*AtFT* (K) and 35S:*AtFT* plants. Leaves were measured three times, and the readings averaged together for each leaf. For events with evenly coloured leaves, a single leaf was measured per ramet. For events with a mixture of leaf colours (mottled and dark), one leaf of each colour category was measured per ramet. Relative chlorophyll measurements were converted to actual units using a standard curve. This curve was generated by comparing SPAD readings to total leaf chlorophyll extracted using an N,N-dimethylformamide (DMF)-based method. Leaves from 409S:*AtFT* (H) plants were used to generate the standard curve data, the same leaves were used for both the SPAD metre readings and total chlorophyll extractions. A standard hole-punch was used to obtain five leaf punches per leaf; these punches were placed into tubes containing 5 mL of DMF. Samples were incubated at 4 °C in the dark for 11 days. The absorbance of each sample at 664.5 and 647 nm was measured using a Beckman-DU 40; total leaf chlorophyll (mg/L) was calculated as  $17.90 \times A_{647} + 8.08 \times A_{664.5}$ . The average leaf punch weight (3.68 mg per punch) per solution volume (1 mL) was used to calculate mg chlorophyll per gram fresh leaf weight. The resulting standard curve was used to calculate the chlorophyll contents of 409S:*AtFT* (K) and 35S:*AtFT* leaves.

### Plant growth measurements

Tree heights were measured from soil level to the highest point of the ramet. For ramets with single stems, this point was the apex of the stem, for ramets with multiple stems, the distance to the apex of the tallest stem or branch was measured. Stem diameter was measured at soil level using digital callipers. For ramets with multiple stems at soil level, data were recorded for the largest stem. All ramets for all events from all constructs were measured.

### Branch counts and apical dominance scoring

Total branches were counted for all ramets for all constitutive *AtFT* constructs. This count included all branches (two cm and longer) found within the first twenty cm of the ramet (soil level up). Apical dominance was scored using a three-category system. Ramets with a single apical shoot were scored category 1, those with two or three apical shoots scored category 2, those with four or more apical shoots were scored as category 3. All ramets for all constructs were scored.

### Pollen staining

Mature anthers were collected and fixed in a 6:3:1 EtOH:chloroform:acetic acid solution. Released pollen grains were stained using a simplified Alexander staining protocol (Peterson

*et al.*, 2010). Slides were sealed with nail polish and viewed using a Keyence digital microscope. Pollen from three flowers from three events each of 409S:*AtFT* (H) and 35S:*AtFT* was stained, as was pollen from three ramets of the only flowering 409S:*AtFT* (K) event.

### Imaging of plants and flowers

Plants and flowers were imaged using a Canon Rebel XSI digital camera. Unopened floral buds were hand-sectioned lengthwise to obtain images of internal floral structures including ovules. Anthers of freshly opened flowers were collected and dried until pollen began to shed. Pollen grains were placed onto microscope slides for imaging. Buds and pollen grains were imaged using a Keyence digital microscope. Floral buds and anthers from three different flowering events each of 409S:*AtFT* (H) and 35S:*AtFT* were imaged as well as the only flowering 409S:*AtFT* (K) event.

### Pollination

Flowering trees from 409S:*AtFT* (H) and 409S:*AtFT* (K) events were allowed to open-pollinate in the glasshouse, leading to the formation of self-pollinated seeds. Other flowers from these events were manually cross-pollinated using *E. grandis* pollen. These flowers were emasculated and tented with plastic wrap to reduce the chance of self-pollination. Seed pods were collected 5–6 months after pollination and were used for seed germination assays. No other flowering *Eucalyptus* was present in the glasshouse at the times of these experiments.

### Seed germination and seedling growth

Mature seed capsules were collected and used for seed germination tests. Pods were dried until valves opened and the seeds shed. Seeds and chaff from each seed pod were placed onto filter paper moistened with sterile distilled water in an individual Petri dish. Dishes were wrapped with parafilm and kept in the dark at room temperature until radicle emergence. Seedlings were transplanted to soil and kept in humid boxes until two or three sets of true leaves were visible. Then, seedlings were transplanted to larger pots and transferred to the glasshouse. For seed yield quantification, plates were photographed immediately after seeds were placed on the filter paper and imaged again after germination was complete. The number of potential seeds per pod was counted using pregermination images. Postgermination images were used to determine whether any viable seeds were missed in the initial tally.

### PCR genotyping of self- and cross-progeny

Genomic DNA was extracted from individual seedlings using a modified CTAB-based protocol (Crowley *et al.*, 2003). Standard PCR analysis was performed with primers specific to *AtFT* (5'-GAATTCATCGTGTCTGTTTA-3') and (5'-AGCCACTCTCCCTCTGAC-3'), with primers specific to *GAPC2* (5'-GGCCTTTCTTAGGGAGAG-3') and (5'-GCAACAATGGCAAAGTGAAAGC-3') serving as a positive control. The PCR cycle consisted of 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 68 °C for 1 min. The final extension was 68 °C for 7 min followed by a hold at 4 °C. Genomic DNA from SP7 control plants and diluted pCAM:409S:*AtFT* plasmid DNA were used as controls. Statistical analysis of inheritance of *AtFT* consisted of a chi-squared test.

### QPCR analysis of *AtFT* expression

Total RNA was isolated from 100-mg samples of young leaves using a rapid CTAB RNA extraction method (Gambino *et al.*, 2008). RNA

was treated with DNase (DNase I, Amplification Grade, Invitrogen, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. SuperScript III Reverse Transcriptase (Invitrogen) was used for cDNA synthesis following the manufacturer's recommendation. QPCR was performed using an Applied Biosystems StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA). The housekeeping gene *GAPC2* was used as a reference, and reactions were run in triplicate. The following gene-specific primers were used as follows: *GAPC2* (5'-ACCTCTTGCCAAGGTCATCA-3') and (5'-TGCTGCTGGGAATGATGTTG-3'), *AtFT* (5'-GAATTCATCGTGTCTGTTTA-3') and (5'-AGCCACTCTCCCTCTGAC-3'). Reactions contained 7.5 µL SYBR Green mix (Platinum<sup>®</sup> SYBR<sup>®</sup> Green qPCR SuperMix-UDG; Invitrogen), 10 ng cDNA, 0.3 µL of primers (10 µM each), 0.3 µL ROX reference dye and water up to 15 µL. The PCR program was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR cycles were followed by melt curve analysis. StepOne Software version 2.2 (Applied Biosystems) was used for data analysis. QPCR analysis was repeated for all ramets, and results from a representative single experiment are shown.

### Accession numbers

The following gene accession numbers were used in this manuscript. *Arabidopsis thaliana* FLOWERING LOCUS T (*AtFT*, At1g65480), *E. grandis* GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2 (*GAPC2*, H04673), *Populus trichocarpa* FLOWERING LOCUS T1 (*PtFT1*, Potri.008G077700).

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### Conflict of interest

The authors have no conflict of interest to declare.

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## Supporting information

Additional Supporting information may be found in the online version of this article:

- Figure S1** Some 35S:*AtFT* ramets flowered when below 5 cm.
- Figure S2** Heat-inducible *PtFT1* led to early flower bud formation.
- Figure S3** Flowering trees had increased leaf chlorophyll.
- Figure S4** Flowering HSP:*PtFT1* trees had normal vegetative growth.
- Figure S5** Flowering trees had reduced apical dominance.
- Figure S6** Flowering trees had increased branches.
- Table S1** Flowering by event for 409S:*AtFT* (H).
- Table S2** Flowering by event for 409S:*AtFT* (K).
- Table S3** Flowering by event for 35S:*AtFT*.
- Table S4** Flowering by event for HSP:*PtFT1*.
- Table S5** Total seeds formed per pod.