Engineering early floral induction in *Eucalyptus* by manipulating the *Flowering Locus T* (*FT*) gene

by

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Submitted in fulfilment of the requirements for the degree

Magister Scientiae

Department of Biochemistry, Genetics and Microbiology
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Under the supervision of **Prof Alexander A. Myburg**

and co-supervision of **Dr Steven Hussey**

2nd December 2022

DECLARATION

I, Vasili Alexander Balios declare that the dissertation, which I hereby submit for the degree M.Sc. Genetics at the University of Pretoria is my own work and has not been submitted by me for a degree at this or any other tertiary institution.

Vasili Alexander Balios

2nd December 2022

DISSERTATION SUMMARY

Engineering early floral induction in *Eucalyptus* by manipulating the *Flowering Locus T* (*FT*) gene

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Eucalyptus species and hybrids include some of the most widely cultivated trees in the forestry industry. They are primarily used to produce pulp and paper, and other bio-based products. *Eucalyptus* trees are ideal for plantation forestry because of their rapid growth rate and wide adaptability. However, *Eucalyptus* species have a relatively long time to flower with widely cultivated species such as *Eucalyptus grandis* and *E. urophylla,* and their hybrids, only flowering at approximately three to five years. Other species such as *E. dunnii* and *E. nitens* only flower after ten years. These long generation times present a bottleneck in *Eucalyptus* breeding programs and the speed with which new cultivars and elite lines can be produced.

This MSc study aimed to identify the functional *FT* ortholog in *Eucalyptus* trees and test its functionality in *Arabidopsis* plants. The putative *Eucalyptus FT* ortholog was selected based on phylogenetic analysis and subsequent 3D protein prediction and comparisons. The gene identified was cloned and transformed into *Arabidopsis* using *Agrobacterium* transformation. Transgenic plants were grown under non-inducing, short-day conditions to test the ability of the *Eucalyptus* FT protein to induce flowering ectopically. To further assess the localisation and graft transmission potential of the *Eucalyptus* FT protein, the GFP protein was translationally fused with the *Eucalyptus* FT protein. Wild-type *Arabidopsis* plants were grafted with transgenic tissues and subjected to fluorescent confocal microscopy. We found that the selected *Eucalyptus FT* gene induced significantly early flowering in *Arabidopsis* when ectopically expressed under short day conditions. We also show that the *Eucalyptus* FT protein is localised to the plant's vasculature and is capable of graft transmissible movement.

An additional aim of this MSc was to determine graft transmissibility of heterologously expressed *Arabidopsis* FT protein and its ability to induce floral development in wild-type *Eucalyptus* tissue and how the application of paclobutrazol affected the transport and floral induction. To assess the graft transmission of *Arabidopsis* FT protein in *Eucalyptus* plants, *Arabidopsis FT* overexpressing *Eucalyptus* lines were grown to a graftable size in naturally lit phytotrons. When the main stem diameters of the plants were approximately 5 cm, a reciprocal grafting trial was performed. After successful grafts produced shoots, a subset of these was treated with paclobutrazol, which is a known floral inducer. We found that the paclobutrazol treated plants had significantly shorter internodal lengths compared to plants which were not treated with paclobutrazol. As of writing this thesis no floral induction has been seen in wildtype *Eucalyptus* tissue.

The work completed in this MSc study has led to the identification of the functional *Eucalyptus FT* ortholog. We confirmed that this protein is capable of graft transmission in a heterologous system. The *Eucalyptus FT* gene can be used in future floral induction studies in *Eucalyptus*, and the graft transmission of the protein assessed in *Eucalyptus* plants. We also showed that the *Arabidopsis* FT protein is capable of inducing early flowering in *Eucalyptus*. However, we found that the *Arabidopsis* FT was not capable of causing early flowering in wild-type tissue grafted onto transgenic material in *Eucalyptus*. We also found that the application of paclobutrazol only resulted in the shortening of the internodal lengths of new shoots on grafted scion material. These results provide a step towards accelerated breeding in *Eucalyptus*.

PREFACE

Due to its fast growth, excellent wood properties and wide adaptability, *Eucalyptus* is one of the most widely cultivated tree genera in the forestry industry. Wood from *Eucalyptus* plantations is primarily used for pulp and paper production, as well as other bio-based products such as textiles, food additives and pharmaceuticals. Depending on the species' wood properties, the solid wood is also used for poles, construction and structural support in mine tunnels, which was the main driver of early import of *Eucalyptus* species into South Africa. Furthermore, *Eucalyptus* is an ideal tree for plantation forestry due to its fast growth rate, which is appealing for crop rotation times and productivity. The various *Eucalyptus* species that are adapted to forest plantation environments are also an advantageous aspect of the genus. At around three to five years, species such as *Eucalyptus grandis* and *E. urophylla,* still exhibit a non-reproductive duration which is considerably extended. This lengthy period where the trees are non-reproductive introduces a major bottleneck in conventional breeding programs, resulting in a long period of time to develop new elite tree lines.

Trees are typically outcrossed crossed in a traditional breeding cycle, and the progeny of that cross are planted and phenotyped at various stages of growth, to look for desirable traits such as growth rate and wood quality. Progeny with desirable characteristics will be cloned or used in more crossing studies. The trees can then also be trialled in various conditions until a tree with a suitable phenotype is obtained. This can take many cycles from seed to seed and is highly reliant on the time it takes for the plants to produce progeny (in other words to flower). Traditional breeding strategies in *Eucalyptus* are hampered by the genus's prolonged time to produce flowers, with exception of *E. occidentalis* which can flower within six weeks of growth. Climate change is occurring at such a rapid pace that by the time a new elite line is generated, it will no longer be suitable for the environment it was originally selected for. All of this does not account for pests and diseases, which may evolve during the period it takes to create a new elite genotype To address these issues, new breeding strategies must be employed.

Recently, biotechnology approaches have been developed to accelerate flowering in plants. Mostly in small herbaceous plants, the overexpression of *Flowering Locus T*(*FT*) has been used to accelerate flowering time. Typically, the 35S promoter is used for overexpression. However, the *Sucrose symporter 2* (SUC2) promoter has also been used as it is constitutively expressed in a tissue type-specific manner, namely in the phloem companion cells. The SUC2 promoter is also an ideal candidate as it shares a similar expression pattern as the *FT* promoter. This approach has also been combined with grafting due to the FT protein's ability to cross a graft junction which has allowed researchers to induce early flowering in wild-type grafted tissue. This allows one to achieve early flowering in wild-type tissue, thus ensuring that the progeny is also non-transgenic. This has great potential to be used in tree breeding where there are lengthy periods of non-reproductive growth. This approach can also be used in conjunction with genomic selection to further accelerate the breeding of new elite genotypes. These can then be used in the field as the product would be completely non-transgenic and thus would have very few ethical considerations.

The main aim of this MSc dissertation was to elucidate and functionally test the *Eucalyptus FT* ortholog in a heterologous system. We also aimed at testing whether the *Arabidopsis* overexpressing *Eucalyptus* lines, which were previously made by our OSU collaborators, demonstrated graft transmissible movement and floral induction in wildtype tissue and whether the application of paclobutrazol would help with signal transmission. To achieve these aims this involved phylogenetic analysis and 3D protein structural prediction followed by cloning of the most likely *Eucalyptus* ortholog and subsequent *Arabidopsis* transformation and phenotyping. I also assess the graft transmission potential of the *Eucalyptus* FT protein using GFP-tagged *Eucalyptus* FT protein and fluorescence confocal microscopy. I also tissue cultured *Arabidopsis FT* overexpression lines, rooted them and grew them with a subsequent grafting trial. Additionally, a subset of successful grafts was then treated with paclobutrazol. The findings from this project will signal the way forward for creating an accelerated *Eucalyptus* breeding strategy.

The dissertation describes the results of a functional gene study in *Arabidopsis* and experiments involving transgenic *Eucalyptus* lines and is structured as follows:

Chapter 1 is a literature review focused on the *Flowering Locus T* gene and protein product. I emphasize the regulation of the *FT* gene and subsequently discuss the potential for biotechnology applications using ectopic expression of *FT* to bypass these regulations. One of the key factors which I highlight in the literature review is the ability of the FT protein to travel long distances. Researchers have taken advantage of the ability for FT to move long distances, to enable long-distance transport over a graft junction. This has shown promise in many smaller herbaceous plant species. However, only recently, as well as during this study, graft transmission of FT has been demonstrated in larger woody perennials. These studies reinforce the concept of using ectopic *FT* expression to bypass its regular expression and induce early flowering in *Eucalyptus*, which has a particularly long juvenile phase which ultimately creates a major bottleneck in breeding programmes. I also write about some biotechnology applications for which FT and grafting can be used. Aspects of grafting and age-related flowering are also touched on during the review.

Chapter 2 describes the results of the identification and functional analysis of the putative *Eucalyptus FT*. I identified the *Eucalyptus FT* gene via phylogenetic analysis and subsequent 3D protein prediction and comparison. I first describe the process taken to identify the putative *Eucalyptus* FT protein by using phylogenetic analysis and 3D protein prediction and comparison. I utilise MegaX, AlphaFold and FATCAT in this bioinformatics section. I then clone the *Eucalyptus* FT CDS and create a *Eucalyptus* FT-GFP fusion CDS for downstream application. I transform *Arabidopsis* with the created constructs, and I subsequently selected true transformants and phenotype them under short-day growth conditions. I report that the *Eucalyptus* FT induces early flowering in *Arabidopsis*. Furthermore, this chapter contains a section which is dedicated to determining the graft transmissibility of the *Eucalyptus* FT protein in a heterologous system. I utilise micrografting and fluorescent confocal microscopy to determine the graft transmission. Ultimately, I characterised the mostly likely functional ortholog of

Eucalyptus. I confirmed that it was responsible for floral induction and that it has graft-transmissible properties.

For **Chapter 3** I describe the process of tissue culturing *Eucalyptus* lines which are overexpressing the *Arabidopsis FT* gene and contain a *LFY* knockout, and subsequently rooting these plants *in vitro* and growing them to a graft-able size in phytotrons. I show the difference in rooting efficiency between wildtype and transgenic *Eucalyptus* lines. I describe the process of performing cleft/wedge graft surgery on *Eucalyptus* plants and the subsequent survival of these grafts. Furthermore, I report the effect that paclobutrazol application has on the successfully grafted *Eucalyptus* plants. As of writing this dissertation, the application of paclobutrazol has only affected the plant's phenotype but unfortunately, no early flowering has yet been seen in the wildtype tissue arising from the application of paclobutrazol.

For Chapter 4 I discuss a few concluding remarks for this MSc dissertation as well as proposed future works and prospective. I describe the formation of an accelerated breeding cycle for *Eucalyptus* with the potential to include a genomic section to further accelerate the cycle.

This dissertation is based on work performed in the Forest Molecular Genetics (FMG) Programme in the Department of Biochemistry, Genetics and Microbiology, and the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria from January 2021 to November 2022.

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TABLE OF CONTENTS

CHAPTER 1 ....

LITERATURE REVIEW ....

FT GENE REGULATION, TRANSPORT, AND BIOTECHNOLOGY APPLICATION IN CROP PLANTS ....

LIST OF TABLES

LIST OF FIGURES

LIST OF SUPPLEMENTARY TABLES

LIST OF SUPPLEMENTARY FIGURES

Chapter 1

Literature Review

FT gene regulation, transport, and biotechnology application in crop plants

1.1 Introduction

Flowering time is a crucial physiological process for successful sexual reproduction, crop yield and productivity (JUNG AND MÜLLER 2009; YEOH *et al.* 2011). The pioneering of the florigen concept and discovery of *FT* was headed by Koorneef and this was later acknowledged as a universal, long-distance, floral initiation signal present in multiple species (KOORNNEEF *et al.* 1991; TURCK *et al.* 2008; ZEEVAART 2008; ANDRÉS AND COUPLAND 2012). Since then, a large amount of research has been done on *FT,* the genes which act upstream and downstream of it, how it is regulated and its involvement in other gene networks.

The regulation of FT expression has been studied in depth. *FT* expression was found to be photoperiodically induced mainly in the leaves of plants (AN *et al.* 2004; WIGGE *et al.* 2005). The expression of FT is controlled by a multitude of proteins which are involved in the circadian clock machinery of the plant, for example, *GIGANTEA* (*GI*)*, CONSTANS* (*CO*), *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*)*, FLAVIN BINDING KELCH REPEAT F-BOX PROTEIN 1* (*FKF1*) and *CYCLING DOF FACTOR 1* (*CDF1*) (IMAIZUMI 2005; MIZOGUCHI *et al.* 2005; SAWA *et al.* 2007; FORNARA *et al.* 2009). The vernalisation pathway is another major way in which *FT* expression is regulated. A critical gene in this pathway is the *FLOWERING LOCUS C* (*FLC*) gene, a Minichrome Maintenance 1, Agamous, Deficiens, Serum response factor (MADS) box transcription factor which acts as a negative regulator of floral induction (MICHAELS AND AMASINO 1999; SHELDON *et al.* 1999; SHELDON *et al.* 2000). *FLC* itself is regulated largely by the gene product of *FRIGIDA* (*FRI*) (JOHANSON 2000; CHOI *et al.* 2011). This regulation takes place on multiple levels (DNA, transcriptional, post-transcriptional etc.), which will be discussed briefly in this review.

The evolutionary history of FT has also been studied. *FT*-like genes are present in all angiosperm plant species and the sequence is highly conserved (DANIEL AND HANZAWA 2015). The function of FT, in the flowering pathway, is also conserved across multiple species, for example, the *Hd3a* and *RFT1* genes in rice are involved in floral induction (TAMAKI *et al.* 2007; KOMIYA *et al.* 2008; KOMIYA *et al.* 2009). However, there is species-specific sub-, neo-functionalisation of the *FT* gene, and in some cases loss of function has also been observed. For example, sugar beet possesses two *FT*-like genes where one acts by repressing flowering (PIN *et al.* 2010). In other plants, *FT* is not involved in floral induction but rather in organ storage differentiation and even roles in seasonal phenology (LEE *et al.* 2013; NAVARRO *et al.* 2015; DING AND NILSSON 2016). Thus, even though *FT*-like genes are present in all angiosperms, it seems that through evolutionary history in specific species, the *FT* genes may have gained new functions or been sub-functionalized.

More recently, with the accumulated understanding of *FT* and its conserved nature across flowering plants, attempts have been made to use FT in biotechnology applications. Ectopic expression of *FT* allows one to induce the plant to flower earlier than usual (ENDO *et al.* 2005; ZHANG *et al.* 2010; YAMAGISHI *et al.* 2011; SRINIVASAN *et al.* 2012b; YAMAGISHI *et al.* 2014; KLOCKO *et al.* 2016; YAMAGISHI *et al.* 2016). Together with the nature of *FT* to be transported long-distances, grafting of wildtype scions to transgenic rootstocks was attempted, to create non-GMO early flowers, with little success in woody perennials (ZHANG *et al.* 2010; SRINIVASAN *et al.* 2012b; WENZEL *et al.* 2013; VELÁZQUEZ *et al.* 2016). This has only been successful in a small number of woody perennials (YE *et al.* 2014; FREIMAN *et al.* 2015; SONG *et al.* 2019; SOARES *et al.* 2020). This application has great potential for accelerated breeding of late flowering plants such as *Eucalyptus nitens,* due to the lengthy minimum time of five years this species takes to reach reproductive maturity (MONCUR AND HASAN 1994).

In this review, I go into detail on the known photoperiodic regulation of *FT* in the leaf. The multiple hypotheses of FT export from phloem companion cells (CC) to phloem sieve tube elements (SE) and long-distance transport of FT are considered. The evidence of specific FT unloading into the shoot apical meristem (SAM) is also discussed. The potential biotechnology application of using FT in breeding is also discussed. The vernalisation pathway is discussed, but not in-depth in terms of epigenetic regulation, reviewed in WHITTAKER AND DEAN (2017). The effect of stress responses on *FT* regulation is not in the scope of this review as well as other non-*FT* related flowering pathways.

1.2 The Plant Circadian Clock and Flowering

The circadian clock underlies the regulation of many pathways in plants. The majority of the circadian clock is made up of transcription factors which constitute a transcription-translation feedback loop which is the basis of clock function (NOHALES AND KAY 2016). Circadian regulation allows plants to predict and adapt to day-night cycles and seasonal changes. Involved in this adaptation are circadian clock-regulated behavioural processes, metabolic pathways, and physiological processes (BARAK *et al.* 2000; MCCLUNG 2019). For example, the nutation of inflorescence stems (NIINUMA *et al.* 2005), the opening and closing of the stomatal openings, plant defence timing (WANG *et al.* 2011b; GOODSPEED *et al.* 2012), and even starch breakdown speed (WEBB *et al.* 2019) are all under control/influenced by the circadian rhythm of the plant. Through the analysis of the *Arabidopsis* transcriptome, many genes were found to be expressed in a cyclic manner (BLÄSING *et al.* 2005; EDWARDS *et al.* 2006). Further evaluation of this transcriptomic data and different processes under the control of the circadian clock were revealed. Briefly, the expression of auxin-signalling genes and *dehydration-responsive element B1/C-repeat-binding factor (DREB1/CBF)* genes (involved in cold-stress tolerance) are also regulated by the circadian clock (HARMER *et al.* 2000; COVINGTON AND HARMER 2007). These findings illustrate the importance of the circadian clock in the regulation of numerous biological pathways.

The *GIGANTEA* (*GI*) gene is involved in a complex translational network and controls different aspects of the circadian clock. GI is a nuclear-localised protein (HUQ *et al.* 2000) and mutational studies of *gi* resulted in the altered period and amplitude of the circadian clock and it was thus hypothesised that functional *GI* is required for proper clock function (PARK 1999). GI possesses different biological functions, one of which is involved in the chaperoning of ZEITLUPE (ZTL) (CHA *et al.* 2017). Thus, GI also affects the number of proteins which ZTL interact with, namely TIMING OF CAB EXPRESSION 1 (TOC1) and PSEUDO-RESPONSE REGULATOR 5 (PRR5) as ZTL is involved in the degradation of these proteins

(MAS *et al.* 2003; KIBA *et al.* 2007). GI also interacts with CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) promoter region and PHYTOCHROME INTERACTING FACTOR (PIF) transcription factors, which ultimately regulates CCA1 expression (NOHALES *et al.* 2019). Thus, GI is required for the precise control of timing of the circadian clock, as well as the transcriptional control of CCA1 through interaction with CCA1 directly and ZTL.

GI regulates other physiological processes, of particular interest is the induction of photoperiodic flower induction. GI binds to and regulates genes such as FLAVIN BINDING KELCH REPEAT F-BOX PROTEIN 1 (FKF1), CYCLING DOF FACTOR 1 (CDF1) and redundantly acting genes which inhibit the transcription of *FLOWERING LOCUS T* (*FT*) and *CONSTANS* (*CO*) (IMAIZUMI 2005; SAWA *et al.* 2007; FORNARA *et al.* 2009) (Figure 1.1). It has been demonstrated that GI also binds to the promoter region of the *FT* gene, suggesting its direct role in the regulation of flowering time (SAWA AND KAY 2011). GI is also involved in the regulation of *microRNA172* processing, although the exact process of regulation is not known, *gi* mutants had reduced miRNA172 compared to control plants, especially when grown under long days, suggesting its role in *miRNA172* processing (JUNG *et al.* 2007) (Figure 1.1). miRNA172 is involved in adult epidermal identity and its increased expression is associated with a plants transition from a juvenile vegetative phase to a mature reproductive phase, it also indirectly upregulates *FT* through interaction with *TARGET OF EAT1 (TOE1)* (JUNG *et al.* 2007; WU *et al.* 2009; WANG *et al.* 2011a; LEVY *et al.* 2014). Lastly, GI has been shown to interact with the N-terminal tetratricopeptide repeats (TPRs) domain of the SPINDLY (SPY) protein (TSENG *et al.* 2004). The interaction of SPY and GI has not been demonstrated in planta, although they both occur in the nucleus, interact in vitro, and participate in common pathways involved in modulating flowering time suggesting multiple possibilities for interaction (HUQ *et al.* 2000; SWAIN *et al.* 2002; TSENG *et al.* 2004). These findings demonstrate how GI connects the circadian clock (photoperiod detection) and the plant's age (phase) to the induction of flowering.

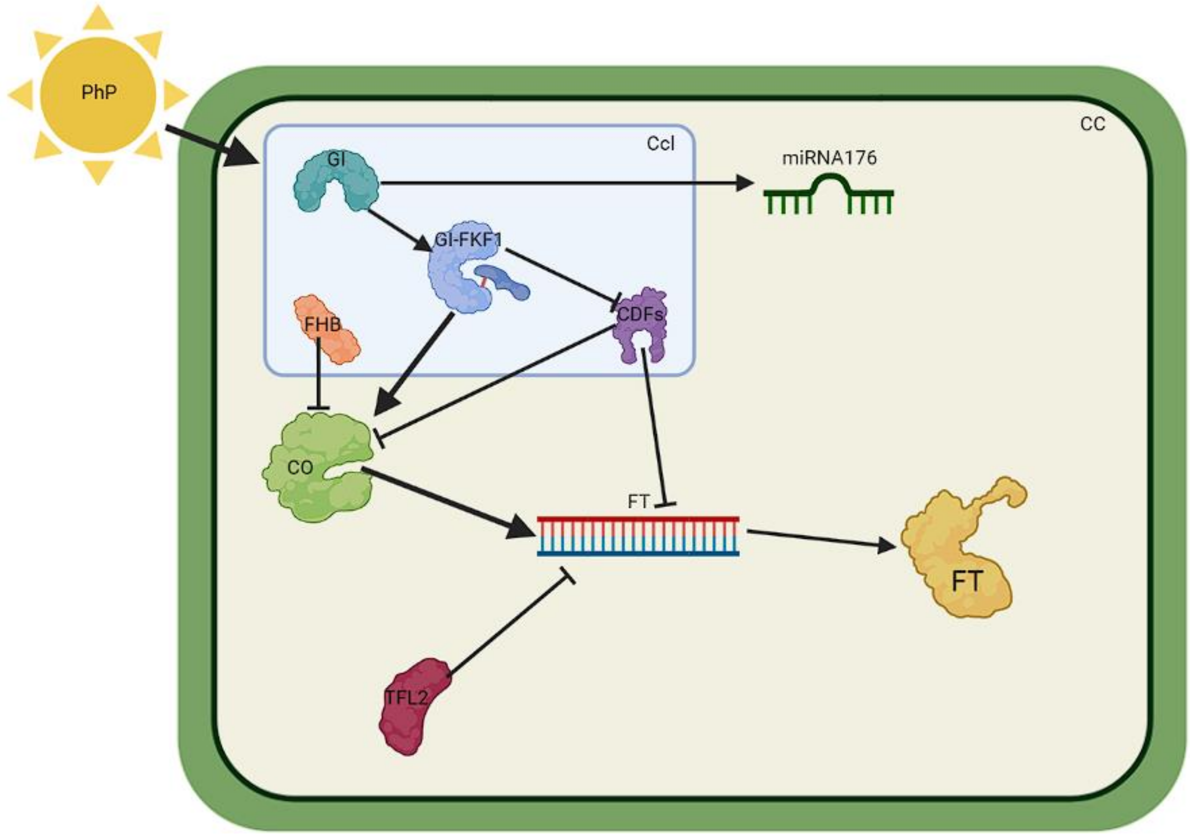


Figure 1. 1 Main interacting genes in *Arabidopsis* which regulate the expression of FT and are primarily influenced by photoperiod. CC = Phloem companion cells. PhP = Photoperiod. Ccl = Circadian Clock.

1.3 FT regulation in leaves

The *Flowering Locus T* (*FT*) gene encodes a small globular protein and is part of the phosphatidylethanolamine-binding protein (PEBP) family which was first described in mammals (Bernier *et al.* 1986). Later it was shown that FT from *Arabidopsis* specifically binds to phosphatidylcholine (PC) (Nakamura *et al.* 2014). Early research suggested that the leaf is the main site of photoperiod detection and that photoperiod detection is linked to florigenic signal production, which is now believed to be encoded by *FT* (Garner and Allard 1920; King and Zeevaart 1973; Koornneef *et al.* 1991; Michaels and Amasino 2000). Thus, it was not by chance that many studies have shown that *FT* is mainly expressed within leaf vasculature (AN *et al.* 2004; WIGGE *et al.* 2005; LIU *et al.* 2012; XU *et al.* 2012; ZHU *et al.* 2016; CHEN *et al.* 2018). A large amount of research has been done on FT and its genetic network mainly in *Arabidopsis*. However, more evidence is being reported that FT is regulated depending on the plant species and thus may have evolved specific roles in different regulatory networks to allow for optimal floral induction timing. For example, the *Phalaenopsis aphrodite FT* gene requires inductive cooling conditions, not related to vernalisation, to flower irrespective of photoperiod (JANG *et al.* 2015). However, *PaFT* showed similar expression patterns when expressed in *Arabidopsis*, and *PaFD* was able to partially rescue *Arabidopsis fd* mutants, demonstrating the conservation of these proteins between species and the distinct regulation of these proteins within species (JANG *et al.* 2015). Thus, FT expression is induced by specific photoperiodic conditions (specifically in *Arabidopsis*), and its expression is regulated directly by *CONSTANS* (*CO*).

CONSTANS (*CO*) is a transcription factor controlled by components of the circadian clock and ultimately regulates the floral transition by interaction with *FT* (Figure 1.1). *CO* is mainly repressed by two genes from the circadian clock, *FLOWERING bHLH* (*FBH*) and *CYCLING DOF FACTOR* (*CDF*) (FORNARA *et al.* 2009; ITO *et al.* 2012) (Figure 1.1). Activation of *CO* is mainly through a complex formed between GI and FKF1 which then promotes the degradation of *CO* repressors such as CDF (SAWA *et al.* 2007). *CO* is also post-translationally regulated. *CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)*, *SUPPRESOR OF PHYA-105 1 (SPA1)* and *EARLY FLOWERING 3 (ELF 3)* all contribute to the degradation of CO and GI respectively through post-translational means (YU *et al.* 2008; ZUO *et al.* 2011). The level of CO may also be increased by its stabilisation by phytochromes and cryptochromes. It was shown that phyB promotes the degradation of CO in the early day but cryptochromes and phyA play an opposite role in the evening, leading to CO stabilisation and increased *FT* expression (VALVERDE *et al.* 2004). The interaction and interplay between phyB and cryptochromes are not known. CO is then responsible for the activation of *FT,* specifically by binding to a cis-regulatory element (a TGTG(N2-3)ATG motif) within the promoter region of the *FT* gene, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (SAMACH *et al.* 2000; TIWARI *et al.* 2010). After this stage, the plant initiated the transition to floral development. From these observations, the conclusion can be made that *FT* expression is regulated by the photoperiod through the interaction with CO and genes which are part of the circadian clock such as GI.

FT is also regulated by the *TERMINAL FLOWER 2* (*TFL2*) gene (Figure 1.1). Earlier research in *Arabidopsis* identified *tfl2* mutants which caused early flowering phenotypes as well as differences in inflorescence phenotype (LARSSON *et al.* 1998). The early flowering phenotype of *tfl2* plants grown under non-inductive photoperiods hinted that *TFL2* played a role in the suppression of flowering under non-inductive conditions. The protein encoded by *TFL2* was found to be homologous to the HETEROCHROMATIN PROTEIN 1 (HP1) in mammals (KOTAKE *et al.* 2003). HP1 proteins bind directly to histone H3K9m and facilitate the formation of higher-order chromatin packaging, thus creating heterochromatin, which represses gene expression in that region (LACHNER *et al.* 2001). The absence of functional TFL2 lead to an increase of ectopic *FT* expression as well as upregulation of floral homeotic genes such as *APETALA3, SEPALLATA3, AGAMOUS, PISTILLATA* and others (KOTAKE *et al.* 2003; TAKADA AND GOTO 2003). Along with evidence that *CO, FT* and *TFL2* are present at the same subcellular locations, it was hypothesised that TFL2 does not repress *FT* expression directly but rather by preventing transcription factors such as *CO* from interacting with *FT* promoter regions (TAKADA AND GOTO 2003).

From the above information, flowering time is tightly regulated by photoperiod, which is linked to seasonal change. The highly regulated flowering time may be a consequence of many years of co-evolution with pollinators (SANDRING AND ÅGREN 2009; RAFFERTY AND IVES 2012). Most studies involved in the functional characterisation of flowering time-related genes have been conducted in *Arabidopsis* and very few studies have been done on large woody perennials such as *Eucalyptus*. Many *Eucalyptus* species begin to flower in response to an increasing photoperiod and an increase in temperature (BOLOTIN 1975; RAWAL *et al.* 2015). The only information on flowering and flowering time genes in *Eucalyptus* comes from a study which investigated the floral transcriptome (VINING *et al.* 2015). It was found that most ABCDE floral whorl genes in *Eucalyptus* such as *AP1, SEP* and *AG*, had similar expression profiles as those in *Arabidopsis*. Of particular interest, the *Eucalyptus FT, TFL3* and *MFT* genes were found to be mainly expressed in the floral buds while expression patterns in *Arabidopsis* would typically resemble an expression pattern more concentrated in the leaf vasculature. However, functional characterisation of these genes has not been conducted in *Eucalyptus* and is needed to further understand woody perennial flowering mechanisms.

1.4 From leaf to SAM: FT long-distance transport

FT is produced in the vasculature of leaves but subsequently acts at the shoot apical meristem (SAM) to induce floral morphogenesis. Early research, involving photoperiodic exposure, conducted on spinach plants showed that the leaf is the main location at which inductive floral signals originated and that the SAM was not involved (KNOTT 1934). Shortly after, a new study demonstrated that when *Perilla frutescens* and *Chrysanthemum* plants were grown in inductive conditions and their leaves were grafted onto respective plants grown in non-inductive conditions, the recipient plants began to produce flower (CHAILAKHYAN 1936). This finding confirmed that a flowering signal was produced in the leaves and thus the florigen theory was born. Subsequently, many studies were conducted to elucidate the transport of florigen (now known to mainly comprise the FT protein as previously discussed).

The first step in FT long-distance transport is the exporting of FT from phloem companion cells (CC) into the sieve tube elements (Figure 1.2). Studies using the expression of *FT* from heterologous promoters suggested that *FT* expression in CC is sufficient to cause floral induction (An *et al.* 2004; Abe *et al.* 2005). Synthetic microRNA, expressed in phloem companion cells, targeting *FT* delayed flowering; YFP-FT fusions, unable to be exported from phloem companion cells also showed delayed flowering. However, once the YFP was cleaved the FT gained movement and was able to induce flowering (Mathieu *et al.* 2007). Both these observations show the requirement of *FT* expression in phloem companion cells and its export to induce flowering in wild-type plants. Studies investigating FT export and transport towards the SAM have led to the implication of FT-INTERACTING PROTEIN 1 (FTIP1) and SODIUM POTASSIUM ROOT DEFECTIVE 1 (NaKR1) proteins to play major roles in these processes, respectively.

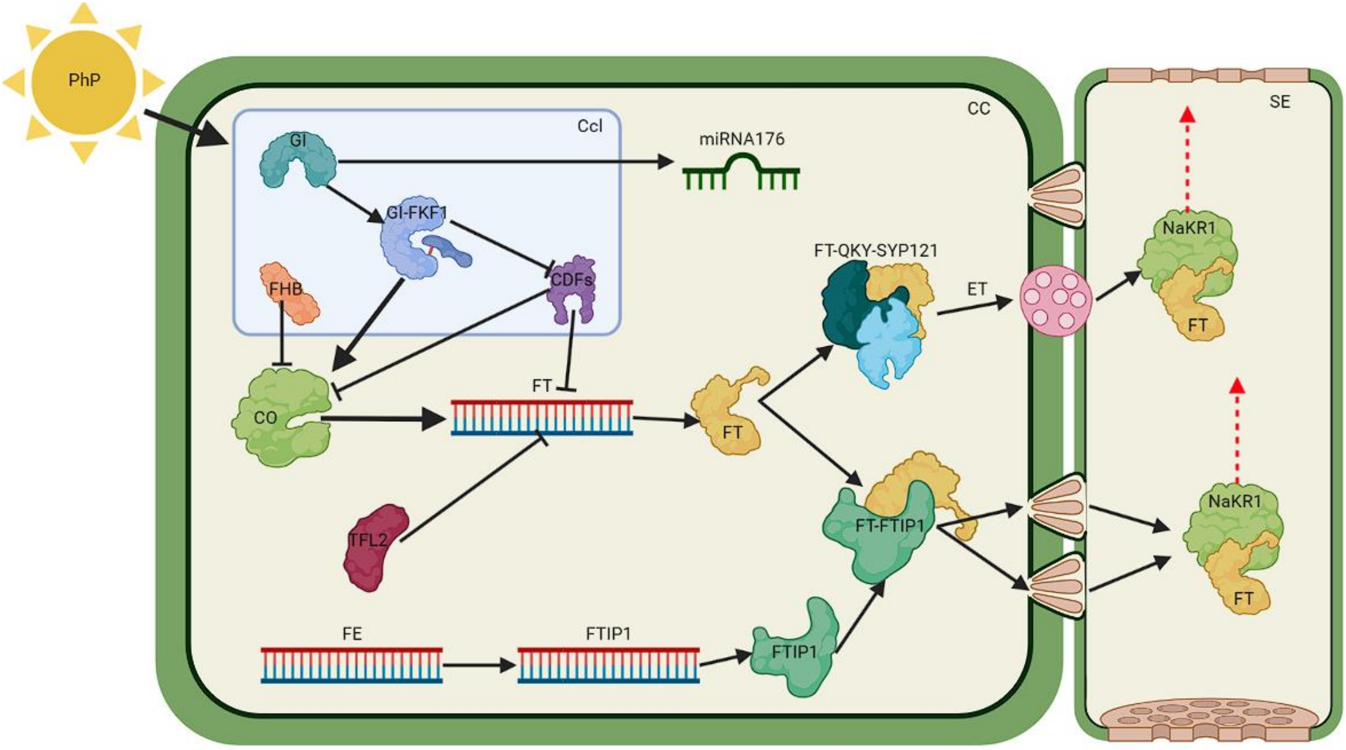


Figure 1.2 FT export from phloem companion cells. This figure is an extension of Figure 1.1 but includes the main modes of FT export from phloem companion cells (CC) to the sieve tube elements (SE). ET = endosomal trafficking pathway. PhP = Photoperiod. Ccl = Circadian Clock. Branched network between CC and SE represents the plasmodesmata.

FTIP1 is an ER-associated protein, part of the multiple C2 domain and transmembrane proteins (MCTPs) family, which is involved in the export of FT from CC to the sieve tube elements in leaf vasculature. *FTIP1* and *FT* were found to have similar expression profiles and their respective proteins were localised to the same tissue, the CC (LIU *et al.* 2012). More specifically, FTIP1 and FT were both localised to the ER. Coupled with the knowledge that the ER networks through the plasmodesmata (PD), which are located at the interface between CC and sieve tube elements (SE), and that FTIP1 and FT directly interact with each other, this forms the basis of the mechanism of FT export from CC to sieve tube elements (MARTENS *et al.* 2006; FITZGIBBON *et al.* 2010; LIU *et al.* 2012). This mode of transport is supported by the observation that plants with mutated *ftip1* displayed a build-up of FT within CC and an absence of FT in sieve tube elements (LIU *et al.* 2012; SONG *et al.* 2017). The FE protein, a phloem-specific Myb-related protein, is involved in the transcriptional regulation of FTIP1 and when mutated, expression data indicated a reduction in *FTIP1* expression ultimately resulting in reduced FT export and late flowering times (ABE *et al.* 2015) (Figure 1.2). These results suggest that the export of FT from CC to sieve tube elements is crucial for flowering time, and FTIP1 plays a major role.

The endosomal trafficking pathway acts redundantly with FTIP1 to achieve FT export from CC. Soluble N-ethylmaleimide-sensitive factor protein attachment protein receptor (SNARE) proteins are involved in intracellular plasma membrane fusion events (CHEN AND SCHELLER 2001; GREFEN AND BLATT 2008). A syntaxin-like Q-SNARE known as SYNTAXIN OF PLANTS121 (SYP121), has a C-terminal transmembrane domain and is involved in mediating vesicle trafficking to the plasma membrane (GEELEN *et al.* 2002; KARNIK *et al.* 2015). QUIRKY (QKY) is part of the MCTPs family, along with FTIP1 (FULTON *et al.* 2009). This information along with mutant *syp121* altering flowering time suggests endosomal trafficking is involved in flowering time (UEMURA *et al.* 2004). Thus, it was recently shown that SYP121 directly interacts with QKY, which in turn interacts with FT to regulate the movement of FT to the plasma membranes of CC via endosomes (LIU *et al.* 2019) (Figure 1.2). Recently, it was shown that FT is sequestered in CC in a temperature-dependent manner (SUSILA *et al.* 2021). According to the work done by SUSILA *et al.* (2021), in CC FT binds to phospholipid phosphatidylglycerol (PG) in a temperate-dependant manner. When PG biosynthesis was disturbed, *Arabidopsis* flowered in a temperature-independent manner. At lower temperatures, FT preferentially binds to PG reducing the level of soluble (and thus transportable) FT and at higher temperatures, it does not. This mechanism of temperature-dependent floral induction is hypothesised to allow plants to flower only under favourable conditions(SUSILA *et al.* 2021). Ultimately, FT is exported from CC to the sieve tube elements, in a temperature dependant manner, via an endosomal trafficking membrane fusion mechanism, which acts redundantly with the FTIP1-ER-mediated exporting pathway of FT.

The NaKR1 protein plays a role in the long-distance movement of the FT protein from leaf to SAM (Figure 1.2). NaKR1 is a soluble metal-binding protein that is involved in the long-distance transport of K^+, Na^+ and Rb^+ (TIAN *et al.* 2010). Additionally, NaKR1 was thought to play a role in the flowering pathway from the observation that plants lacking a functional copy of NaKR1 or if *NaKR1* transcripts were knocked down using micro RNA, resulted in a delayed flowering time (ZHU *et al.* 2016). Overexpression of *NaKR1* promoted early flowering (ZHU *et al.* 2016). These observations, along with evidence that *NaKR1* is expressed in CC (localised with FT), NaKR1 and FT directly interact with each other and that the amount of FT protein present at the SAM is correlated with NaKR1, strongly suggest NaKR1's role in the long-distance movement of FT from sieve tube elements to SAM (ZHANG *et al.* 2008; ZHU *et al.* 2016). More recently it was found that NaKR1 may regulate the expression of *FT* through the microRNA 156-Squamosa Binding Protein-Like3 (SPL3) process in response to the K^+ conditions the plant experiences, where increased K^+ conditions would delay flowering (NEGISHI *et al.* 2018). This suggests that NaKR1, in addition to FT transport, may play a role in floral timing depending on potassium conditions. Thus, the photoperiodically induced florigenic signal produced in the leaves is exported to the sieve tube elements by FTIP1, and relies on proteins such as NaKR1 to be transported to the SAM.

Plasmodesmata (PD) play a key role in the cell-to-cell transport of molecules. As previously discussed FTIP1 and NaKR1 interact with the ER, which networks through the PD, indicating the importance of PD in FT transport. PD that form a junction between CC and SE are branched on the CC side and merge on the SE side to form one large pore (LEE AND FRANK 2018). These junctions have a molecular size exclusion limit which only allows the diffusion of molecules up to 70kDa in size (OPARKA AND TURGEON 1999). This branched format accommodates large selective traffic loads to be exported into the SE. FT is a protein that is approximately 20kDa and was shown to readily diffuse out of the CC and into the SE (YOO *et al.* 2013a). However, observations indicate that entry into the phloem may also be shape-dependent as FT-GFP(cylindrical,27kDa) freely diffused but FT-glutathione S-transferase (GST)(spherical,26kDa) did not (YOO *et al.* 2013a). Other evidence may suggest that non-cell-autonomous proteins (NCAPs) may dilate the CC-SE PD junction to allow for the movement of certain proteins (LUCAS AND JUNG-YOUN 2004; LIN *et al.* 2009). These findings demonstrate the importance of PD in regard to the trafficking of molecules out of specific cells.

After long-distance transport, the FT protein needs to be specifically unloaded into the SAM and transported to the nucleus. Due to FT's homology with mammalian PEBP it was thought that FT may bind to the plasma membrane of SAM cells to be unloaded (BERNIER *et al.* 1986). It was demonstrated that FT specifically binds to phosphatidylcholine (PC), a phospholipid present in the plant plasma membrane (NAKAMURA *et al.* 2014). The amount of PC present in the plasma membrane directly correlated to flowering time and PC composition showed diurnal oscillation (NAKAMURA *et al.* 2014). Thus, PC may preferentially attract FT from the phloem stream and may additionally attract FT that is in the cytosol of SAM to be transported into the nucleus. Previously, it has been shown that the FT protein may bind to 14-3-3 proteins, which are a family of highly conserved proteins that participate in many protein interactions (PNUELI *et al.* 2001; LIFSCHITZ *et al.* 2006) (Figure 1.3). The name 14-3-3 originates from the particular elution and migration pattern of 14-3-3 proteins in chromatography (14th fraction of bovine brain homogenate) and gel electrophoresis (position 3.3 in the gel) (MOORE 1967; AITKEN 2006). Later, it was shown in rice that the FT protein interacted with the GF14c protein in rice, which is a 14-3-3 protein (TAOKA *et al.* 2011). This led to the hypothesis that 14-3-3 proteins may act as intracellular receptors for FT or FT-like proteins (TAOKA *et al.* 2011). Essentially, when FT is unloaded from the sieve tube elements into the SAM, FT will bind with 14-3-3 proteins and be transported to the nucleus of the cell to act as a transcription factor (Figure 1.3). Once FT reaches the nucleus of SAM cells, it will initiate the transcription of floral meristem identity genes which is a commitment to flowering.

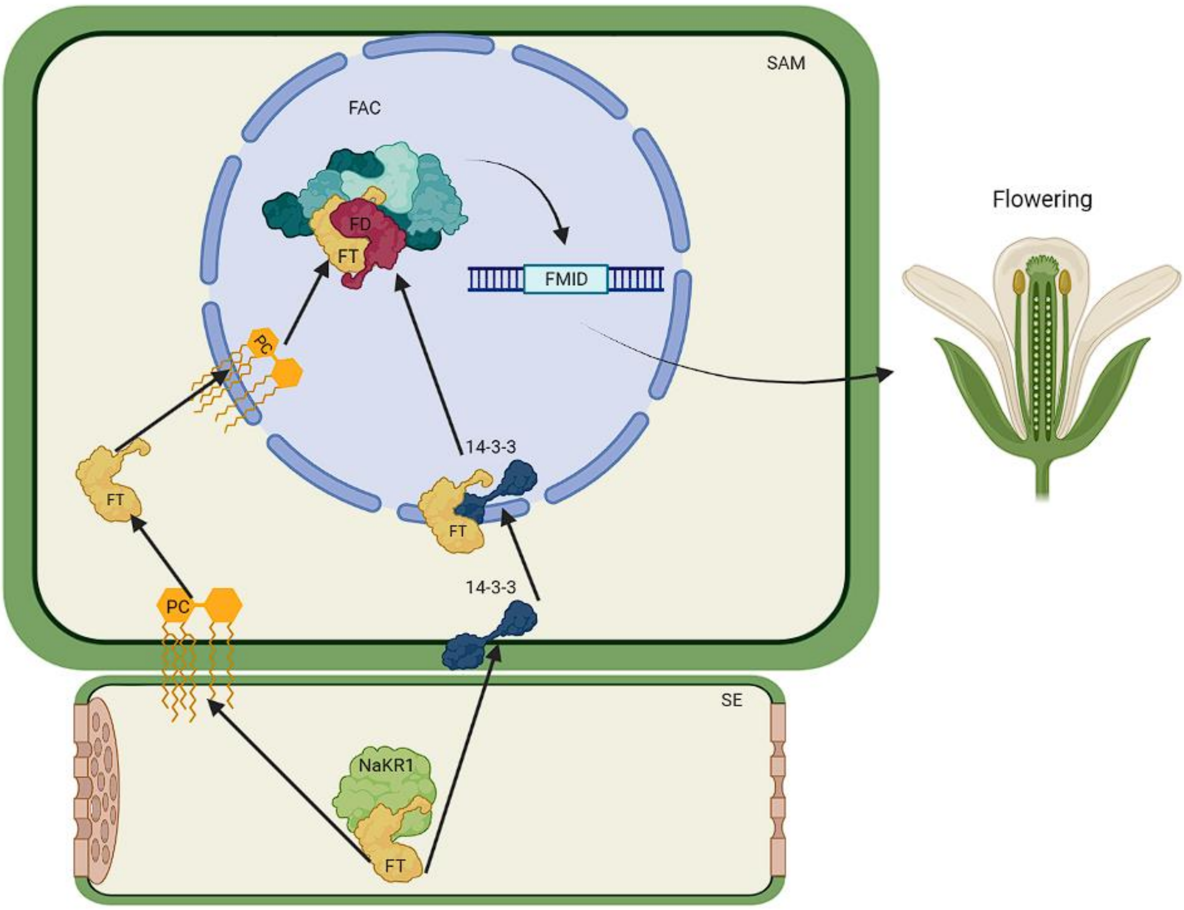


Figure 1. 3 Unloading of FT from the SE to the SAM. PC and 14-3-3 proteins facilitate FT unloading and transport to the nucleus. The florigen activation complex (FAC) is formed facilitating FT FD interaction and allowing activation of Floral meristem identity genes (FMID), such as *LFY, AP1, CAL* and *FUL.*

1.5 Floral induction at the SAM

Once FT reaches the nucleus of SAM cells it must interact with other proteins to initiate floral development. The *Flowering Locus D* (*FD*) gene was first thought to be part of the flowering time pathway after mutation of the *FD* gene produced late flowering phenotypes (KOORNNEEF *et al.* 1991). *FD* encodes a basic leucine zipper (bZIP) transcription factor, which is primarily expressed in the SAM (ABE *et al.* 2005). Since *fd* mutant plants almost suppressed the early flowering phenotype of 35S:*FT*, FDs role in the flowering pathway was investigated. It was determined that FT and FD proteins transiently interact with each other and that this interaction is needed for FT to function (ABE *et al.* 2005; WIGGE *et al.* 2005; ABE *et al.* 2019). The interaction between FT and FD may not be direct but mediated through 14-3-3 proteins (discussed earlier) as well as phosphorylation of FD by various SAM expressed proteins, altogether this forms a complex, known as the florigen activation complex (FAC), which initiates the floral transition by activating floral meristem identity genes (TAOKA *et al.* 2011; KAWAMOTO *et al.* 2015; COLLANI *et al.* 2019) (Figure 1.3). Ultimately, an interaction between FT and FD in the SAM is required for the activation of floral meristem identity genes such as *AP1, FUL, CAL* and *LFY*.

Many genes act downstream of the FAC, and all contribute to floral development. *FRUITFULL (FUL), APETELA1 (AP1), CAULIFLOWER (CAL,* a paralog of *AP1)* and *LEAFY (LFY)* act directly downstream of the FAC which activates these genes (FERRÁNDIZ *et al.* 2000; ABE *et al.* 2005; TEPER-BAMNOLKER AND SAMACH 2005; WIGGE *et al.* 2005; GOSLIN *et al.* 2017). These floral meristem identity genes form a complex gene regulatory network. *LFY* and *AP1*, are thought to play important roles in meristem identity, both are transcription factors and mutation of either greatly affects floral morphogenesis, while double mutants (*lfy ap1*) almost completely abolish floral production (IRISH AND SUSSEX 1990; WEIGEL *et al.* 1992). *FRUITFULL (FUL)* and *CAULIFLOWER (CAL)* are closely related to *AP1* and have been shown to act redundantly with other floral meristem identity genes (FERRÁNDIZ *et al.* 2000). The redundant manner of regulation as well as evidence of interaction of floral meristem identity genes, specifically *LFY, AP1* and *CAL*, demonstrates the importance of accurate flowering time (William *et al.* 2004; Cara *et al.* 2011).

LFY and AP1 also contribute to the maintenance of meristem conversion to floral meristem by repressing negative floral regulators. To do this LFY and AP1 repress the expression of other genes which are known to be negative regulators of the flowering pathway. AP1 represses the expression of *TFL1*, *TARGET OF EAT1 (TOE1)* and *TOE3* while also being involved in the down-regulation of *TEMPRANILLO1 (TEM1)* and *TEM2*, all of which have negative effects on the expression of *FT* (Castillejo and Pelaz 2008; Kaufmann *et al.* 2010). LFY has also been shown to directly suppress the expression of the *TFL1*, *TOE3* and *TEM1* genes, again emphasising the redundancy between floral meristem identity gene function (Parcy *et al.* 2002; Cara *et al.* 2011). LFY and AP1 have also been implicated in the regulation of hormone pathways in emerging floral meristematic cells, for example, auxin biosynthesis and gibberellin metabolism respectively (Kaufmann *et al.* 2010; Li *et al.* 2013; Yamaguchi *et al.* 2013). From these results, it seems that floral meristem identity genes function in a multitude of pathways to ensure the development of floral-associated cell types.

1.6 FT evolution

The origins and evolution of the FT gene have been partly elucidated through sequencing, phylogenetics and protein analysis. Through phylogenetic analysis of gymnosperms (Norway spruce and pine) and angiosperms (various species), it is clear that there are two groups of phosphatidylethanolamine-binding protein (PEBP) in gymnosperms, *MOTHER OF FT AND TFL1 (MFT)*-like and *FT/TERMINAL FLOWER1*-like *(TFL1)* respectively (KLINTENÄS *et al.* 2012). It is also hypothesised that after the gymnosperm-angiosperm split *FT*-like genes evolved from *FT/TFL1*-like genes and acquired the role of a flowering time integrator, this argument is supported by a functional study of spruce *FT/TFL1*-like genes and phylogenetic analysis (KLINTENÄS *et al.* 2012). This evidence suggests that *MOTHER OF FT AND TFL1 (MFT)*- like and *FT/TERMINAL FLOWER1*-like *(TFL1)* genes in gymnosperms evolved into what is now known as *FT*-like genes which play a large role in the timing of floral development.

Since the gymnosperm-angiosperm split, FT-like genes can be found in all angiosperms (including basal and modern lineages). Most of these FT-like genes have retained their function as a flowering time integrator, however, some have gone through duplication events and subsequently, the homologs have met different fates. For example, in sunflower, a recent duplication event resulted in four highly similar FT genes (BLACKMAN *et al.* 2010). Each paralog was found to meet a different fate. HaFT2 nonfunctionalized and was found to be more responsive to an earlier, long day flowering time due to *trans- and cis*-regulatory effects. HaFT1 is sub-functionalized, as HaFT1 was found to be associated with a flowering QTL, which delays plant floral induction through interaction with HaFT4. HaFT3 is hypothesised to have gone through a process of non-functionalisation as none of its transcripts was able to be detected (BLACKMAN *et al.* 2010). There are two *FT*-like homologs in *Beta vulgaris ssp. vulgaris (*sweet beet), *BvFT1* and *BvFT2* respectively. *BvFT2* is the functional *FT* ortholog in beets while *BvFT1* represses flowering, thus the *BVFT* genes have gone through a process of neofunctionalization in terms of the function of the protein each gene produces (PIN *et al.* 2010). There are many soybean

(*Glycine max*) *FT* homologs, *GmFT2a* and *GmFT5a* promote flowering, while *GmFT7* has no significant impact on flowering time (ZHANG *et al.* 2021). These findings suggest that the FT homologs still largely play a role in floral timing, but some FT genes have sub-functionalised, neo-functionalised or become non-functional after duplication events.

FT and TFL1 are similar proteins which act antagonistically to each other with regard to flowering time. FT and TFL1 share 57.5% nucleotide identity and 60% protein identity (KLINTENÄS *et al.* 2012), however despite their similarity they act oppositely when controlling flowering time (KOBAYASHI 1999). In species which have paralogs of FT that act as repressors or inducers (more than one FT due to duplication), the inducer FT typically has a tyrosine at position 134 based on protein alignment with the *Arabidopsis* FT protein (DANIEL AND HANZAWA 2015), and repressor FTs contain a non-tyrosine aa at position 134. This is supported by experiments where transgenic *Arabidopsis* which contains an *AtFT* which has a substitution at position 134 gains the functionality of a floral repressor (HO AND WEIGEL 2014). Four other critical residues namely, Glu-109, Trp-138, Gln-140 and Asn-52 can convert FT from a floral promoter into a TFL-like floral repressor (HANZAWA *et al.* 2005; AHN *et al.* 2006; PIN *et al.* 2010; HO AND WEIGEL 2014). Mutations at these sites have a significant impact on the surface charge of the FT protein potentially affecting its ability to interact with specific co-factors (HO AND WEIGEL 2014). These results show that the proteins are so similar that a single mutation converts the FT protein into a TFL1-like protein.

FT protein evolution in angiosperm large woody perennials has not been widely investigated, as in the above-mentioned examples. In *Populus, FT1* is involved in initiating reproductive development and is maximally expressed in the winter while *FT2* is involved in vegetative growth and reproductive structure development (HSU *et al.* 2011). Interestingly, a specific FT in citrus is associated with long-distance graft transmissible movement (SOARES *et al.* 2020). Seven FT-like genes have been identified in *Eucalyptus (VINING et al. 2015),* however extensive phylogenetic and protein analysis of these FT-like genes have not been conducted, and each gene's specific role in flowering time has not been elucidated. Thus, there is a gap in knowledge with regard to the *Eucalyptus* FT homolog's function and evolution.

1.7 The miRNA156 and miRNA172 age related flowering pathway

MicroRNAs (miRNAs) are small RNA molecules that are typically between 21 and 24 nucleotides in length and are found in most organisms. The general function of miRNAs is to regulate gene expression through transcript cleavage or the blocking of translation (BARTEL 2009). Of particular interest is the plant miRNA156, which targets a family of transcription factors known as SQUAMOSA PROMOTER BINDING LIKEs (SPLs), these transcription factors share a highly conserved DNA binding domain known as the SBP domain (CARDON *et al.* 1999; RHOADES *et al.* 2002). There are multiple SPL genes which have been identified in *Arabidopsis* and range from *SPL1* to *SPL13-like* (CARDON *et al.* 1999; XING *et al.* 2010). The *SPL3* gene has been shown to confer an early flowering phenotype in *Arabidopsis* when constitutively expressed (CARDON *et al.* 1997). The 3'UTR of the *SPL3* gene contains a miRNA-responsive element which is the location where miR156 binds and regulates *SPL3* expression via transcript cleavage as well as translational inhibition (GANDIKOTA *et al.* 2007). *SPL* genes are also involved in leaf morphogenesis, and the levels of miRNA156 dictate whether the leaves produced will have juvenile or mature characteristics, this principle is easily observable in the leaves of trees (GANDIKOTA *et al.* 2007; USAMI *et al.* 2009; WU *et al.* 2009; LEVY *et al.* 2014; WANG 2014). These findings suggest that miRNA156 is a key regulator of the juvenile phase of plants and declines as the plant matures and transitions into a reproductive phase.

miRNA172 acts downstream from miRNA156. The transcription factor *SPL9* binds to the regulatory region of miRNA172 which consequently increases its expression (WU *et al.* 2009). Mature miRNA172 (and its homologs) target 6 *APETALA (AP)* like genes, including *TARGET OF EAT1,2* and *3* (*TOE1, TOE2, TOE3*), and *AP2* itself (AUKERMAN AND SAKAI 2003). miRNA172 binds to *TOE1* and *TOE2* transcripts and mainly blocks translational machinery, thus regulating its expression (AUKERMAN AND SAKAI 2003;

SCHWAB *et al.* 2005; JUNG *et al.* 2007). miRNA172 is integrated into the photoperiodic pathway via *GIGANTEA* (*GI*), where miRNA172 processing is regulated by *GI,* thus miRNA172 acts downstream of GI (JUNG *et al.* 2007). Overexpression of miRNA172 (or its homologs) results in an early flowering phenotype, in many different species, mostly due to the repression of *TOE1, TOE2* and *AP2* which are floral repressors (JUNG *et al.* 2007; SHIVARAJ *et al.* 2018; LI *et al.* 2019). However, overexpression of miRNA172 and its homologs have varying effects. When *MIR172e* and *MIR172e*' from *Brassica* were overexpressed in *Arabidopsis* they produced an early flowering phenotype with functional flowers (SHIVARAJ *et al.* 2018). While *MIR172d* and *MIR172d'* overexpressing plants flowered extremely early but flowers were defective (SHIVARAJ *et al.* 2018). In another study overexpression of *miRNA172* from *Arabidopsis* in gloxinia (*Sinningia speciosa*) produced extremely early flowering times along with normal flowers (LI *et al.* 2019). The ability of miRNA172 and miRNA156 to perform their endogenous functions in other species demonstrates the highly conserved nature of these microRNAs, along with other studies showing the similarity of these microRNAs and their targets across the plant kingdom (ZHANG *et al.* 2006a). These results suggest that miRNA156 and miRNA172 expression is temporally coordinated and that miRNA156 plays a critical role as a key regulator of the juvenile phase of plants, while miRNA172 takes over in the mature/reproductive phase of a plants life.

1.8 Phloem transport and grafting

In terms of FT long-distance transport, it is important to understand the directional flow of the sieve tube elements (the phloem stream) since FT is loaded into the sieve tube elements from phloem companion cells. The first hypothesis of long-distance phloem transport, the pressure flow hypothesis, was modelled on an osmotically generated pressure differential between (leaves) and sink tissue (roots, shoot apex) (MUNCH 1930). This hypothesis was largely accepted due to its simplicity and plausibility, with little evidence. However, more intricate studies have been done which support this hypothesis, by measuring internal pressure in sieve tube elements, and how the pressure scales in accordance with plant size (KNOBLAUCH *et al.* 2016). An earlier study used a CO_2 isoform ($^{11}CO_2$) to study the movement of photoassimilates through the phloem to sinks (shoot apex and roots) (THORPE *et al.* 2011). It was found that generally, labelled sources (leaves) closer to a specific sink led to faster and higher accumulation of labelled photoassimilates at the respective sink. However, labelled photoassimilates were always detected both in the shoot apex and the roots, suggesting a bidirectional flow of phloem, which was also seen in earlier studies (ST-PIERRE AND WRIGHT 1972; DICKSON 1989; JAHNKE *et al.* 1998; THORPE *et al.* 2011). Interestingly, in trees, the transport of photoassimilates towards the shoot apex was only seen from younger leaves closer to the shoot apex (JAHNKE *et al.* 1998). However, in some cases, photoassimilates accumulated in sink tissues which were distant from the source tissues that they were produced in (THORPE *et al.* 2011). This may be explained by formations of orthostichy, where leaves on one side of the stem are connected by common vasculature, and to a lesser extent to leaves on the opposite side of the stem (MURRAY *et al.* 1982; WATSON AND CASPER 1984; ORIANS *et al.* 2004). Even if only leaves were left on one side of the stem, bidirectional flow was observed and can be explained by opening of anastomoses allowing enhanced linkage of a vertical row of leaves, one above the other, on a specific side of the plant and molecular hopping (phloem leakage and reloading) (ALONI AND PETERSON 1990; VAN BEL *et al.* 2011). These findings from photoassimilate transport via phloem translocation stream may lay out the path which the FT protein may travel to reach the shoot apex.

Grafting is an ancient technique which is still widely used today, mostly for the generation of a plant which is an amalgam of two different plants each with desirable traits. For example, it can be used to combine a scion which has the potential to produce a high yield with a rootstock that has stress-resistant properties. The success of grafting is due to the innate regenerative ability that plants possess. Xylem-pole pericycle cells possess the potential to produce root apical meristem cells and are the cell type responsible for regenerating organs from roots and hypocotyl when placed in specific tissue culture media (ATTA *et al.* 2009). However, it is the cambium cells in the vasculature of leaf explants which are responsible for the regeneration of roots mediated by *WUSCHEL RELATED HOMEOBOX11* (*WOX11*) and *WOX12* genes (LIU *et al.* 2014; CHEN *et al.* 2016). Organ regeneration may also be initiated from other somatic cells such as endodermal cells, these cells were shown to regenerate roots in tissue culture (FALASCA *et al.* 2004). This demonstrates the potential of cells to dedifferentiate, proliferate and subsequently differentiate into a different cell type.

One of the first steps in plant regeneration is callus formation in response to wounding, this is exploited in grafting. Cytokinin plays a major role in wound-induced callus formation. When a plant is wounded cytokinin biosynthesis genes such as *ISOPENTENYL TRANSFERASE 3* (*IPT3*), *LONELY GUY 1* (*LOG1*), *LOG4*, and *LOG5* are transcriptionally activated, and when these genes are mutated the plants show defects in wound-induced callus formation (IKEUCHI *et al.* 2017). Another set of reprogramming regulator genes known as *WOUND INDUCED DEDIFFERENTIATION* genes (*WIND1, 2, 3 and 4*) are also activated upon wounding (IWASE *et al.* 2011). The cytokinin biosynthesis and *WIND* pathways both facilitate the activation of cytokinin signalling by activating type-*B ARABIDOPSIS RESPONSE REGULATOR 1* (*ARR1*) and *ARR12* which ultimately leads to upregulation of *CYCLIN D3;1* (*CYCD3*;*1*) and re-entry into the cell cycle (IWASE *et al.* 2011; IKEUCHI *et al.* 2017). In the case of grafting, the cells from scion and rootstock both transiently proliferate forming a callus which bridges the gap between them.

After cell proliferation and callus formation, hormones play a key role in establishing cell re-proliferation and vascular re-joining. Auxin and ethylene both play a role in promoting the transcription of *NAC DOMAIN CONTAINING PROTEIN 71* (*ANAC071*), this regulation is asymmetrical as it is more prominent above incision sites (i.e. in the scion tissue) (ASAHINA *et al.* 2011). ANAC071 is involved in the regulation of the tissue repair pathway and has been shown to upregulate *XYLOGLUCAN ENDOTRANSGLUCOSYLASES/HYDROLASE 19* (*XTH19*) and *XTH20* which are involved in pith cell proliferation (PITAKSARINGKARN *et al.* 2014). While *RELATED TO APETALA2 6L* (*RAP2.6L*) expression is increased below the incision site (i.e. in the rootstock tissue) and is needed for tissue repair (ASAHINA *et al.* 2011). Studies have also shown the order of events, the phloem usually reconnects first followed by the xylem within a few days (MELNYK *et al.* 2015). Particularly, cytokinin does not affect phloem reconnection, but several genes involved in auxin signalling or response do.

AUXIN RESISTANT 1 (*AXR1*) and *ABERRANT LATERAL ROOT FORMATION 4* (*ALF4*) were shown to perturb phloem reconnection (MELNYK *et al.* 2015). These genes were only important in cells below and near the graft junction (MELNYK *et al.* 2015), a similar asymmetric regulation that was also by other researchers (ASAHINA *et al.* 2011). Recently it was shown that the *WUSCHEL-RELATED HOMEOBOX 4* (*WOX4*) gene is needed in at least the scion or rootstock for xylem reconnection in tomatoes (THOMAS *et al.* 2021). Again, it seems that an asymmetric regulation of genes near or at the graft junction is needed for successful graft formation. There are still many unknowns to the regulation of genes at the graft junction, but there are many parallels to wound healing such as cell dedifferentiation, proliferation, and re-differentiation.

1.9 Biotechnology applications using FT

FT can be used to alter the flowering time of plants. In many of the previous studies mentioned in this review, overexpression of *FT* from a constitutive promoter resulted in an early flowering phenotype. With this knowledge and evidence that the FT protein moves long distances, grafting experiments were conducted to determine graft transmissibility of the florigen signal, indeed FT protein produced in rootstocks was able to rescue late flowering time of *ft* scions in *Arabidopsis* (CORBESIER *et al.* 2007; NOTAGUCHI *et al.* 2008). Thus, the effects of *FT* overexpression and its long-distance movement have been well studied in *Arabidopsis* and other small herbaceous plants, such as Cucurbits (LIN *et al.* 2007; LIN *et al.* 2009) (Figure 1.4a). However, much less knowledge is available for larger woody perennials.

The control of flowering time of woody perennial plants is of great value due to their long juvenile phase before flowering. Shortening the juvenile phase of woody perennials using FT would have a benefit in breeding programmes. This prompted the investigation of *FT* ectopic expression in multiple woody perennials, apple, pear, poplar, citrus, *Eucalyptus* and plum, which all resulted in early flowering time (Table 1). However, when testing the graft transmissibility of FT in poplar, apple, plum and citrus, no floral induction was seen in wild-type plants (Table 1). No, graft transmissibility may be attributed to loss of rootstock leaves, other reasons may be due to problems with the unloading of FT from CC or upload into the SAM, as many of these studies have used non-endogenous *FT* for early induction. Long-distance graft transmissibility has been achieved in smaller woody perennials such as blueberry and *Jatropha* (Table 1). Interestingly, ectopic expression of a pear FT-like protein (PcFT2), promoted early flowering tobacco and was graft transmissible, the movement was also detected in apples, however, it did not induce flowering (Freiman *et al.* 2015). Graft transmissibility was achieved in *Citrus* using a specific citrus FT homolog (*CcFT3*) (Soares *et al.* 2020). More recently, it was shown that young *Eucalyptus* scion grafted onto old *Eucalyptus* rootstocks (18 years and older), eventually produced flowers and fruit within two years of grafting, thus accelerating the time to flowering in the younger material (De Oliveira Castro *et al.* 2021; Castro *et al.* 2022). The earliest floral induction in young material (4 months), was only achieved when paclobutrazol was applied to the rootstocks. Thus, some aspect of a florigenic signal being produced in the mature rootstocks is being transported past the graft junction into the young material and is amplified by the application of paclobutrazol, ultimately inducing flowering.

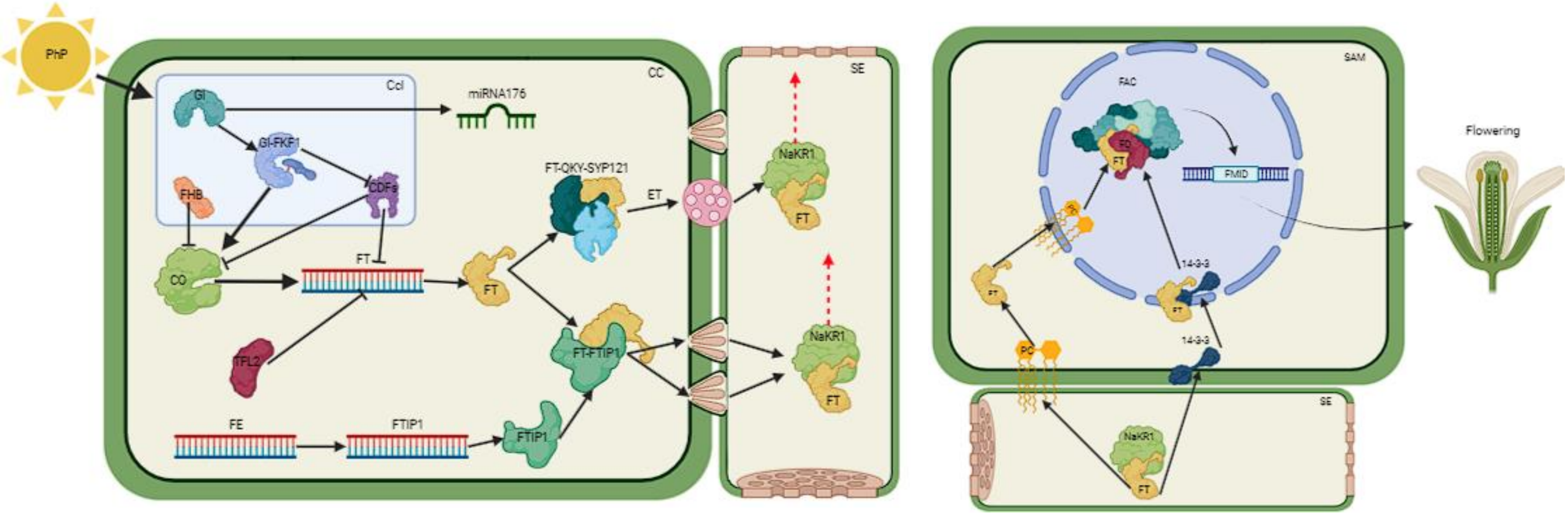
Table 1. 1. Experiments attempting graft transmission of FT in multiple species

Species of graft	FT gene used	Promoter used	Transmission (resulting in earlier flowering)	Early flowering	Graft	Reference
Populus (*tremula x tremuloides*) or (*tremula x alba*)	*AtFT*	GmHsp17.6-L (HSP)	No graft transmission	Only on transgenic rootstock	Cleft-graft (3-6mm d succulent shoots)	(Zhang *et al.* 2010)
Populus (*tremula x tremuloides*) or (*tremula x alba*)	*Populus trichocarpa FT1* and *FT2*	GmHsp17.6-L (HSP)	Not tested	Only on transgenic rootstock		
Tomato (*Solanum*) (*sft* mutant scions)	*SINGLE FLOWER TRUSS* (*SFT*)	35S	Graft transmission (only from scion to rootstock)	Yes	classic wedge-shaped/slit	(Lifschitz *et al.* 2006)
Arabidopsis thaliana	*AtFT:GFP*	SUCROSE TRANSPORTER 2 (SUC2)	Graft transmission	Yes	Y-grafts and butt grafts	(Corbesier *et al.* 2007)
Arabidopsis thaliana	*AtFT*	35S	Graft transmission	Yes	Y-grafts	(Notaguchi *et al.* 2008)

***Arabidopsis thaliana* (co-mutants)**	Endogenous *FT*	Endogenous FT	Graft transmission	Yes	Y-grafts	(An *et al.* 2004)
Rice (*Oryza sativa*)	*Hd3a*	Hd3a promoter	Not tested (long-distance movement detected)	No	None	(Tamaki *et al.* 2007)
(Plum) *Prunus domestica*	*Populus trichocarpa FT1*	35S	Not tested	Only on transgenic rootstock	None	(Srinivasan *et al.* 2012a)
Apple (*Malus domestica*) (Pinova)	*Populus trichocarpa FT1* and *FT2*	GmHsp 17.5-E (HSP)	No graft transmission	Only on transgenic rootstock	“Micrograft” (no further detail)	(Wenzel *et al.* 2013)
Citrus hybrids	*AtFT, CiFT*	CLBV-based vector expression	Graft transmission of virus	Yes	Not stated. Figure looks like wedge/cleft	(Velázquez *et al.* 2016)
Jatropha	*JcFT*	double CaMV35S promoter	Graft transmission	Yes	Shoot grafting	(Ye *et al.* 2014)
Blueberry (*Vaccinium corymbosum*)	*VcFT*	35S	Graft transmission	Yes	Splice grafting	(Song *et al.* 2013; Song *et al.* 2019)
Apple and Tobacco	Pear (*Pyrus communis*) FT2 (*PcFT2*)	35S	Graft transmission in apple and tobacco	Yes (only in tobacco)	Wedge graft	(Freiman *et al.* 2015)
Arabidopsis thaliana	*AtFT*	*AtSUC2*	Graft transmission	Yes	Cot graft	(Zhu *et al.* 2016)
Arabidopsis thaliana	*AtFT*	35S	Graft transmission	Yes	Cot-, Y-,Butt-grafting	(Yoo *et al.* 2013b)
Citrus	*CcFT3*	*AtSUC2*	Graft transmission	Yes	“Budwood grafted onto 6-month-old rootstock”	(Soares *et al.* 2020)
Eucalyptus species	*None*	*None*	Graft transmission	Yes	Cleft grafts	(De Oliveira Castro *et al.* 2021; Castro *et al.* 2022)
Populus tremula xPopulus tremuloides	*Populus trichocarpa FT1* and *FT2*	35S/409S	Graft transmission	Yes	Not stated (likely cleft graft)	(Miskolczi *et al.* 2019)

Early flowering graft transmissible plants can be used to avoid the production of transgenic progeny while still exploiting transgenic material (rootstock/scion). Wildtype scions induced to flower early by being grafted onto transgenic rootstocks are non-GMO, but as a result, have a much shorter time to flower. One advantage to this approach is that DNA marker-assisted selection to breed superior plant lines will be more efficient as many early flowering scions may be crossed with many parents, producing many offspring in a shorter amount of time. For example, this method was used to breed flood-tolerant maize (MANO *et al.* 2016). Another advantage of using ectopically expressed FT rootstocks is that the plants are dwarfed, which saves greenhouse space, allowing more plants to be experimented with, dwarfing of plants was reported in multiple studies (GAO *et al.* 2016; KLOCKO *et al.* 2016; VELÁZQUEZ *et al.* 2016). This approach combined with genetic containment of transgenic rootstock via CRISPR-Cas knockout of *LFY* recently investigated in *Eucalyptus* (ELORRIAGA *et al.* 2021), would ensure that progeny produced by wild-type scion are completely non-GMO, which is pleasing to a sceptical public.

A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FTIP1
FTIP1
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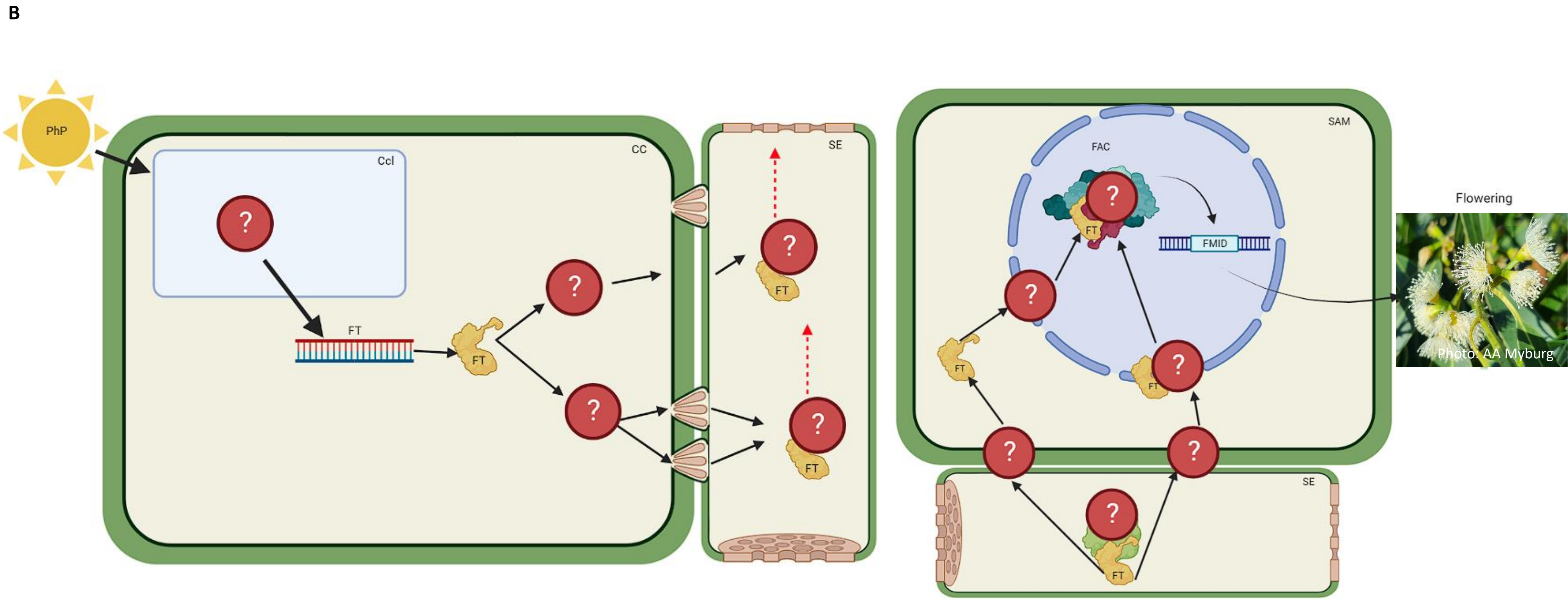

Figure 1.4 Comparison of known FT regulation and transport in *Arabidopsis* and *Eucalyptus*. A) The main pathways of FT induction, transport from phloem companion cells to the shoot apical meristem and how it induces flowering in *Arabidopsis,* that have been functionally determined. B) Known flowering pathway of *Eucalyptus*, only RT-qPCR data available on flowering *Eucalyptus* plants (Vining *et al.* 2015), no genes have been functionally characterised. A BLAST search of the *Eucalyptus grandis* proteome against the *Arabidopsis* NaKR1, FTIP1 and CO proteins yields potential homologs: A0A059A8G7_EUCGR, A0A059DIR1_EUCGR and A0A059ARP7_EUCGR respectively. This suggests that *Eucalyptus* may regulate flowering in a similar manner as *Arabidopsis*. However, it is known that *Arabidopsis FT* overexpression is sufficient to induce *Eucalyptus* flowering (Klocko *et al.* 2016).

1.10 Conclusions

A lot of information and knowledge has been accumulated in terms of *FT* regulation in the leaf. Specifically, photoperiodic induction of *FT* has been studied in depth, however, most of these studies have been conducted using *Arabidopsis* and this knowledge does not directly translate to other organisms such as large woody perennials, even though *FT* is relatively highly conserved. However, more evidence is coming about identifying the regulation on a finer scale.

Accelerated flowering has only been successful in a few woody plants which hold economic value and has not been fully explored in *Eucalyptus*. *Eucalyptus spp.* are an economically important crop due to the value of its wood and wood-derived products. Thus, there is a strive to breed trees with higher quality wood, disease-resistant traits, and abiotic-resistant traits (drought tolerance, cold tolerance etc). However, *Eucalyptus* trees are known to take up to at least eight years to flower, causing a bottleneck in breeding programmes. There have been a limited number of studies on the use of *FT* in *Eucalyptus,* and *Eucalyptus*-specific orthologs of *FT, FTIP1, NaKR1* and promoters such as *SUC2* have not been tested for accelerated flowering of *Eucalyptus*. Therefore, there is a need to develop a platform in which FT and its transport can be studied in *Eucalyptus*.

Engineering the induction of early flowering of woody plants, via long-distance graft transmissible signals that produce early flowering phenotypes, will be highly beneficial in terms of accelerated breeding programmes of economically important crops such as *Eucalyptus* and *Citrus*. With the breakthrough results demonstrating FT long-distance movement and floral induction of wild-type scions in *Citrus*, the goal of achieving this in *Eucalyptus* species becomes one step closer (SOARES *et al.* 2020). Combined with the genetic containment technique, this has the potential of serving as the basis of an accelerated breeding approach in *Eucalyptus* (ELORRIAGA *et al.* 2021). Subsequent DNA marker analysis of the progeny may be performed and progeny with genotypes of interest may be selected to grow, saving time, money, and space. Progeny produced from scions will not be considered GMO and will be able to be deployed in commercial plantations without ethical consequences. I hypothesize that there is a FT homolog in *Eucalyptus* which has a similar function in floral induction as is present in many other species, and thus responsible for floral induction in *Eucalyptus* itself. Expanding on this, I hypothesize that the FT protein of *Eucalyptus* (or the FT derived from other species), will be a mobile protein and capable of long-distance graft transmissible movement and that ectopically expressed FT in *Eucalyptus* will allow the induction of flowering of wildtype material after a successful graft union is formed.

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Chapter 2

Functional Characterisation of the *Eucalyptus* FT protein in *Arabidopsis*

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This research chapter has been prepared in the format required for submission to a peer-reviewed journal (Genetics). I performed all experiments and analyses described in this manuscript and drafted the manuscript. Dr Greg Goralogia provided the destination backbone used in this study. Dr Steven Hussey co-supervised the project and provided technical support and advise on data analysis as well as valuable inputs into the manuscript revision. Prof A.A Myburg conceived and supervised the study and provided valuable revisions for this manuscript

2.1 Abstract

The *Flowering Locus T* (*FT*) gene or *FT-like* genes have been cloned in many plant species and functionally characterised in those species, or in a heterologous system such as *Arabidopsis thaliana*. Despite the fact that *Eucalyptus* species often produce abundant amounts of flowers and it is known that top-grafting of juvenile shoots on older rootstock induces flowering in juvenile material, the *FT* gene in *Eucalyptus* has not yet been functionally characterised. This study aimed to characterise the putative *FT* ortholog of *Eucalyptus* in *Arabidopsis thaliana* with emphasis on its ability to traverse a graft junction. Phylogenetic analysis and 3D protein prediction and comparison were used to identify the most likely *Eucalyptus FT* candidate. The putative *Eucalyptus FT* coding sequence was cloned into and transformed into *Arabidopsis* via *Agrobacterium*-mediated floral dip transformation. The resultant transgenic plants flowered on average three weeks after germination when grown under short-day conditions and flowered significantly earlier than the wildtype controls. Fluorescence confocal microscopy and a GFP tagged *Eucalyptus* FT protein was used to show that the *Eucalyptus* FT protein localised to the plant's vasculature. Using the same approach, transgenic *Arabidopsis* hypocotyl and cotyledons were grafted to wildtype plant's lower hypocotyl and root demonstrating that the *Eucalyptus* FT-GFP protein was capable of crossing the graft junction and traveling into the wildtype roots. This study functionally demonstrates the floral induction and graft transmissible properties of the *Eucalyptus* FT protein.

2.2 Introduction

The *Flowering Locus T* (*FT*) gene is a small globular protein, which in early research was found to be mainly expressed in leaf tissue and was linked to photoperiodic detection and subsequently confirmed to be a florigenic signal which initiates the transition to flowering (GARNER AND ALLARD 1920; KING AND ZEEVAART 1973; KOORNNEEF *et al.* 1991; MICHAELS AND AMASINO 2000). Later, it was shown on a molecular level that *FT* is expressed in leaf material and more specifically, specialised phloem companion cells (AN *et al.* 2004; WIGGE *et al.* 2005; LIU *et al.* 2012; XU *et al.* 2012; ZHU *et al.* 2016; CHEN *et al.* 2018). *FT* expression in phloem companion cells is sufficient to initiate floral induction (AN *et al.* 2004; ABE *et al.* 2005). *FT* is linked to photoperiod (and the circadian clock) via *CONSTANS* (*CO*), a transcription factor which is itself controlled by components of the circadian clock and initiates floral transition by activation of transcription of the *FT* gene (SAMACH *et al.* 2000; TIWARI *et al.* 2010). CO activates *FT* specifically by binding to a cis-regulatory element (a TGTG(N2-3)ATG motif) within the promoter region of the *FT* gene which recruits the transcriptional machinery. CO also interacts with other floral related genes such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), which also interact with *FT* (SAMACH *et al.* 2000; TIWARI *et al.* 2010). The detection of favourable conditions for flowering in the leaf is crucial for many flowering plants to flower at optimal times.

After the *FT* gene is expressed in the phloem companion cells, the FT protein must be exported into the sieve tube elements to be transported to the shoot apical meristem (SAM). Studies have shown that *FT-INTERACTING PROTEIN 1* (*FTIP1*) and *SODIUM POTASSIUM ROOT DEFECTIVE 1* (*NaKR1*) are two genes that are involved in the transport of FT. *FTIP1* and *FT* have similar expression profiles and their respective proteins are localised to the endoplasmic reticulum (ER) of phloem companion cells (LIU *et al.* 2012). It is hypothesised that both FTIP1 and FT directly interact with each other and are localised to the ER, suggesting a path taken to export FT from phloem companion cells (MARTENS *et al.* 2006; FITZGIBBON *et al.* 2010; LIU *et al.* 2012). This theory is supported by the observation that *ftip1* mutants displayed a build-up of FT within phloem companion cells (CC) and an absence of FT in sieve tube elements (LIU *et al.* 2012; SONG *et al.* 2017). NaKR1, is a soluble metal binding protein which has been shown to be involved in the long distance transport of K^+, Na^+ and Rb^+ (TIAN *et al.* 2010). NaKR1 and FT directly interact with each other, and this interaction been shown to be involved in the transport of FT to the SAM, due to *nakr1* mutants showing a decreased amount of FT at the SAM(ZHANG *et al.* 2008; ZHU *et al.* 2016).

The long-distance movement of FT and its ability to alter flowering time has been exploited, for the early flowering of plants in a non-transgenic fashion. Experiments have shown that FT can move long distances, from leaves near the SAM and potentially multiple internodes, through a plant and is also graft transmissible (AN *et al.* 2004; CORBESIER *et al.* 2007; NOTAGUCHI *et al.* 2008; SOARES *et al.* 2020; DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022). FT protein produced in transgenic rootstocks was able to rescue the late flowering phenotype of *ft* mutant scions in *Arabidopsis* (CORBESIER *et al.* 2007; NOTAGUCHI *et al.* 2008). The effects of *FT* overexpression and its long-distance, graft-transmissible movement have been well-studied in *Arabidopsis (AN et al. 2004; CORBESIER et al. 2007; NOTAGUCHI et al. 2008; YOO et al. 2013a; ZHU et al. 2016)* and other small herbaceous plants, such as Cucurbits (LIN *et al.* 2007; LIN *et al.* 2009). The ability to control the time when woody perennials flower is of great value because they take a relatively long time before they can produce flowers. Shortening the time to flower of woody perennials using FT would confer a great advantage to breeding programmes that are often hampered by their long generation times. This prospect led to the investigation of *FT* ectopic expression in multiple woody perennials such as apple, pear, poplar, citrus, *Eucalyptus,* plum and *Jatropha* which all resulted in early floral induction (ZHANG *et al.* 2010; SRINIVASAN *et al.* 2012a; SONG *et al.* 2013; WENZEL *et al.* 2013; YE *et al.* 2014; FREIMAN *et al.* 2015; KLOCKO *et al.* 2016; VELÁZQUEZ *et al.* 2016; SONG *et al.* 2019; SOARES *et al.* 2020). Due to the FT protein's ability to move over a graft junction, it may act on the recipient scion and induce floral induction without affecting the genome of the recipient scion. The flowers produced due to the induction by FT (regardless of its source, i.e. from rootstocks expressing *FT* ectopically in a transgenic fashion), are non-transgenic and thus will produce non-transgenic pollen and ultimately non-transgenic seed. This approach can be used to accelerate the generation time of plants which have a long time to flowering such as woody perennials (SOARES *et al.* 2020).

When testing the graft transmissibility of FT in poplar, apple, plum, citrus, and *Eucalyptus* no floral induction was seen in wild-type plants. Long-distance graft transmissibility in woody perennials using a transgenic approach has been achieved in *Citrus, Jatropha* and blue berry (YE *et al.* 2014; SONG *et al.* 2019; SOARES *et al.* 2020). SOARES *et al.* (2020), showed that the CcFT3 homolog could induce early flowering in rootstocks expressing the *CcFT3* coding sequence via the *Arabidopsis SUC2* promoter.

Additionally, Soares *et al.* (2020), demonstrated the graft transmission of CcFT3 from transgenic rootstocks to wildtype scions, which induced early flowering of the wildtype tissue, and produced non-transgenic fruit and seed. Recently it was shown that top grafting of juvenile *Eucalyptus* material onto much older rootstocks (18+ years) resulted in early floral induction in the young scions (De Oliveira Castro *et al.* 2021; Castro *et al.* 2022), implying that the older material is producing florigenic signals, whether it be an *FT* homolog and/or other age-related co-factors, capable of moving across graft junctions and inducing flowering (De Oliveira Castro *et al.* 2021; Castro *et al.* 2022). No molecular work on the *Eucalyptus FT* homolog (EgFT) has been reported.

In this study, we aimed to identify the putative *Eucalyptus* FT homolog using phylogenetic analysis and 3D protein prediction methods and determine whether this protein is involved in floral induction and capable of being transmitted through a graft junction into relatively distant tissue in *Arabidopsis* (Jumper et al. 2021). To track the location and long-distance movement of the putative *Eucalyptus* FT protein, an EgFT-GFP fusion protein was used to facilitate the use of fluorescent confocal microscopy.

2.3 Materials and Methods

2.3.1 Phylogenetic analysis

All homologous protein sequences for the *Arabidopsis thaliana FT* and *FT*-like genes were retrieved from Dicots PLAZA 4.5 (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_5_dicots/) and NCBI (https://www.ncbi.nlm.nih.gov/gene). Amino acid sequences for *Eucalyptus grandis*, *Arabidopsis thaliana*, *Populus trichocarpa*, *Vitis vinifera*, *Citrus clementina* and *Jatropha curcas* were combined into a single FASTA file. These sequences were imported into Mega-X™ (Kumar *et al.* 2018). The sequences were aligned using ClustalW with the following parameters: Gap opening penalty of 15 and a gap extension penalty of 6.66. These were used for pairwise alignment and multiple alignment parameters during the phylogenetic analysis. A protein weight matrix is used as a scoring table to describe the similarity of each amino acid to each other. The aligned sequences were used for phylogenetic analysis using the maximum likelihood algorithm. The default parameters of Mega-X™

were used with a bootstrap value of 1000. The phylogenetic tree produced was visualised in Mega-X™.

2.3.2 Protein structure prediction

The putative protein sequence of the *Eucalyptus* FT was used to predict its protein structure. The known 3D structure of the *Arabidopsis* FT protein was obtained from the RCSB protein data bank (https://www.rcsb.org/). The 3D structure of the putative *Eucalyptus* FT protein was predicted using AlphaFold2 (Jumper *et al.* 2021). The following parameters for AlphaFold2 were used: msa_mode = MMseqs2 (UniRef+Environmental), pair_mode = unpaired+paired, model_type = auto, num_recycles = 3, rank_num = 1 and color = IDDT. The known and predicted 3D structures were then visualized using ChimeraX™ (Pettersen *et al.* 2021). The proteins were superimposed and compared to check for overall similarity (explained in the next section). For the prediction of the *Eucalyptus* FT protein fused to the green fluorescent protein (GFP), the same process was followed as for the putative *Eucalyptus* FT protein.

2.3.3 Protein structure comparisons

To compare the predicted 3D protein structures to the available X-ray crystallography structures, the FATCAT flexible structural comparison algorithm (see section 2.4.2) was used (Ye and Godzik 2004) (https://fatcat.godziklab.org/fatcat/fatcat_pair.html). ChimeraX™ (Pettersen *et al.* 2021), or standard output was used to visualize FATCAT superimposed 3D structural output.

2.3.4 Cloning of EgFT and EgFT-GFP

The *CcFT3* gene (Soares *et al.* 2020) was generously gifted to us from the Manjul lab (University of Florida). The *CcFT3* coding sequence was inserted into a modified pTRANS200HL (similar to pTRANS210 in addGene https://www.addgene.org/91108/) vector, downstream of the *Arabidopsis SUC2* promoter. The *EgFT* coding sequence was synthesized at Macrogen™ (Amsterdam, Netherlands). Primers were designed (Table **S2.1**) to perform an In-Fusion (Takara, Shiga, Japan) reaction to replace the *CcFT3* gene with the *EgFT* gene. No restriction enzyme sites were found to flank the *CcFT3* gene, therefore an inverse PCR was performed to remove it and linearize the modified pTRANS200HL destination vector (Figure S3). The *EgFT* CDS was amplified with suitable overhangs for the In-Fusion (Takara) reaction according to the manufacturer's instructions. A double In-Fusion reaction was also designed to fuse the *GFP* coding sequence (sourced from in-house constructs) with the *EgFT* coding sequence, ultimately replacing the *EgFT* stop codon with a short linker (gly-ser-gly-ser) to the *GFP* coding sequence. The PCR products were resolved using gel electrophoresis (1% agarose gel) and subsequent excision and purification of the desired amplicons. The amplicons were used in the In-Fusion reactions which were subsequently transformed into competent DH5α *E.coli*. The bacteria were grown on kanamycin (50µg/ml) supplemented LB plates (10g Tryptone/L, 5g Yeast extract/L, 10g NaCl/L, 15g Agar/L) to select for successful transformants. Successfully transformed colonies were grown in liquid LB media overnight and plasmid DNA was subsequently extracted using a GeneJET Plasmid miniprep kit (ThermoFisher Scientific, Massachusetts, United States). The plasmid DNA was sent to Macrogen™for Sanger sequencing using the appropriate primers (Table S1).

2.3.5 Agrobacterium transformation and Arabidopsis floral dip

Agrobacterium strain LBA4044 were used for the floral dip experiment. *Agrobacterium* was transformed with sequence confirmed constructs. Chemically competent *Agrobacterium* cells were transformed using heat shock. The cells then spread on YEP agar plates (10g Bacto/L peptone, 5g NaCl/L, 10g Yeast extract/L) supplemented with 50 µg/ml of rifampicin, 50 µg/ml of kanamycin and 50 µg/ml of streptomycin. The plates were incubated at 28°C until colonies appeared. Each positive colony was screened via a colony PCR (Table S2.1). A confirmed colony for each construct was grown in 5 ml of liquid LB medium (10g Tryptone/L, 5g Yeast extract/L, 10g NaCl/L) with rifampicin, kanamycin, and streptomycin all with a final concentration of 50 µg/ml. Flowering *Arabidopsis* plants were used for the floral dip experiment (ZHANG *et al.* 2006b). Plants were grown, and seeds were harvested and screened for the DsRed marker and hygromycin resistance (Figure S2.5). The GeneJET plant genomic DNA purification mini kit (ThermoFisher Sientific, Massachusetts, United States) was used to extract genomic DNA from plant material, using the manufacturer's guidelines.

2.3.6 *Arabidopsis* grafting and fluorescent confocal microscopy

All seeds were surface sterilized and subsequently germinated on MS media. T1 transgenic seeds were plated on hygromycin supplemented MS media. Four to five days after germination, grafting surgery (TURNBULL *et al.* 2002; NOTAGUCHI *et al.* 2009) was performed on the seedlings using a small tweezer and sterile injection needles as a blade. Grafted seedlings were grown for an additional 4-5 days before confocal microscopy. Un-grafted transgenics, un-grafted wildtypes and grafting experiments were used for confocal analysis. Seedlings were placed on a glass microscope slide and covered with a 50% glycerol solution. A cover slip was placed on top of the seedlings and secured to the microscope slide with regular transparent tape prior to confocal microscopy (Zeiss LSM 880 Elyra, AxioOserver). For GFP detection we used an excitation wavelength of 488 nm with a MBS 488 filter, to obtain an emission wavelength window of 495-552 nm. For dsRed detection we used an excitation wavelength of 561 nm with a MBS 488/561 filter, to obtain an emission wavelength window of 560-632 nm.

2.4 Results

2.4.1 Phylogenetic analysis of *Eucalyptus* FT and TFL1 homologs

To determine the most likely FT homolog in *Eucalyptus* we performed a maximum likelihood phylogenetic analysis comprising *Arabidopsis FT* and its close homologs, as well as other species in which successful *FT* graft transmission (Figure 2.1) has been demonstrated. We found that each *Arabidopsis* FT and FT-like homolog clustered into a separate clade consisting of at least one copy from each species. For *Eucalyptus*, each clade only contained one copy of each homolog except for the *TFL1*-like clade in which *Eucalyptus* had two homologous sequences. This may suggest that the original homolog in *Eucalyptus* was duplicated and subsequently sub-functionalised. The branches leading to the MFT and FT clades both had bootstrap support of 100. The common node between the TFL1 and BFT clades only had a bootstrap support of 72, suggesting that these two clades are less diverged than the MFT and FT clades. A second phylogenetic analysis was done including only FT protein sequences and that of the gymnosperm ancestor of *FT* (KLINTENÄS *et al.* 2012; JESUS *et al.* 2021; RODRÍGUEZ-PELAYO *et al.* 2022), to analyse the similarity between the FT proteins in more depth (Figure S2.1). The results resembled Figure 2.1, due to the conserved nature of *FT* protein sequences in angiosperms. The ancient FT-TFL1-like protein sequences of *Picea abies* formed an outgroup. The duplication and subsequent sub-functionalisation of this protein group from gymnosperm ancestors may have given rise to FT and TFL1 like proteins in angiosperms and may be responsible for the flowering reproductive mechanism common to all angiosperms, including crucial components such as FT and FT-like proteins (KLINTENÄS *et al.* 2012; JESUS *et al.* 2021; RODRÍGUEZ-PELAYO *et al.* 2022).

2.4.2 Prediction of 3D structure of Putative *Eucalyptus* FT homolog and GFP fusion protein

To determine whether the similarity between the putative *Eucalyptus* FT homolog (from the above phylogenetic analysis) and the *Arabidopsis* FT protein of known function is significant, the 3D protein structure was predicted and subsequently compared. The *Arabidopsis* FT 3D protein (Protein ID 1WKP), known from previous X-ray crystallography was used as a reference as the FT protein structure and function is conserved across angiosperm lineages. The predicted protein structures produced by AlphaFold2 were also superimposed using ChimeraX™ (PETTERSEN *et al.* 2021) to compare similarity (Figure 2.2). The predicted 3D protein structures were compared to the known *Arabidopsis* 3D structures using FATCAT (YE AND GODZIK 2004). The p-value (Table 2.1) is calculated based on the FATCAT similarity score between two unrelated structures following the extreme value distribution (an internal calculation used by the FATCAT algorithm, LI *et al.* (2020)). The FATCAT similarity score itself is made up of the FATCAT chaining score, the root mean square deviation (RMSD) of the superimposed structures, the number of equivalent positions in the protein alignment and number of allowed twists in the 3D structure (0 twists for all calculations in this experiment). The FATCAT similarity score is calculated as:

$$S = cs \times \sqrt{\frac{L}{RMSD(N)}}$$

Where cs is the FATCAT chaining score, L is the number of equivalent positions in the alignment, RMSD is the overall RMSD between the two superimposed structures and N is the number of blocks in the alignment. The p-value of s is then calculated as:

$$P(X > s) = 1 - exp\left(- exp\left(\frac{s - \mu}{\lambda}\right)\right)$$

Other important outputs of the FATCAT algorithm such as the graph of the FATCAT chaining results, superimposed FATCAT protein structure files, transformation matrices of alignment blocks and differential distance matrix decomposition maps can be found in Figure S2.2.

The *Citrus* FT1 and the *Citrus* FT3 were compared due to the observation that only the *Citrus* FT3 protein induced early flowering and was graft transmissible (SOARES *et al.* 2020). Based on the FATCAT analysis we found that the *Eucalyptus* and *Citrus* FT1 and FT3 proteins were significantly similar to the *Arabidopsis* FT protein with p values < 0.05 (approximately 0). However, based on the RMSD values, the *Citrus* FT1 protein has slightly higher similarity to the *Arabidopsis* FT than the *Citrus* FT3. When comparing the *Eucalyptus* and *Citrus* FT proteins to each other, we found that they were also highly similar with a p value of < 0.05 (approximately 0). Again, like the *Arabidopsis* comparison, *Citrus* FT 1 was slightly more similar to *Eucalyptus* FT than the *Citrus* FT 3 protein, based on RMSD values. We compared the known 3D *Arabidopsis* FT protein structure (determined from X-ray crystallography, AHN *et al.* (2006)) to itself (also the X-ray derived 3D structure) for a reference to a “perfect alignment/similarity”. We also compared other related proteins from the PEBP protein family, to explore a wider range of similarities given that the FT and FT-like proteins are so similar to each other.

The PEBP protein family is an umbrella classification of proteins which bind to phosphatidylethanolamine. FT and FT-like proteins fall into this family (BERNIER *et al.* 1986) .

We also compared the *Arabidopsis* FT, known to induce flowering, with the *Arabidopsis* TFL1 protein, known to inhibit floral induction. AtFT and AtTFL1 are significantly similar to each other, even though they have opposite effects on floral induction. Interestingly, CcFT3 is more similar to AtTFL1 than it is to AtFT, even though it has been shown that CcFT3 acts as a floral inducer (SOARES *et al.* 2020). However, we also know that due to the high homology between AtFT and AtTFL1, even one targeted base change can convert a AtFT floral inducer into a TFL1-like floral repressor (HANZAWA *et al.* 2005; HO AND WEIGEL 2014). We found that based on this analysis the putative *Eucalyptus* FT protein is actually slightly more similar to the *Citrus* FT1 protein than the *Citrus* FT3 protein based on RMSD scores, even though statistically they are both significantly similar. We also include comparisons with the gymnosperm FTL1 and FTL2 proteins from spruce (hypothesized to have evolved from the same common ancestor of FT and FT-like proteins in gymnosperms and angiosperms) (KLINTENÄS *et al.* 2012). We found that none of the FT proteins of interest (EgFT, AtFT, CcFT1 and CcFT3) were significantly similar to either SpFTL1 or SpFTL2 (Table 2.1). As stated previously, it was shown that even single amino acid changes can result in the change of the FT protein from acting as a floral inducer to a floral suppressor, for example mutation of Tyr-85, Tyr-134, Trp-138, Gln-140 or Asn-152 is sufficient to convert a FT floral inducer into a TFL1 mimic which represses flowering (HANZAWA *et al.* 2005; HO AND WEIGEL 2014) making it difficult to predict the precise biological functions of these closely related proteins, even with accurate 3D protein structural predictions. This analysis combined with transcriptional data from VINING *et al.* (2015), suggest that EgFT is the most likely *Eucalyptus* FT homolog involved in the floral induction pathway. Functional studies of the putative *Eucalyptus* FT protein are needed to confirm its role in the flowering pathway. We also used the 3D protein prediction to assess whether the addition of a relatively large GFP protein to the EgFT protein would cause any obvious steric hindrance (Figure 2.2E). This bioinformatics analysis showed that there is only one *Eucalyptus* FT protein which groups with the functional FT homologs of other species. We also showed that the 3D structures of all of the FT homologs did not differ significantly except for gymnosperm FT/TFL1-like proteins and other distantly related PEBP proteins. Ultimately, these results pointed to EgFT as the most likely *Eucalyptus* FT protein.

2.4.3 Cloning of the *Eucalyptus* FT coding sequence and the *Eucalyptus* FT-GFP fusion protein

The destination vector used was a pTRANS 200 modified plasmid, that was received from collaborators at Oregon State University (OSU), which already contained the *CcFT3* coding gene kindly provided by the Manjul lab (SOARES *et al.* 2020). This destination vector also contained the hygromycin resistance gene and DsRed gene as selection markers (Figure S2.3). The CDS of the predicted *Eucalyptus FT* was synthesised at Integrated DNA Technologies (https://eu.idtdna.com/pages). Subsequently, custom designed primers were used to prepare DNA fragments for In-Fusion cloning (Takara Bio). A plant optimised GFP (sGFP S65T, emission maxima of 510 nm, HEIM *et al.* (1995)) subcloned from (HBT95::sGFP(S65T)-NOS , CHIU *et al.* (1996)) was used to create the *EgFT-GFP* fusion. PCR amplicons were subjected to agarose gel electrophoresis to confirm the expected sizes (Figure S2.4). After PCR, two separate In-Fusion reactions were set up to create pTRANS 200 destination vectors containing the *Eucalyptus FT* coding sequence and the *Eucalyptus FT-GFP* fusion coding sequence (Figure S2.5). The reaction was transformed into competent *E.coli DH5α* cells which yielded positive colonies on kanamycin selective media. These colonies were grown in liquid LB media and plasmids were extracted using a GeneJET Plasmid miniprep kit (ThermoFisher Scientific, Massachusetts, United States) and sent for sequencing at Macrogen (Europe). All sequencing results can be found in the supplementary materials (Figure S2.6). We found that no PCR mutations were induced in any of the cloned coding sequences, and that the cloned sequences were identical to the reference sequences.

2.4.4 *Arabidopsis* transformation, isolation of transgenic seed and phenotypic data collection

Agrobacterium LBA4404 was transformed with the sequence confirmed constructs mentioned above. Colonies which grew on selective media were then confirmed with PCR and subsequently visualized by gel electrophoresis (Figure S2.7). All colony PCRs produced bands of expected sizes. *Arabidopsis* plants that were flowering, were dipped into bacterial solutions established from the confirmed colonies. Subsequently, seeds were harvested and screened for dsRed expression (Figure 2.3).

The seeds that were fluorescing were separated and germinated on MS media supplemented with hygromycin to ensure that true positive transgenic seeds were selected (Figure 2.4), all germination and subsequent plant growth was done under short day conditions in order to identify plants that flowered early due to the ectopic expression of CcFT3 or EgFT. After 7 to 10 days on MS media, positive transgenic plants were moved to soil. Each construct's data was combined to determine statistical significance using the mann-whitney-wilcoxon test two-sided test (Figure 2.5). We found that when each transgenic line was grouped by construct, they flowered significantly earlier than the wildtype plants (Figure 2.5A). There were also significantly fewer leaves on average at each rosette compared to wildtype rosettes (Figure 2.5B). Interestingly, three of the four dsRed empty vector lines produced a single inflorescence, with no viable flowers, earlier than the wild type plants, which is most likely due to stress on the plants due to hygromycin selection and transplantation into soil after being germinated on MS media. To confirm that the EgFT, EgFT-GFP and CcFT3 lines were not flowering due to hygromycin stress, we selected transgenic seeds from the second (T2) generation based on dsRed fluorescence alone and grown under short day lengths to test for early induction of flowering time by the transgenes (Figure 2.6). DNA was extracted from each line (Figure S2.8), and subsequently used as template for confirmatory PCR (Figure 2.7). Extra PCRs were conducted on DNA extracted from dsRed empty vector lines to confirm that they did not contain the FT transgenes. We only observed amplification products (Figure 2.7). This suggests that no cross contamination occurred during floral dipping of the *Arabidopsis* plants.

The phenotypes of transgenic and wildtype plants were striking, with transgenic lines flowering when the rosettes were still small, and no sign of flowering in the wildtype plants (Figure 2.8A). At the time of wildtype bolting and flowering the transgenics had already produced seeds. The wildtype plants had large rosettes with many leaves (Figure 2.8B) as is expected for wildtype *Arabidopsis* plants grown under short day conditions. In contrast, at the same time, the transgenics had small rosettes with few leaves, but with large, bushy inflorescences (Figure 2.8B). Due to the bushy nature of the transgenics, we counted the number of branch points on the inflorescence and measured the length of the longest/main inflorescence at a single timepoint. There was a negative correlation between the number of branch points and the length of the main inflorescence (Figure S2.9). This is most likely due to the high amount of FT being produced influencing the plant to produce many secondary inflorescences, rather than, a single large inflorescence which terminated in a clump of flowers like in the wildtype plants. Using the T2 generation of the EgFT-GFP lines we investigated the localisation of EgFT-GFP protein in *Arabidopsis* plants. We found that the EgFT-GFP protein was mainly localised in the *Arabidopsis* vasculature and cells adjacent to the vasculature, most likely phloem companion cells due to our EgFT-GFP coding sequence being driven by the *Arabidopsis* SUC2 promoter (Figure 2.9).

To investigate the number of insertions of the construct during *Agrobacterium* mediated transformation, we analysed the progeny of each line, counting the number of seeds per silique which were showing dsRed fluorescence and those which had none (wildtype). We found that the ratio of dsRed to wildtype seed per silique varied from 90% transgenic to 50% transgenic (Figure S2.10A). Siliques from transgenic lines varied in size, some were similar to the typical large siliques of wildtype plants whereas others were small with few seeds. However, when totalling the seeds from all siliques for each transgenic line we found that the ratio was approximately 3:1 (dsRed : Wildtype), which is representative of the classic Mendelian inheritance ratio obtained when investigating a single locus (Figure S2.10B).

We also investigated whether the EgFT-GFP fusion protein and thus EgFT itself is capable of long-distance graft transmissible movement. To investigate this, we grafted EgFT-GFP expressing *Arabidopsis* lines onto wildtype *Arabidopsis*. In total, 120 butt grafts were performed (including reciprocal grafts), of which only six were successful (Figure 2.10). Other literature reported a 30%- 50%

graft success rate when performed by experienced micro-grafters, whereas we only achieved around a 5% success rate (TURNBULL *et al.* 2002; NOTAGUCHI *et al.* 2009).These successful grafts were then analysed under confocal microscopy to determine whether EgFT-GFP was moving through the graft junction, into the wild-type tissue (Figure 2.10). Based on the green fluorescence observed beyond the graft junction, we conclude that the EgFT-GFP fusion protein moved across the graft junction, but the dsRed was not observed beyond the graft. Weak GFP signal was also found in the vasculature of the root of wildtype rootstock, which was grafted to a plant expressing the EgFT-GFP construct, (Figure 2.10B), suggesting the long-distance movement of EgFT-GFP via the plant's vasculature.

2.5 Discussion

One of the major bottlenecks in conventional forest tree breeding is their long generation times. This is also a bottleneck for genome-assisted breeding, since the time from seed-to-seed imposes a biological constraint to realizing the genetic gain from approaches such as genomic selection. Some of the earliest flowering *Eucalyptus* tree species such as *Eucalyptus grandis* and *E.urophylla* take at least five years to flower. While later flowering species such as *Eucalyptus dunnii* and *E. nitens* take up to 10 years to flower. Thus, breeders have to wait 5 to 10 years before they can make crosses and then (a year or more later) establish the next generation of breeding trials. Approaches to induce early flowering such as top-grafting onto mature plants ((DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022), or chemical treatment (e.g. Paclobutrazol,(GRIFFIN *et al.* 1993; MONCUR AND HASAN 1994)) have great potential to accelerate breeding cycles and the rate of genetic gain over time. Similarly, approaches that target floral induction genes hold great promise to accelerate tree breeding (ZHANG *et al.* 2010).

In this study we investigate the *Eucalyptus* FT proteins' involvement in floral induction and its ability to move through a graft junction in a heterologous system. We predicted the most likely *Eucalyptus* FT protein using phylogenetic analysis and 3D protein prediction and comparisons. We also functionally test the ability of the putative *Eucalyptus* FT to induce flowering as well as its graft transmissible potential using a GFP tagged *Eucalyptus* FT protein. Our phylogenetic analysis revealed that there is only one likely FT homolog in *Eucalyptus* (Figure 2.1). The amino acid sequence of the *Eucalyptus* FT clustered with those of the main FT proteins from other species. All the angiosperm FT proteins grouped into a single clade which is understandable given the highly conserved nature of the FT protein among angiosperms. Three other distinct clades were also seen, representing TFL1, BFT, and MFT like proteins. These results are in agreement with a previous study on the floral transcriptome of *Eucalyptus* (VINING *et al.* 2015). When focusing only on the FT and FT-like proteins (Figure S2.1), we found that the *Eucalyptus* FT is related to all the other species' FT proteins , one of which is the *Jatropha* FT protein, which has been shown to be able to induce flowering in a graft transmissible fashion (YE *et al.* 2014). It also seems that the *Citrus* FT3 protein diverged from the *Citrus* FT1 and FT2 proteins which are at the same locus (SOARES *et al.* 2020). This is interesting as only the *Citrus* FT3 protein was shown to induce early flowering via graft transmission in *Citrus* (SOARES et al. 2020). This suggests that the *Citrus FT3* gene could have sub-functionalised to tackle on a more specific role in long-distance transport and floral induction, or that it retained the ancestral state, while the *Citrus FT1* and *Citrus FT2* lost this function. *Eucalyptus* only has one copy of the putative *FT* gene which predicts that it will be graft-transmissible since floral induction via top-grafting has been demonstrated in *Eucalyptus* (DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022).

We used AlphaFold2 to predict the 3D structure of the putative *Eucalyptus* FT protein and then subsequently compared the prediction with other proteins of interest (Table 1.1) (JUMPER *et al.* 2021). First, we found that the 3D structures of all of the proteins closely related to AtFT (Figure 2.1) were significantly similar to that of the putative *Eucalyptus* FT protein. This is due to the highly conserved nature of FT and FT-like proteins throughout the angiosperm lineage. Interestingly, the AtTFL1 protein structure was significantly similar to AtFT and EgFT, 77.91% and 73.86% similarity respectively(Table 2.1), even though TFL1 proteins act antagonistically to floral initiation (HANANO AND GOTO 2011). However, some studies have shown that TFL1 and FT in *Arabidopsis* is so similar that even single codon changes can result in FT acting like a TFL1 protein and thereby suppress floral initiation (HANZAWA *et al.* 2005; HO AND WEIGEL 2014). Even though the EgFT is statistically similar to AtFT and AtTFL1, the

RMSD values differ by 0.45Å (Table 2.1). This shows that there is a difference between the proteins even if they are small, however this small difference may be the difference between floral induction and floral repression, just as was reported for the conversion of AtFT to a AtTFL1-like protein (HANZAWA *et al.* 2005; HO AND WEIGEL 2014). Similarly, we found that the CcFT1 and CcFT3 are significantly similar to EgFT. Again, there is still a difference in the RMSD values, and based on this EgFT is actually more similar to CcFT1 than CcFT3 (Table 2.1). This supports the hypothesis that CcFT1 and CcFT2 have taken on a more specific role (while the CcFT3 protein has kept the more conserved role i.e. CcFT3 is more ancestral) in its evolutionary history while EgFT retained a more general function, similar to CcFT3. The *Arabidopsis, Eucalyptus* and *Citrus* proteins were all significantly different from the gymnosperm Spruce FTL proteins, which is hypothesised to share an ancestor with the angiosperms FT and FT like protein precursors (KLINTENÄS *et al.* 2012). Thus, our bioinformatic analysis showed that the putative *Eucalyptus* FT protein is structurally significantly similar to FT proteins with confirmed function, although it remained to be functionally tested.

To functionally test the putative *Eucalyptus* FT protein, we cloned the coding sequence alone, as well as the coding sequence fused via a linker to GFP. After *Agrobacterium* mediated floral dip transformation of *Arabidopsis,* we screened transgenic events firstly via dsRed fluorescence (Figure 2.3), which is a selection marker incorporated into the pTRANS200 T-DNA, and secondly via germination on hygromycin supplemented MS media (Figure 2.4). Germination and growth of all plants were conducted under short day conditions to prevent the activation of the endogenous *Arabidopsis FT* gene. The EgFT, CcFT3 and EgFT-GFP lines all flowered significantly earlier than the wildtype plants. EgFT-GFP lines flowered somewhat later (4 weeks vs 3 weeks) than the EgFT and CcFT3 lines, which may be a result of the GFP fusion. The increase in size of the FT fusion protein caused by the fusion was also shown in other studies to delay flowering time slightly compared to the FT protein alone (CORBESIER *et al.* 2007; NOTAGUCHI *et al.* 2008). This may be linked to plasmodesmata, connecting phloem companion cells and sieve tube elements, which limit the size of molecules which can freely diffuse up to 70 kDa (OPARKA AND TURGEON 1999). FT with a size of 20 kDa has been shown to readily diffuse as well as FT-GFP (27kDa), but at a slower rate, explaining the slightly longer time to induce flowering (CORBESIER *et al.* 2007; YOO *et al.* 2013a). The EgFT-GFP fusion protein may also be less active than the EgFT alone due to a small amount of steric hindrance. Additionally, the overall shape may prevent efficient transport of the EgFT-GFP fusion protein (YOO *et al.* 2013a).

We found that the EgFT and CcFT3 expressing lines flowered within three weeks after germination, when grown under short-day conditions. Similar observations were made when other *FT* genes were expressed via the SUC2 promoter including when ectopically expressing the *Citrus FT3* in *Arabidopsis* (AN *et al.* 2004; ABE *et al.* 2005; CORBESIER *et al.* 2007; SOARES *et al.* 2020). We found that constitutive expression of the *Eucalyptus FT* and *Citrus FT3* via the *SUC2* promoter produced shorter but highly branched inflorescences (Figure 2.8 B). The EgFT-GFP expressing plants produced longer, less branched inflorescences, which was likely a consequence of the decreased activity and/or transport due to the larger size of the fusion protein as discussed above. We found that in the T1 generation, some of the empty vector dsRed expressing plants flowered earlier than the wildtype controls. To exclude the possibility that some of these plants could have been transformed with the incorrect vector, we confirmed the absence of the EgFT and CcFT3 gene via PCR (Figure 2.7). The early flowering of these lines may be explained either by stress-induced flowering due to the hygromycin selection, or that the T-DNA from the *Agrobacterium*-mediated transformation integrated into a floral repressor, although the latter is highly unlikely due the fact that we observed this phenotype in multiple independent lines. The inflorescences of the empty vector controls were not highly branched and looked similar to that of the wildtype plants.

We investigated the localisation and graft transmissibility of the *Eucalyptus* FT protein by using the EgFT-GFP fusion protein. We found that the EgFT-GFP localised around and within the plant vasculature. This is in agreement with the expression pattern driven by the SUC2 promoter, it is phloem companion cell-specific and the EgFT confers phloem-specific transport (TRUERNIT AND SAUER 1995; STADLER AND SAUER 1996; IMLAU *et al.* 1999; CHEN *et al.* 2018). It is also evident, especially from confocal analysis of hypocotyl tissue (Figure 2.9 EFGB01), that the 35S driven dsRed marker is expressed in more tissues than the EgFT-GFP which only shows expression where the plant vasculature is located. To evaluate the long-distance graft transmissible movement of EgFT-GFP, we grafted AtSUC2::EgFT-GFP *Arabidopsis* onto wildtype *Arabidopsis* and found that were was GFP signal in the transgenic tissue as well as in wildtype tissue by the graft junction as well as in the roots of wildtype tissue (Figure 2.10 A). This was also seen in other studies which used GFP tagged FT proteins to evaluate long distance FT movement (CORBESIER *et al.* 2007; NOTAGUCHI *et al.* 2008; YOO *et al.* 2013a; ZHU *et al.* 2016). Interestingly, dsRed was only found in transgenic tissue and did not move across the graft junction (Figure 2.10). However, these results suggest that the EgFT protein is capable of long-distance transport (in *Arabidopsis* at least). Two recent studies conducted top grafting experiments on *Eucalyptus*, which showed that younger tissue does start flowering when grafted onto mature trees (DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022). Together, our results suggest that the EgFT protein that we tested may be a large part of the florigenic signal which crosses these graft junctions and subsequently induces flowering in the younger tissue.

2.6 Conclusion

In this study we identified the putative *Eucalyptus* FT protein using phylogenetics and 3D protein prediction. The putative *Eucalyptus* FT coding sequence was then cloned downstream of the *Arabidopsis* SUC2 promoter and subsequently used to transform *Arabidopsis* via *Agrobacterium* mediated floral dip. We then showed that the *Eucalyptus* FT shortened the time to flowering of the *Arabidopsis* growing under short day conditions. This is comparable to the effect that the *Citrus* FT3 protein had on flowering time. Using a GFP fusion protein with the *Eucalyptus* FT protein, we showed that the *Eucalyptus* FT protein (EgFT-GFP) is localised to the plant vasculature. We also show that the EgFT-GFP has long-distance graft transmissible movement in *Arabidopsis*. The results we show suggest that the *Eucalyptus FT* that we identified and functionally tested may play a large role in the florigenic signal in *Eucalyptus*. It may also be the signal that is causing younger material to flower in top grafting experiments that was discussed previously. It may also be the protein needed in the future to enable early flowering of *Eucalyptus* in the lab/greenhouse environment.

2.7 Figures and tables

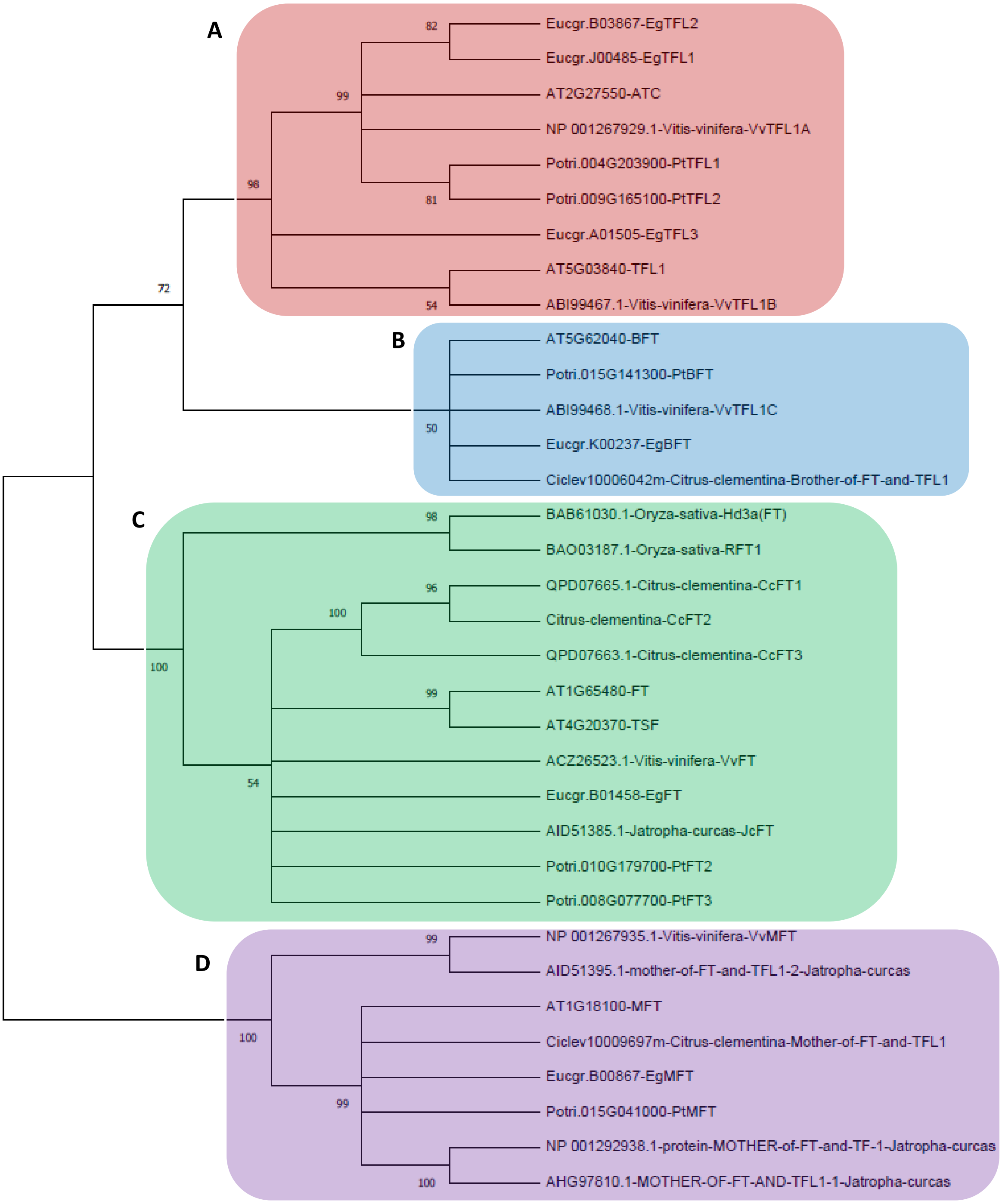

Figure 2. 1 Phylogenetic analysis of *Eucalyptus FT homologs*. Phylogram of the phylogenetic analysis of *FT* and *FT* homolog protein sequences. A) *TFL1* clade, B) *BFT* clade, C) *FT* clade, D) *MFT* clade.

Eucalyptus has a single copy of each corresponding *Arabidopsis* homolog, except in the case of the *TFL1* clade which has two *Eucalyptus* homologs. The maximum likelihood tree was constructed using MegaX (KUMAR *et al.* 2018). Bootstrap values from 1000 replicates were used to assess support for the clustering. Clades with low bootstrap support were collapsed.

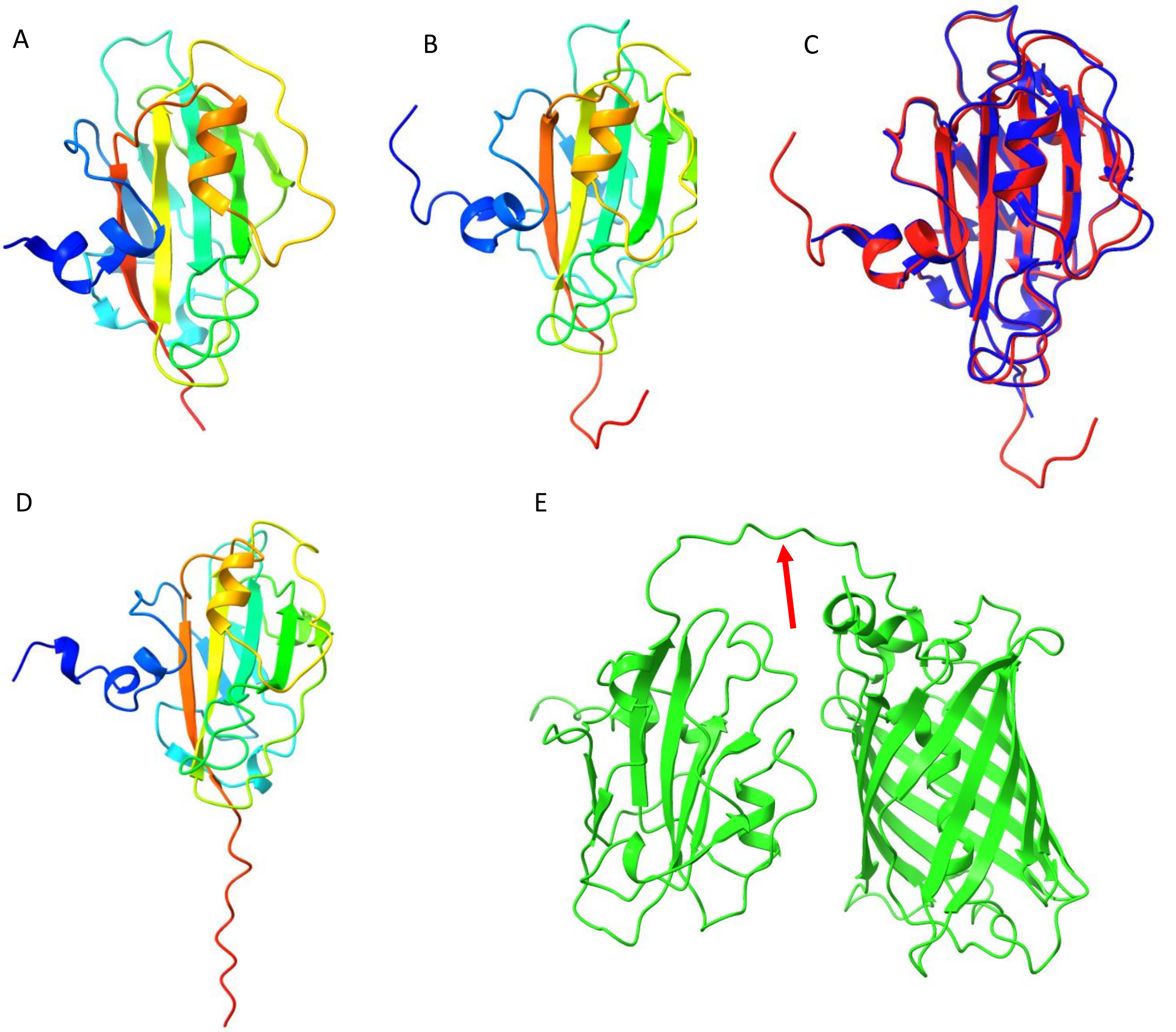

Figure 2. 2 Overlay of 3D protein predictions. The predicted ribbon 3D structures of A) *Arabidopsis* FT, B) putative *Eucalyptus* FT homolog (EgFT), C) superimposed images of A and B, the *Eucalyptus* FT is highlighted in red, and the *Arabidopsis* FT is highlighted in blue. D) Predicted ribbon 3D structure of the *Citrus* FT3 protein and E) *Eucalyptus* FT protein fused with the GFP protein via a short linker (red arrow). These images were produced using ChimeraX version 1.4 (PETTERSEN *et al.* 2021).

Table 2. 1 Protein similarity comparison based on FATCAT algorithm

Comparison	Number of Equivalent positions	P-Value	RMSD (Å)	Raw FATCAT score	Significantly similar
AtFT vs AtFT	163	0	0.00	480.00	Y
AtFT vs AtTFL1	163	~0	0.69	473.52	Y
AtFT vs EgFT	163	~0	0.69	473.52	Y
AtFT vs CcFT3	177	~0	1.41	503.40	Y
AtFT vs CcFT1	174	~0	0.43	503.17	Y
EgFT vs CcFT3	174	~0	0.43	503.17	Y
EgFT vs CcFT1	170	~0	0.36	503.05	Y
AtTFL1 vs EgFT	163	~0	1.14	448.14	Y
AtTFL1 vs CcFT3	174	~0	0.43	503.17	Y
AtTFL1 vs CcFT1	170	~0	0.36	503.05	Y
AtFT vs VFT	163	~0	0.66	473.58	Y
AtFT vs JcFT	163	~0	0.63	474.07	Y
AtFT vs CcFT1	163	~0	0.65	473.88	Y
AtFT vs PotFT1	163	~0	0.66	473.08	Y
AtFT vs PotFT2	163	~0	0.67	472.68	Y
AtFT vs RFT1	163	~0	0.82	465.25	Y
AtFT vs HD3A	163	~0	0.83	465.50	Y
AtFT vs TSF	163	~0	0.66	472.27	Y
AtFT vs SpFTL1	34	7.21e-01	3.29	47.32	N
AtFT vs SpFTL2	84	8.63e-01	3.31	47.08	N
EgFT vs SpFTL1	58	9.19e-01	4.43	49.48	N
EgFT vs SpFTL2	66	8.60e-01	2.88	51.34	N
CcFT3 vs SpFTL1	58	9.22e-01	4.43	48.94	N
CcFT3 vs SpFTL2	66	8.62e-01	2.87	51.30	N
CcFT1 vs SpFTL1	58	9.25e-01	4.49	48.64	N
CcFT1 vs SpFTL2	77	8.99e-01	3.94	50.33	N
CcFT3 vs CcFT1	177	~0	1.41	503.40	Y
CcFT1 vs EgFT	170	~0	0.36	503.05	Y
AtFT vs AtFRL5	18	9.99e-01	3.44	36.90	N
AtFT vs AtBFT	162	~0	0.76	449.28	Y
AtFT vs AtPCS1	71	9.39e-01	3.81	64.40	N
AtFT vs EUGRSUZ_A00595	124	1.63e-05	3.11	160.13	Y
EgFT vs EUGRSUZ_A00595	122	2.52e-05	3.05	159.67	Y
CcFT3 vs EUGRSUZ_A00595	122	2.50e-05	3.04	159.66	Y
CcFT1 vs EUGRSUZ_A00595	122	2.68e-05	3.05	159.12	Y

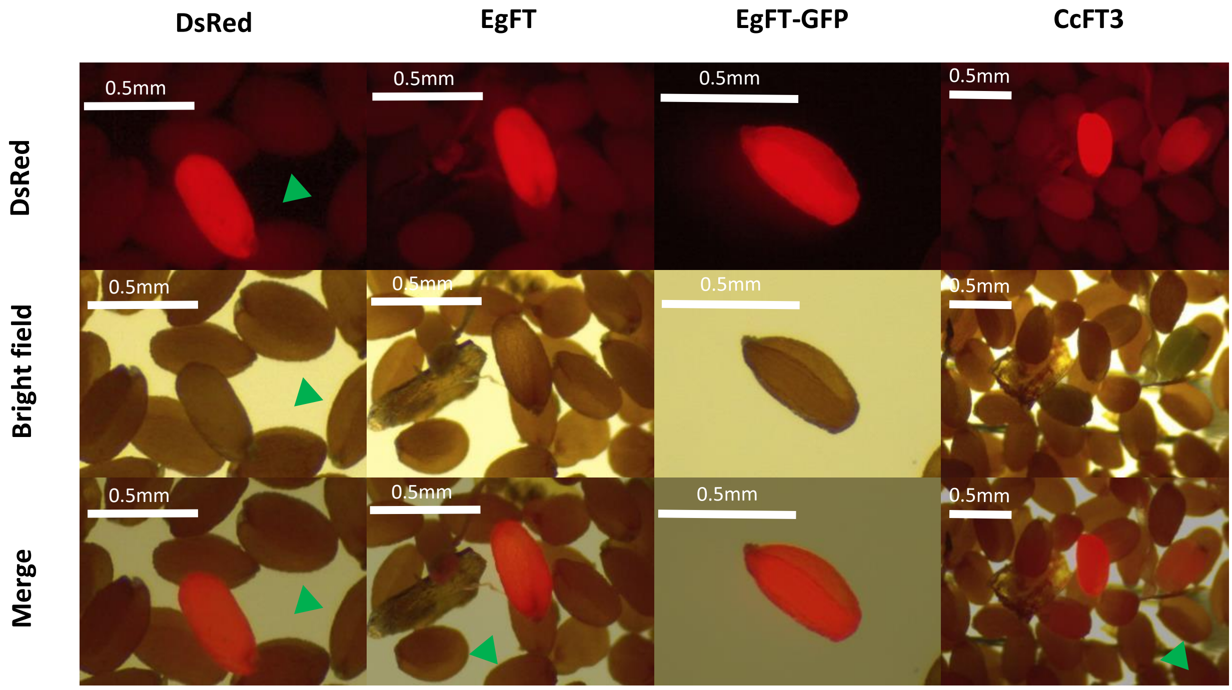

Figure 2. 3 T1 generation seeds screened for DsRed expresssion. Seeds collected from transformed plants were visualised under a light source emitting light at a wavelength of approximately 558 nm. The seeds were viewed under a bright field microscope with a filter only allowing a small range of wavelengths to pass through, including 583 nm, the emission maximum of the DsRed fluorescent protein. Green arrows indicate wildtype seeds with no sign of dsRed fluorescence. Merge is a overlap of the DsRed and bright field views. Images were taken using a Leica EZ4 W dissection microscope.

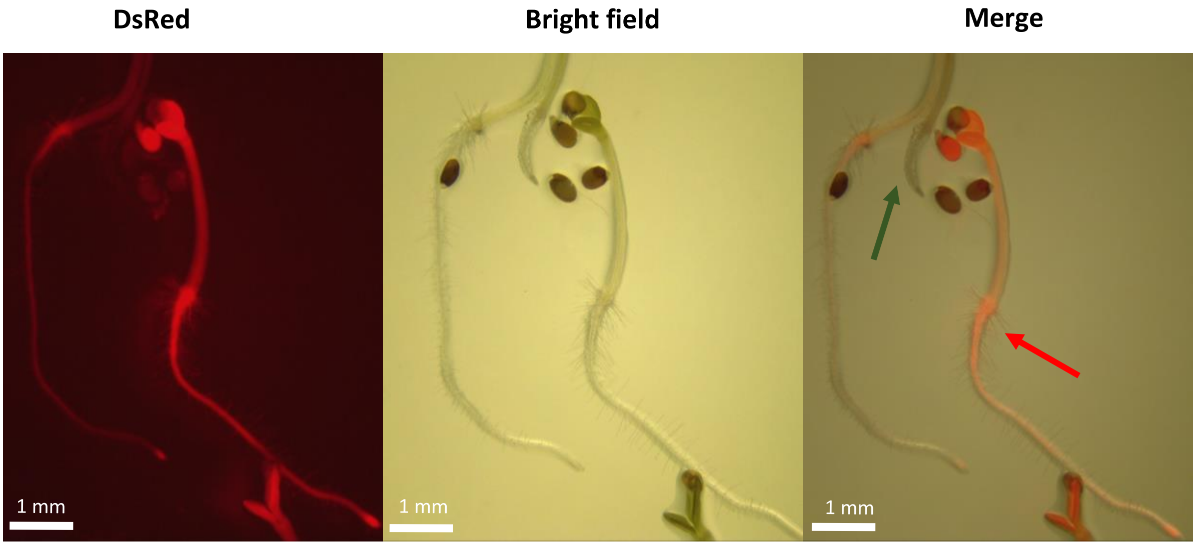

Figure 2. 4 Germination of transgenic seeds on hygromycin supplemented MS media. Transgenic seedlings show strong DsRed fluorescence. True transgenic lines develop root hairs in the presence of hygromycin while wild type plants do not produce root hairs and eventually die off. Red arrow

indicates transgenic seedling with root hairs. Green arrow shows a wildtype plant without root hairs and stunted growth in presence of hygromycin. Merge is a overlap of the DsRed and bright field views. Images were taken using a Leica EZ4 W dissection microscope.

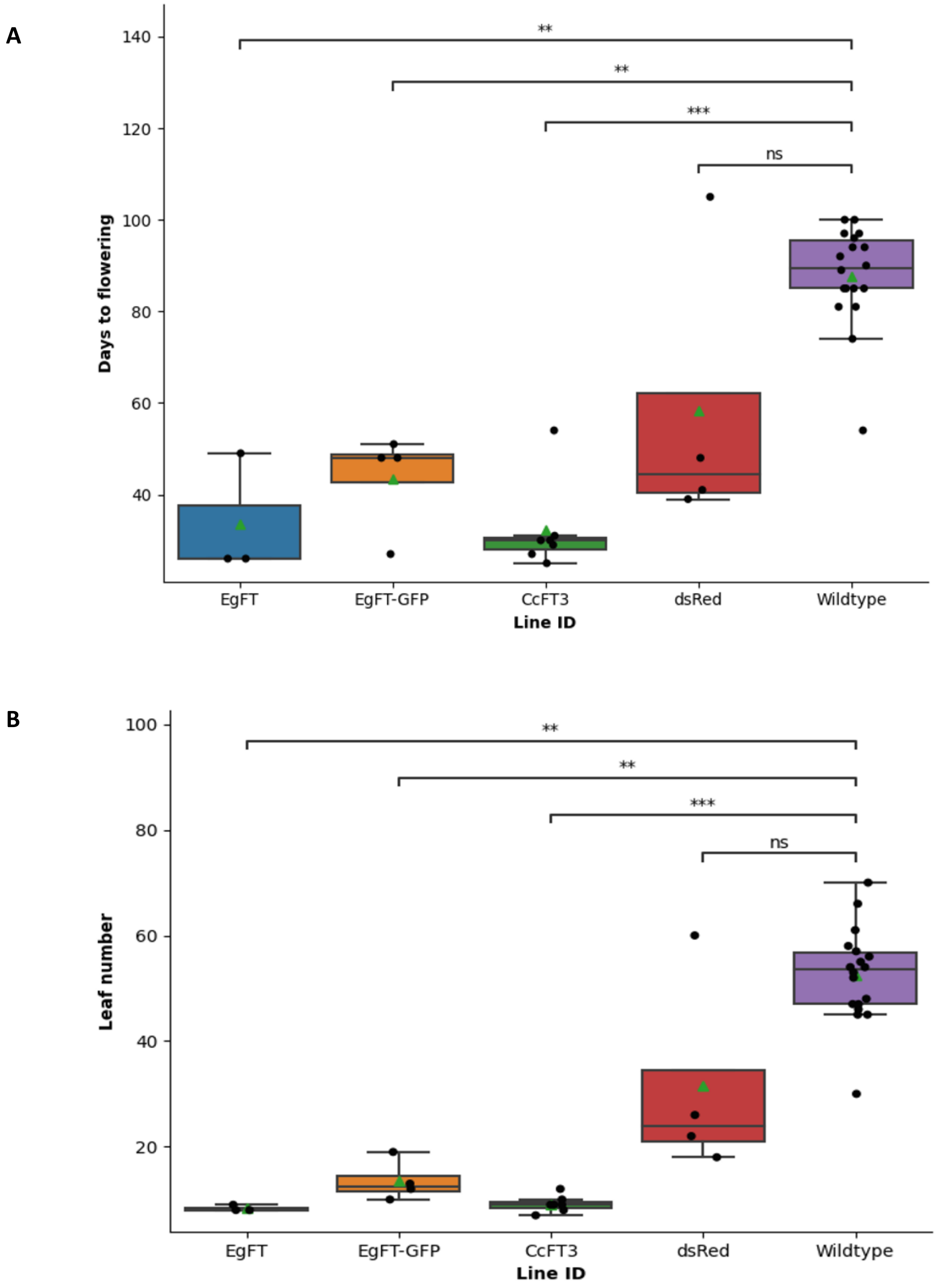

Figure 2. 5 Effects of FT constructs on flowering time in *A. thaliana*. A) Days to flowering for transgenic and wildtype lines. A two-sided Mann-Whitney-Wilcoxon test to determine the difference in means of flowering times between the transgenic lines and the wildtype plants was conducted. All the transgenic lines flowered significantly earlier than the wildtype plants except for the dsRed empty vector lines. The test statistic and p values can be found in table S2.2. . B) Number of rosette leaves at time of flowering. A two-sided Mann-Whitney-Wilcoxon test to determine the difference in means of rosette leaves between the transgenic lines and the wildtype plants was conducted. All the transgenic lines had significantly less leaves than the wildtype plants except for the dsRed empty vector lines. The test statistic and p values can be found in table S2.2. A minimum of 3 biological replicates were used for each construct. The box shows the quartiles of the dataset while the whiskers extend to show the rest of the distribution. Green triangles indicate averages. p-values indicated as ns not significant, * p <= 5.00e-02, ** p <= 1.00e-02, *** p <= 1.00e-03, **** p <= 1.00e-04.

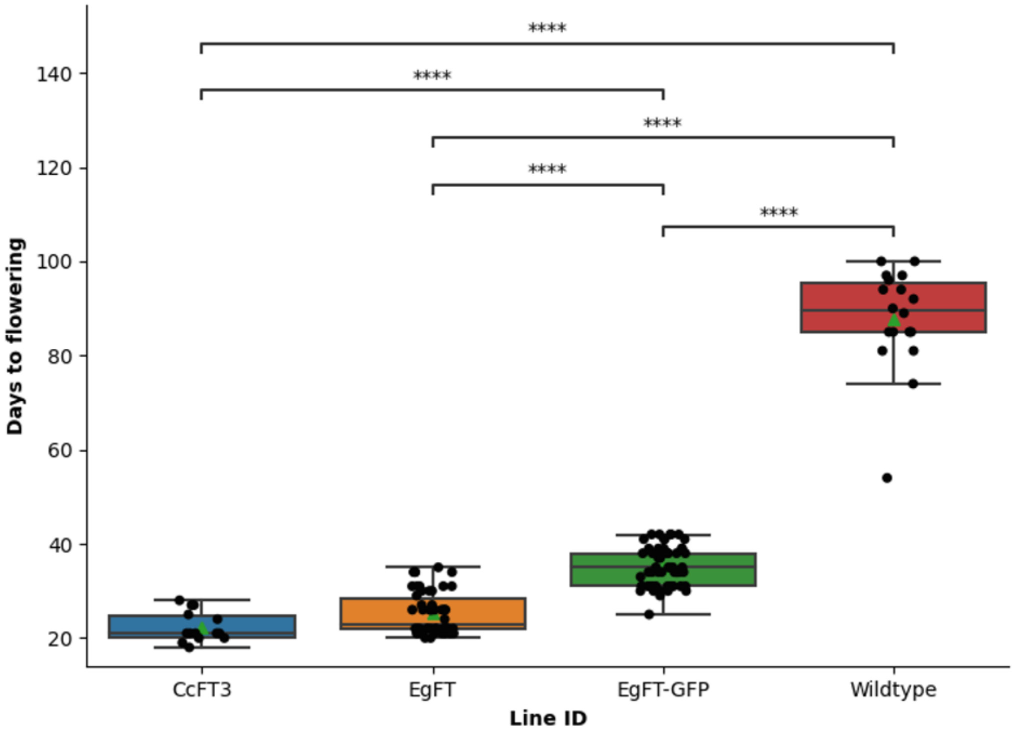

Figure 2. 6 Flowering time of transgenic progeny. A Mann-Whitney-Wilcoxon test two-sided was conducted for each pair of constructs. All transgenic lines flowered significantly earlier than the wildtype plants. Both the CcFT3 and EgFT expressing plants flowered significantly earlier than the EgFT-GFP expressing plants. The test statistic and p values can be found in Table S2.3. A minimum of 20 biological replicates were used for each transgenic construct and wildtype plants. The box shows the quartiles of the dataset while the whiskers extend to show the rest of the distribution. Green dots indicate averages (n=20). p-values indicated as ns not significant, * p <= 5.00e-02, ** p <= 1.00e-02, *** p <= 1.00e-03, **** p <= 1.00e-04.

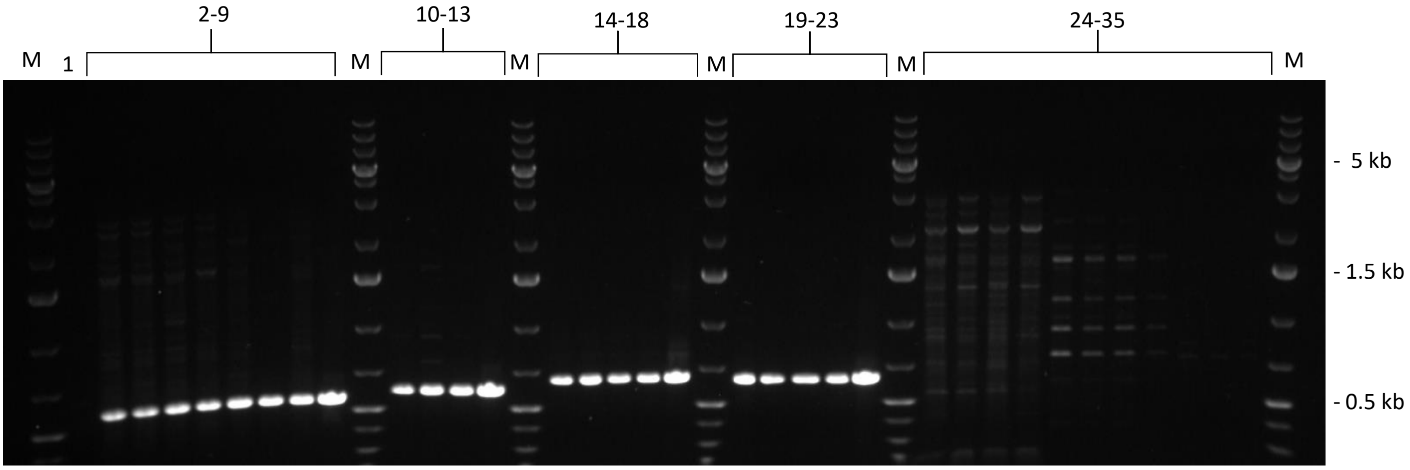

Figure 2. 7 PCR confirmation of transgenic constructs. M = 1 kb plus DNA molecular weight marker (ThermoFisher Scientific, Massachusetts, United States). 1 = Wild type genomic DNA amplified with DsRed confirmatory primers (Table S2.1). 2-8 = *Citrus FT3* lines' genomic DNA PCR with *CcFT3* confirmatory primers. 9 = pTRANS200 containing *CcFT3* amplified with *CcFT3* confirmatory primers (Table S2.1). 10-12 = *Eucalyptus FT* lines amplified with *EgFT* confirmatory primers (Table S2.1). 13 = pTRANS200 containing *EgFT* amplified with *EgFT* confirmatory primers (Table S2.1). 14-17 = *EgFT-GFP* lines amplified with *EgFT-GFP* confirmatory primers (Table S2.1). 18 = pTRANS200 containing *EgFT-GFP* amplified with *EgFT-GFP* confirmatory primers (Table S2.1). 19-22 = *DsRed* lines amplified with *DsRed* confirmatory primers (Table S2.1). 23 = pTRANS200 containing *DsRed* amplified with *DsRed* confirmatory primers (Table S2.1). 24-35 = *DsRed* lines ampified with all other confirmatory primers (Table S2.1).

Figure 2. 8 Plant growth and flowering in transgenic and wildtype *Arabidopsis*. A) Phenotype of all transgenic lines at time of bolting of the transgenics (3 weeks after planting). B) Phenotype of all transgenic lines at time of bolting of the wildtipe plants (12 weeks after planting). At this time most of the CcFT3, EgFT and EgFT-GFP plants had already made flowers and seeds. All scale bars are 10 mm.

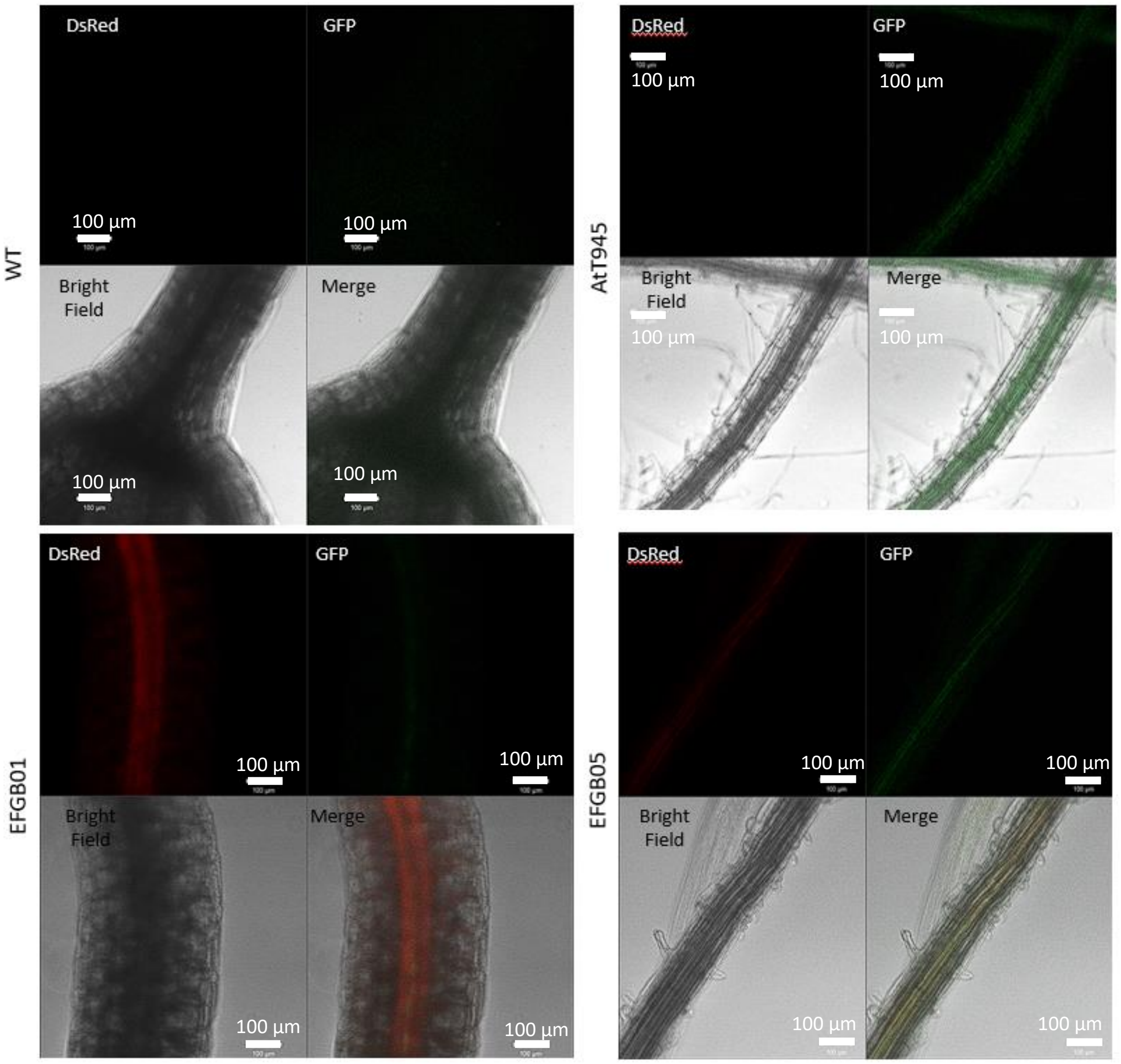

Figure 2. 9 Fluorescent confocal microscopy in *A. thaliana*. A positive control for constitutive GFP expression (under control of 35S promoter) was used, labelled AtT945. No dsRed signal was observed in the GFP positive control plants. Negative control (wildtype, WT) plants showed no dsRed or GFP signal. The GFP signal seen in the positive control was very weak. Plants from transformation events EFGB01 and EFGB05 are shown. GFP seems to be localised to the centre of the plant root and hypocotyl, where the plant vasculature is located.

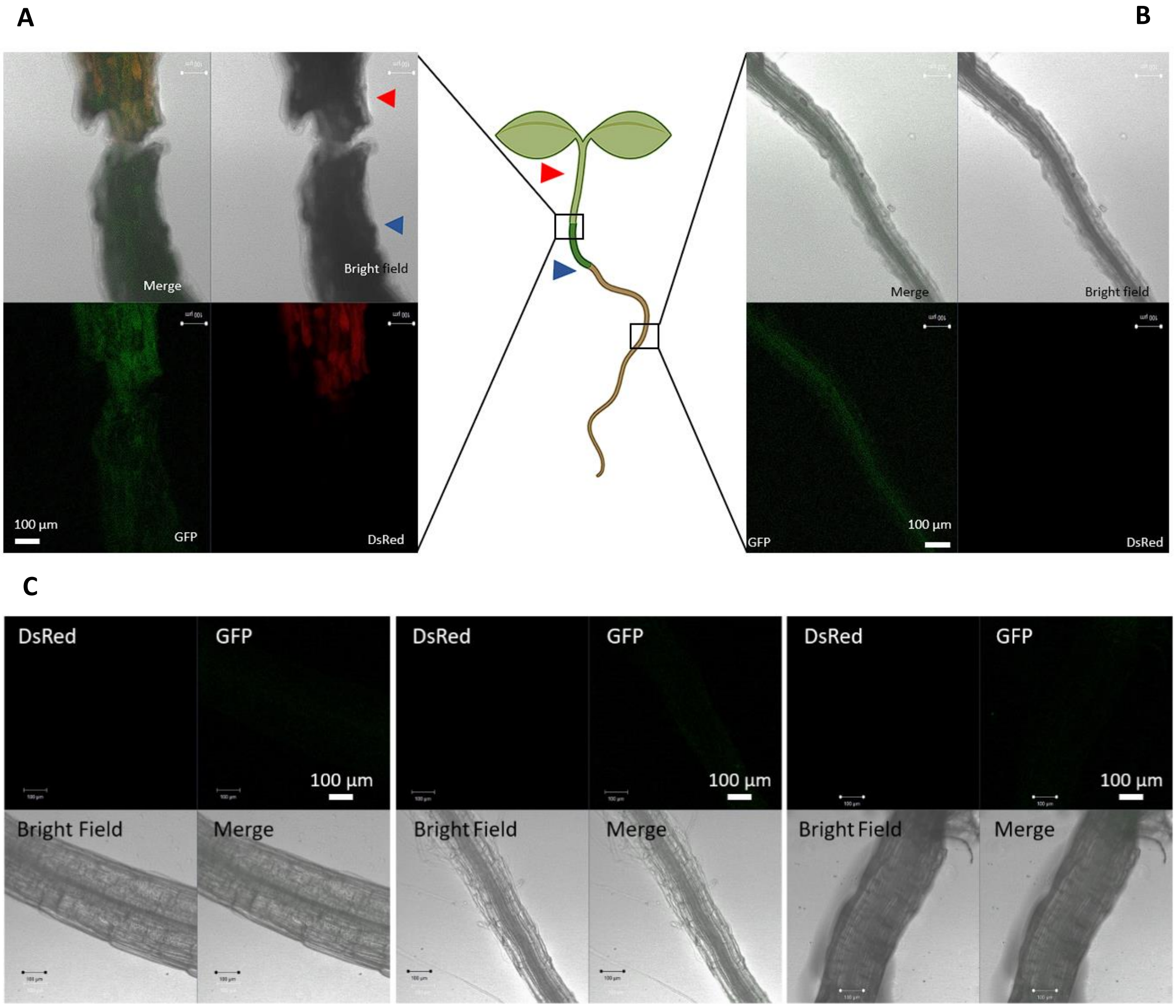

Figure 2. 10 Graft transmission of EgFT-GFP in *A. thaliana*. A) Graft junction between a transgenic EgFT-GFP expressing scion (red arrow) and a wildtype rootstock (blue arrow). Transgenic scion is distinguishable by the absence of a clear dsRed signal and does not move into the wildtype tissue. B) Root section of the wild-type rootstock. There is a weak GFP signal that is localised to the plant's vasculature, there is also no dsRed signal, indicating that the root is not transgenic. C) Wildtype tissues showed no dsRed or GFP fluorescence. It seems that EgFT-GFP is moving from the transgenic scion into the wildtype rootstock.

2.8 Supplementary tables and figures

Table S2. 1 Primers used in study

Primer name	Primer sequence (5'--->3')	Purpose	Length (bp)
CcFT3conReverse	AGTTGAAGTAGACAGCGGCC	Colony and gDNA PCR	573
CcFT3conForward	CTACAACCACCGCAACAACC	Colony and gDNA PCR	
DsRed-EVconReverse	GCTCCACGATGGTGTAGTCC	Colony and gDNA PCR	630
DsRed-EVconForward	CCTCCGAGAACGTCATCACC	Colony and gDNA PCR	
EgFTconReverse	CGCTCTCCCTCTGACAGTTG	Colony and gDNA PCR	600
EgFTconForward	CCTCCACCACTACAACCACC	Colony and gDNA PCR	
EgFT-GFPconReverse	TTGCCGTCCTCCTTGAAGTC	Colony and gDNA PCR	645
EgFT-GFPconForward	CTTCGGGCAGGAGATTGTGT	Colony and gDNA PCR	
Oligo 1 (Destination Vector) Forward	ACTAGTGTCGACGATATGAAGATG	In-Fusion cloning	13 267
Oligo 2 (Destination Vector) Reverse	GGATCCATTTGACAAACCAAGAAAG	In-Fusion cloning	
Oligo 3 (Insert 1 (EgFT)) Forward	TTGTCAAATGGATCCATGCCGAGGGACAGAGATAGAG	In-Fusion cloning	561
Oligo 4 (Insert 1 (EgFT)) Reverse	ATCGTCGACACTAGTTCATCGTCGTCGACCACC	In-Fusion cloning	
Forward primer (EgFT linker)	ATGCCGAGGGACAGAGATAGAGATCC	In-Fusion cloning	540
Reverse primer (EgFT linker)	GCTCCCGCTCCCTCGTCGTCGACCACCAGAGC	In-Fusion cloning	
EgFT double insert Forward	TTGGTTTGTCAAATGGATCCATGCCGAGGGACAGAGATAGAGATC	In-Fusion cloning	570
EgFT double insert Reverse	TGCTCACCATGCTCCCGCTCCCTCGTCG	In-Fusion cloning	
GFP Insert Forward	GAGCGGGAGCATGGTGAGCAAGGGCGAGG	In-Fusion cloning	750
GFP Insert Reverse	TTCATATCGTCGACACTAGTTTACTTGTACAGCTCGTCCATGCC	In-Fusion cloning	
GOISeqAtSUC23p	CCACCGCAACAACCACCAAA	Sequencing downstream SUC2 promoter	NA

EgFT-GFPCon	GGAGCATGGTGAGCAAGGG	Sequencing of GFP fusion	NA

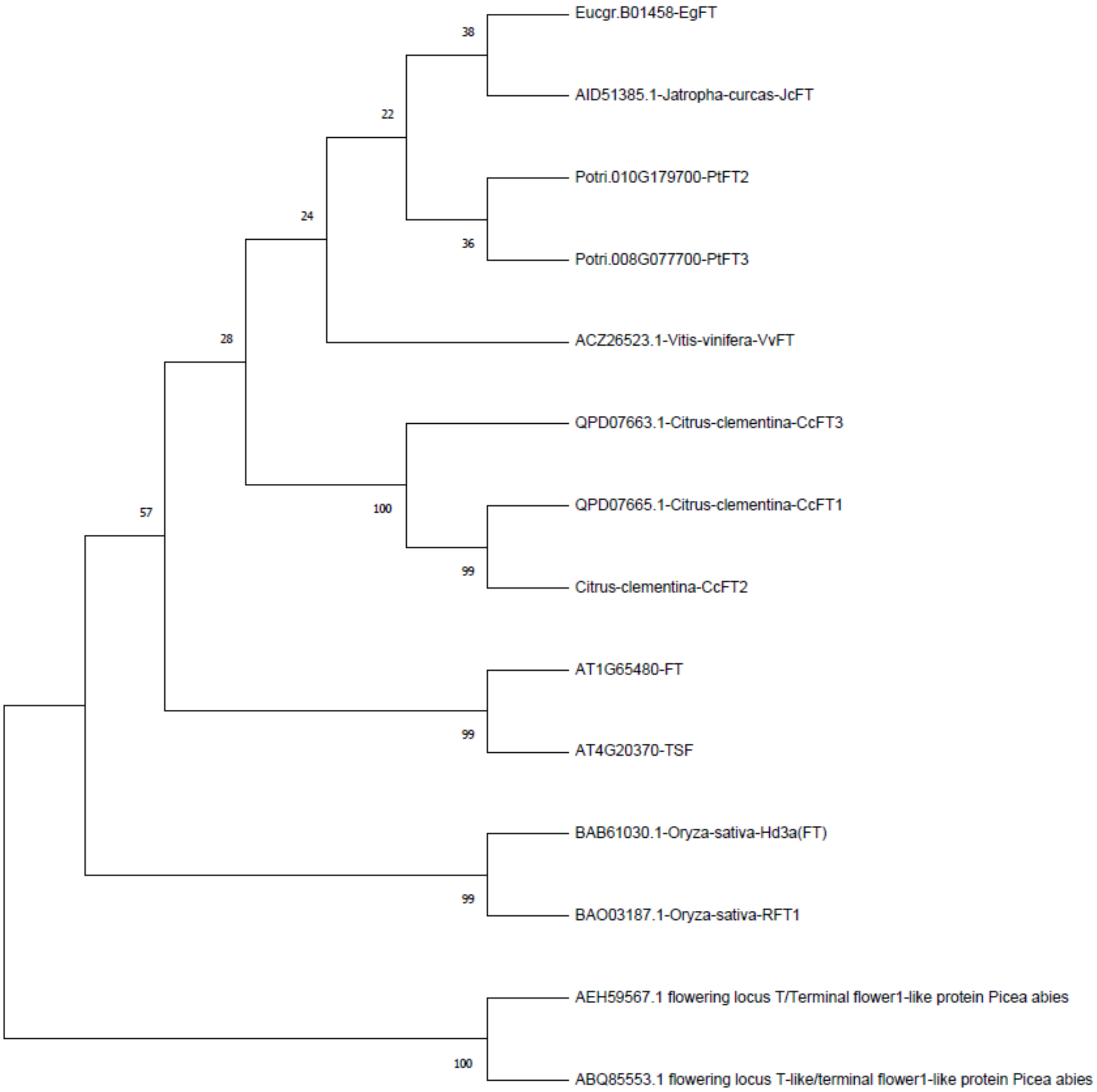

Figure S2. 1 Phylogenetic analysis of FT homologs in angiosperms. The *Eucalyptus FT* clusters closely with the *Jatropha curcas* homolog. However, this clustering is not strongly supported (only a shared node with a bootstrap value of 38). Naturally, the homologs in species which most likely arose from lineage-specific duplication events cluster together closely. The gymnosperm FT-TFL1 like proteins from *Picea abies* form an outgroup. The maximum likelihood tree was constructed using MegaX (KUMAR *et al.* 2018). Bootstrap values from 1000 replicates were used to assess support for the clustering.

***Arabidopsis* FT vs *Eucalyptus* FT**

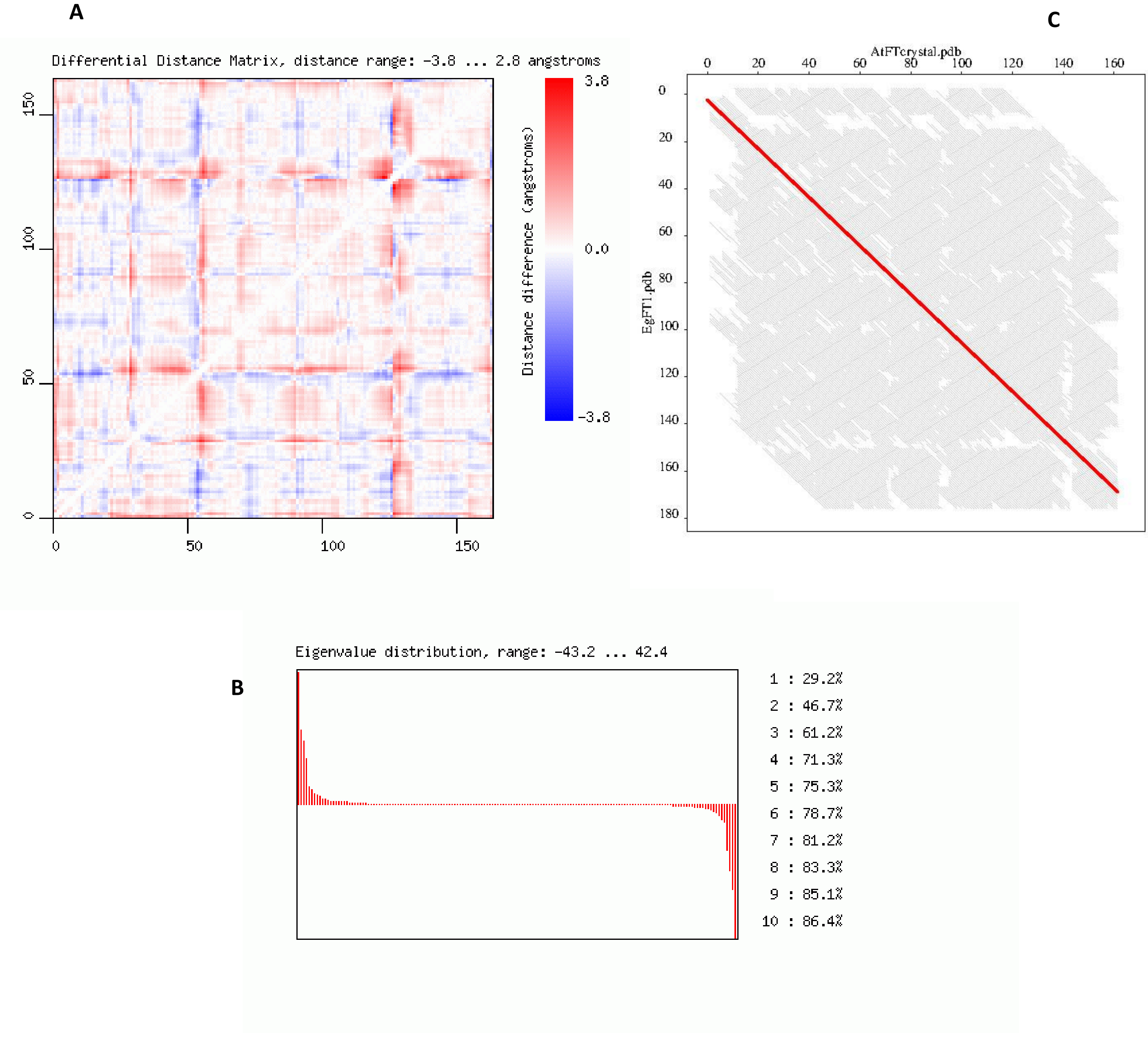

Figure S2. 2 Differential distance matrix, FATCAT chaining graph and eigenvalue distribution for each protein comparison respectively. A) The differential distance matrix contains all the distance difference values, resulting from the subtraction of the corresponding elements in the two distance matrices between two proteins. Such a distance matrix represents a protein structure by the distances between the Cα-atoms of every pair of the amino acids common in the protein structures being compared. The two protein structures are different when the distance difference values deviate from zero, indicating structural differences. B) Eigenvalue distribution of the corresponding differential distance matrix. C) FATCAT alignment graph with aligned fragment pairs in the optimal alignment depicted as a red line, all other aligned fragment pairs between the two structures are shown as grey segmented lines.

***Arabidopsis* FT vs *Citrus* FT3**

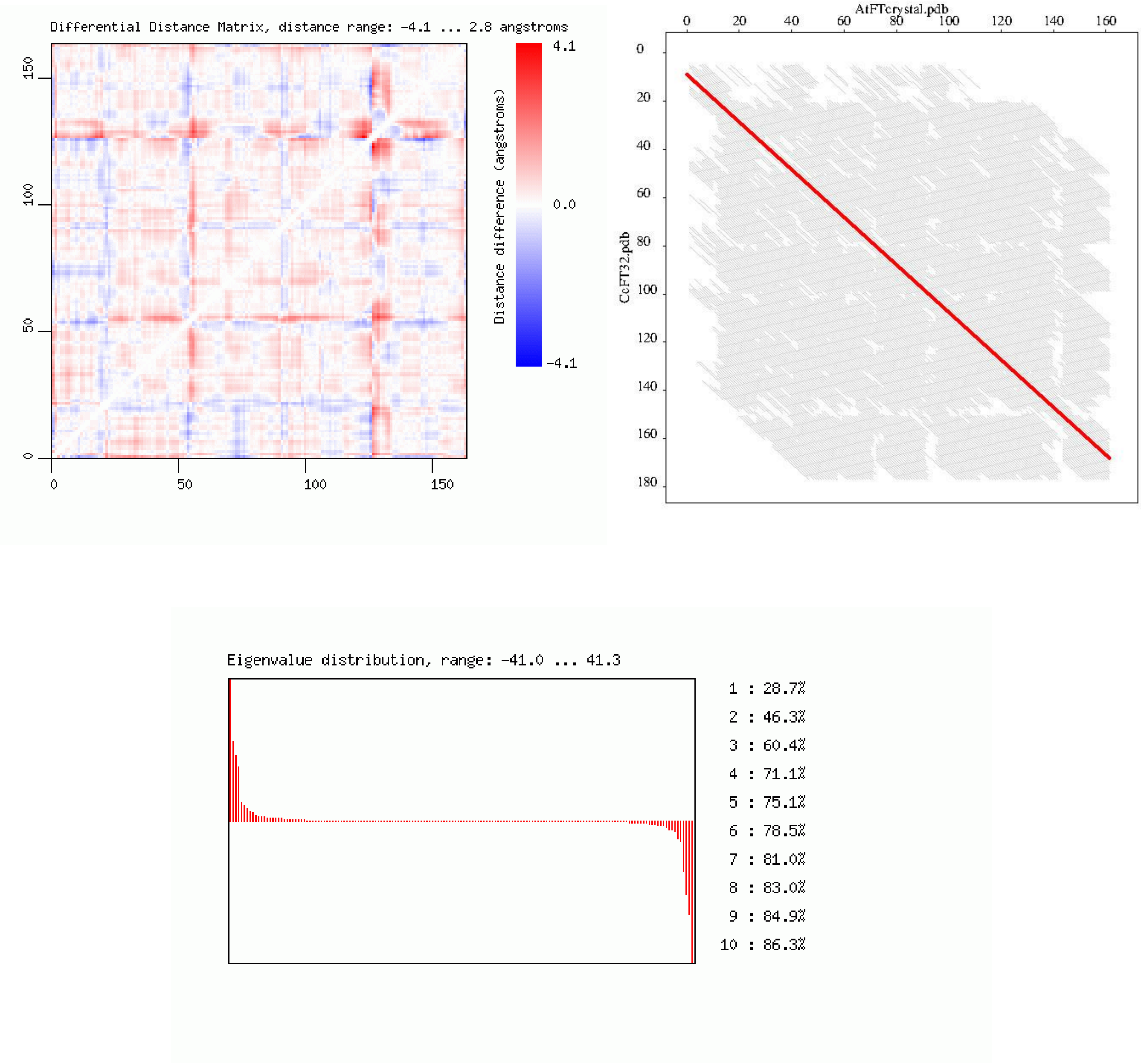

Figure S2.2 (Cont.)

Eucalyptus FT vs *Citrus* FT3

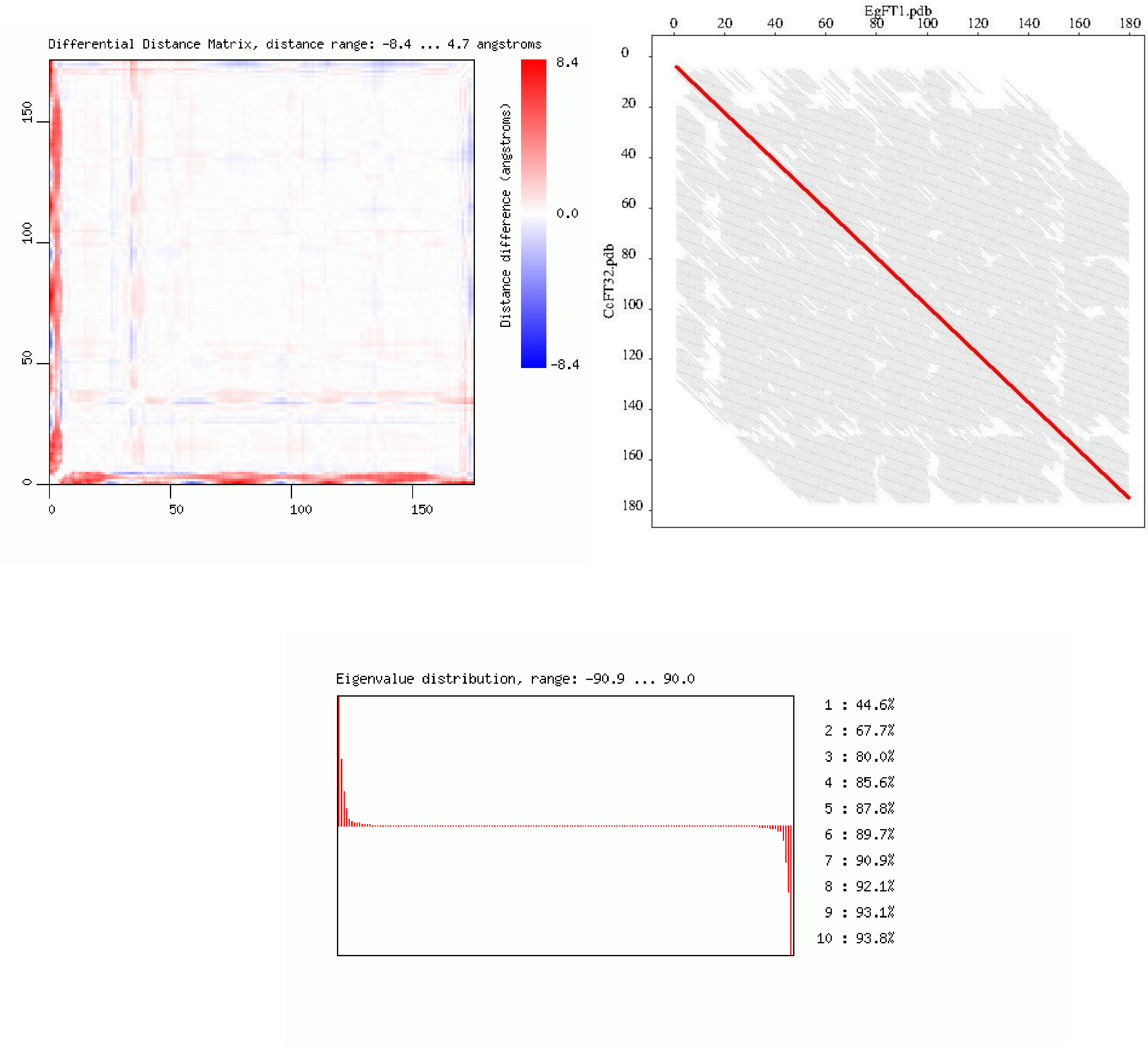

Figure S2.2 (Cont.)

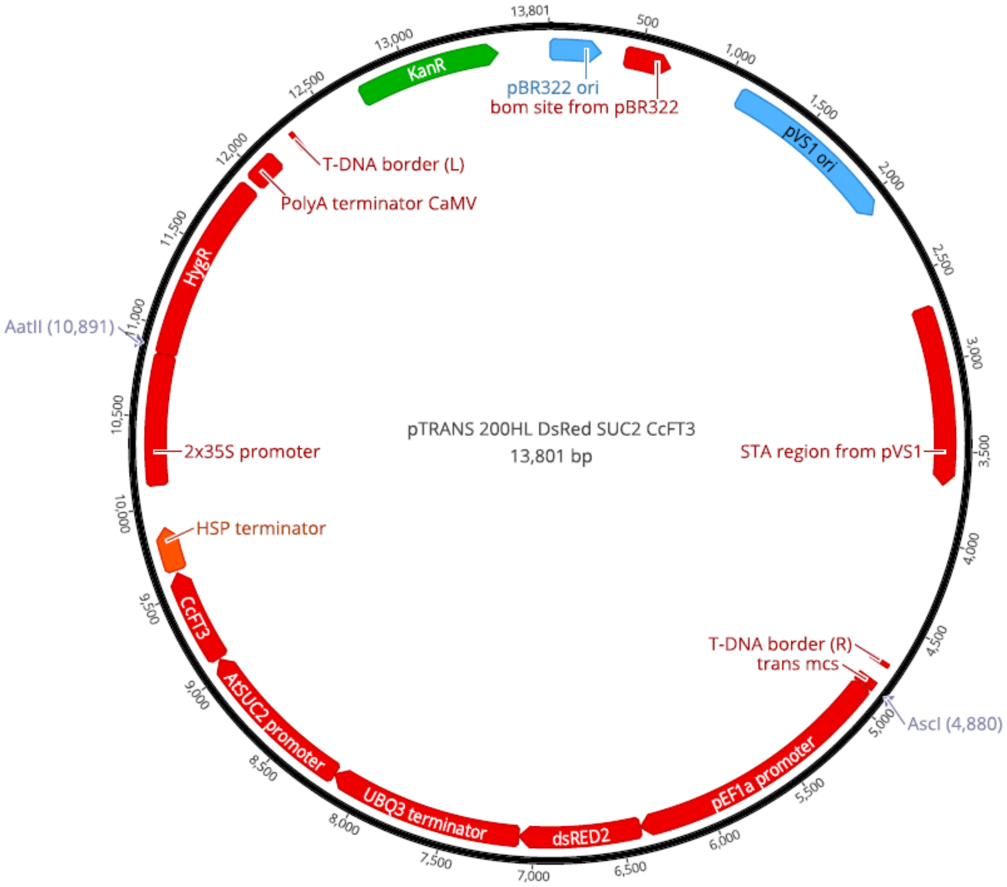

Figure S2. 3 Modified pTRANS200 plasmid map. The pTRANS200 plasmid (ČERMÁK *et al.* 2017) modified to contain the *CcFT3* gene driven by the *Arabidopsis* SUC2 promoter, hygromycin resistance gene driven by a 2x35S promoter and a DsRed gene driven by a pEF1a promoter, all within the T-DNA boarders. Kanamycin resistance gene is present for bacterial selection. Other important regions are labelled on the plasmid map**.**

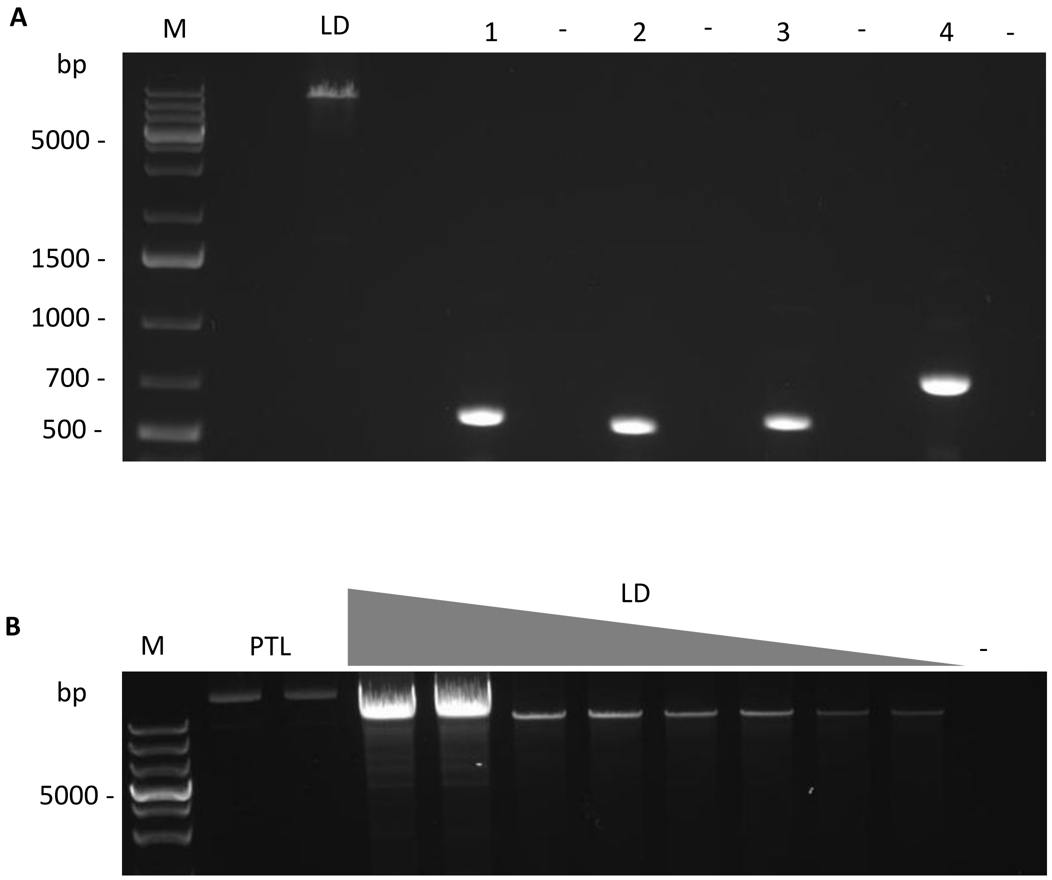

Figure S2. 4 PCR amplicon gel electrophoresis of PCR amplicon fragments needed for InFusion™ cloning reaction. A) All PCR products that were used for the In-Fusion cloning reaction. M = Molecular Weight DNA marker (ThermoFisher Scientific, Massachusetts, United States) , LD = Linearised pTRANS200 destination vector after PCR with primers Oligo 1 (Destination Vector) Forward and Oligo 2 (Destination Vector) Reverse, 1 = PCR product from primers Oligo 3 (Insert 1 (EgFT)) Forward and Oligo 4 (Insert 1 (EgFT)) Reverse, 2 = PCR product from primers Forward primer (EgFT linker) and Reverse primer (EgFT linker), 3 = PCR product from primers EgFT double insert Forward and EgFT double insert Reverse, 4 = PCR product from primers GFP Insert Forward and GFP Insert Reverse. All primers produced the expected sized bands. B) Comparison between the linearized pTRANS200 destination vector and the linearized destination vector after PCR to remove CcFT3. M = DNA marker, PTL = linearized pTRANS200 destination, and LD = linearized destination vector after PCR with primers Oligo 1 (Destination Vector) Forward and Oligo 2 (Destination Vector) Reverse. A decreasing concentration (from 5ng of template to 0.25 ng) of template was used to determine the optimal amount of template to get clean bands which assisted in gel extraction. All primers used can be found in Table S2.1.

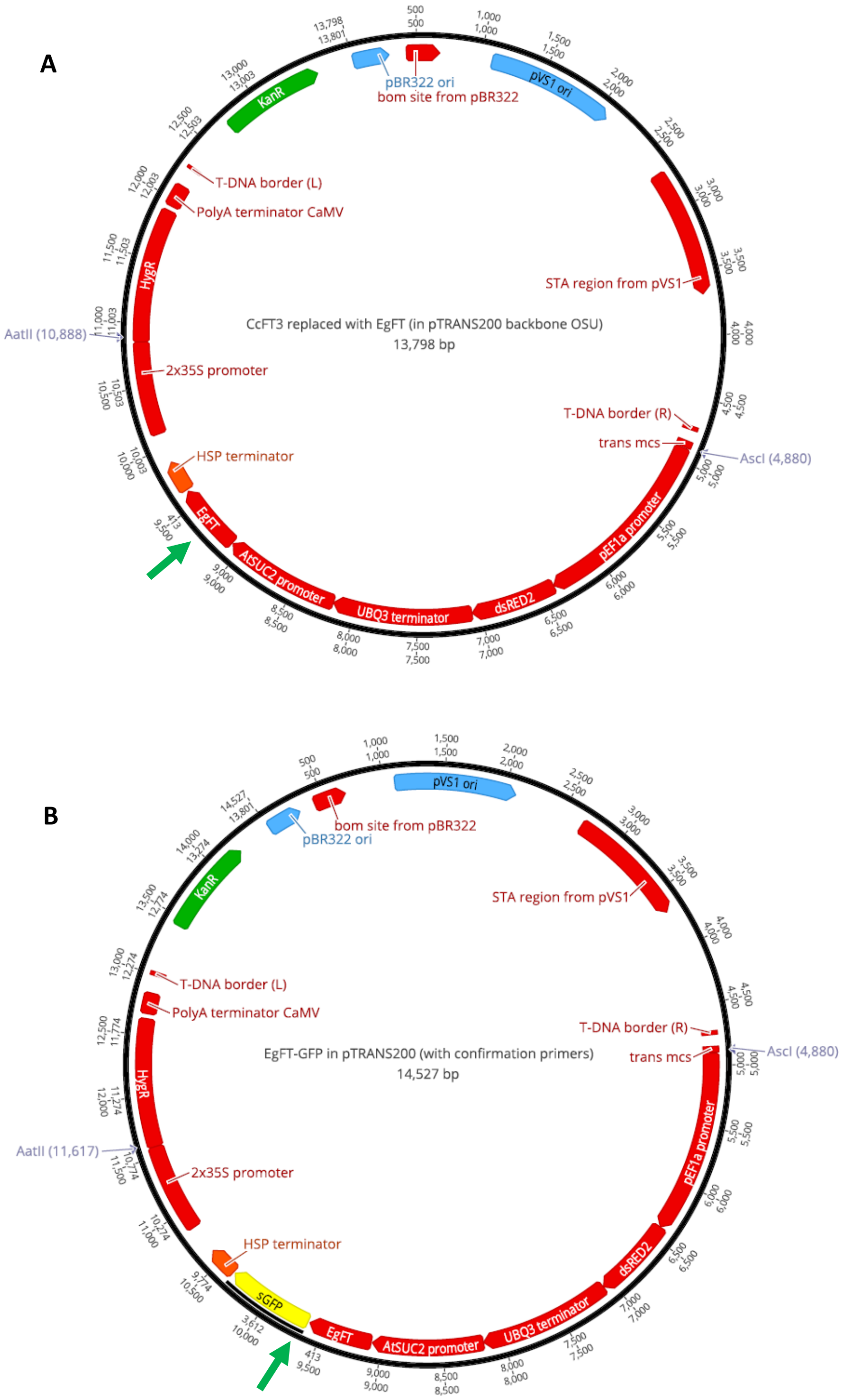

Figure S2. 5 Destination plasmids produced from In-Fusion reactions. A) pTRANS200 (ČERMÁK *et al.* 2017) destination plasmid, the *CcFT3* gene (Figure 3), was replaced by the synthesised *Eucalyptus FT* coding sequence. B) pTRANS200 destination plasmid, the *CcFT3* gene (Figure 3), was replaced by the *Eucalyptus FT-GFP* fusion coding sequence. Both are indicated with green arrows. All other elements in the final constructs remain the same as the initial pTRANS200 destination vector as received by OSU.

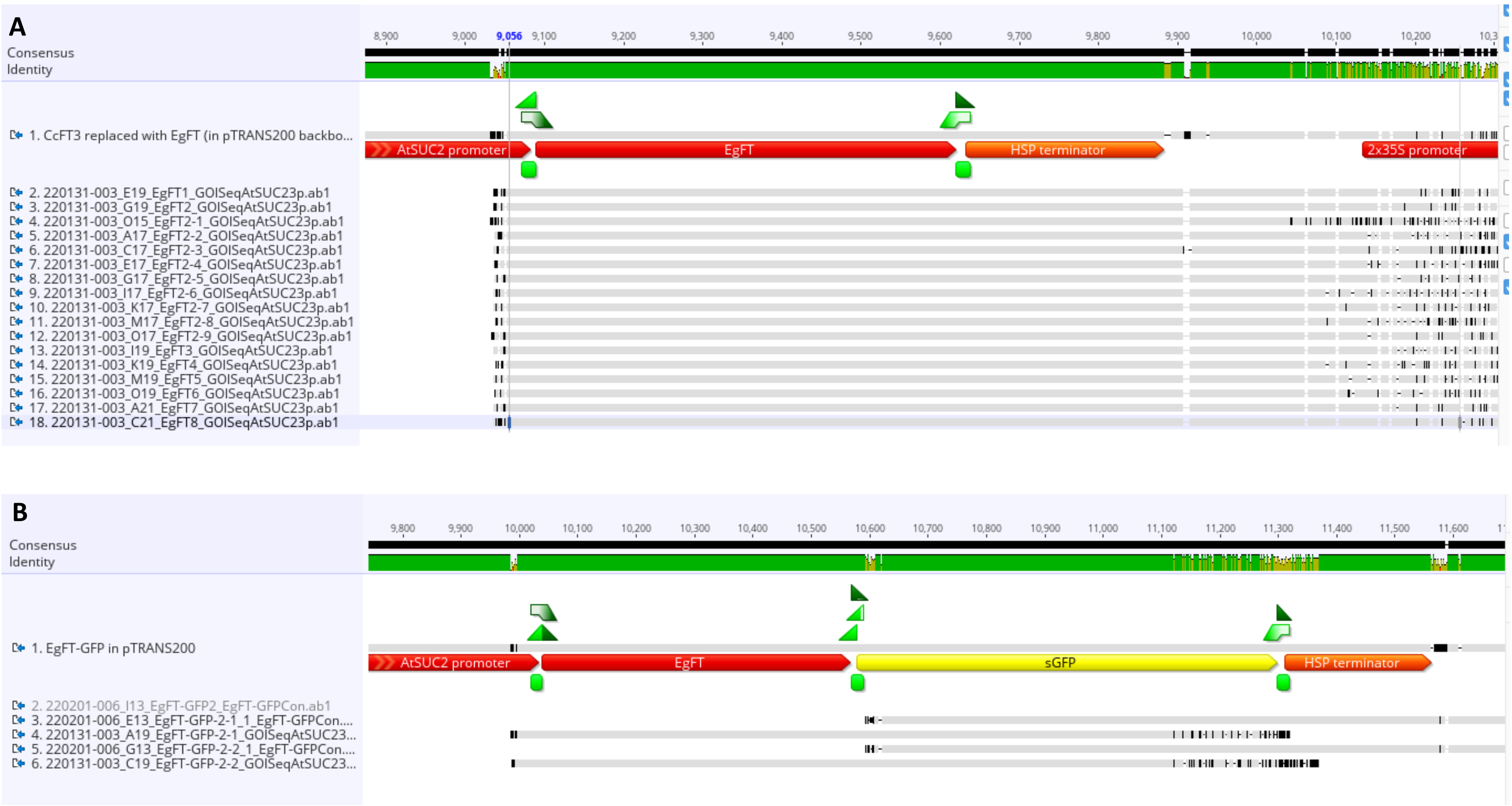

Figure S2. 6 Alignment of sequencing results. A) All alignments were based on sanger sequencing results for EgFT cloned constructs. B) Alignments from sequencing results for EgFT-GFP cloned constructs. Black lines indicate base pairs which deviate from the consensus.

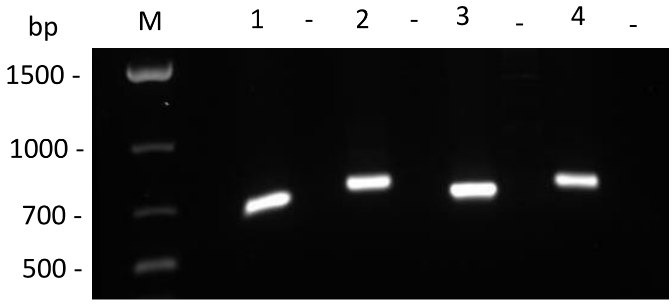

Figure S2. 7. Colony PCR of transformed *Agrobacterium*. Primers used for colony PCR conformation can be found in Table S1. M = Molecular Weight DNA marker (ThermoFisher Scientific, Massachusetts, United States). Lanes labelled 1,2,3 and 4 represent PCR amplicons from colony PCR of colonies containing *Citrus FT3, dsRed* (empty vector), *Eucalyptus FT* and *Eucalyptus FT-GFP* fusion respectively. Each PCR also had a negative control.

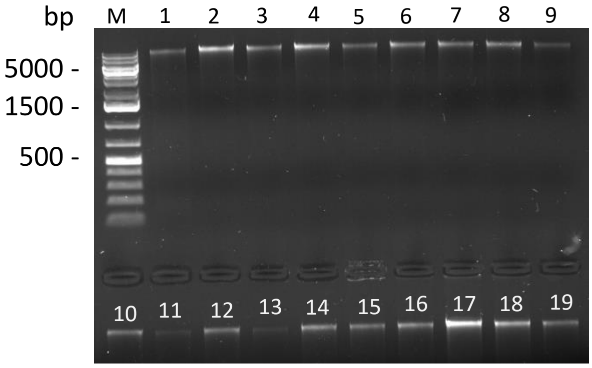

Figure S2. 8 Genomic DNA from T1 transgenic lines. M = Molecular Weight DNA marker (ThermoFisher Scientific, Massachusetts, United States), 1 = Wild type, 2-19 represents all T1 transgenic lines DSRA01, DSRA03, DSRA04, DSRA05, CFA01, CFA03, CFA04, CFA05, CFA06, CFA07, CFA08, EgFTA02, EgFTA04, EgFTA05, EFGA01, EFGA04, EFGA05 and EFGA06 respectively.

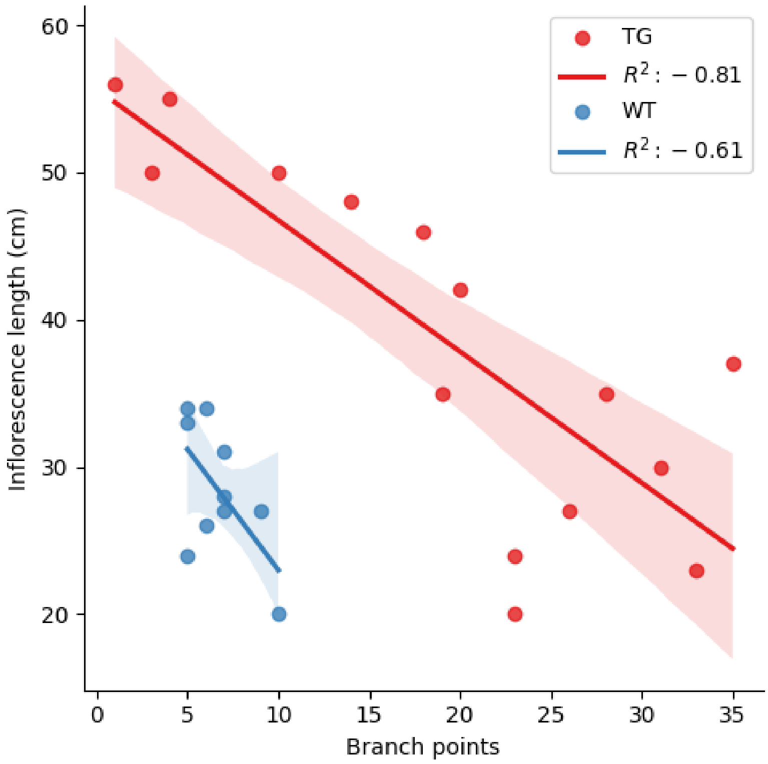

Figure S2. 9 Correlation between inflorescence length and branch points. TG = transgenic, WT = wildtype. The shaded area shows the size of the confidence interval for the regression estimate. Red shaded components are linked to data from transgenic plants and blue shaded components are linked to wildtype plants. R^2 is the square of regression coefficient of each line.

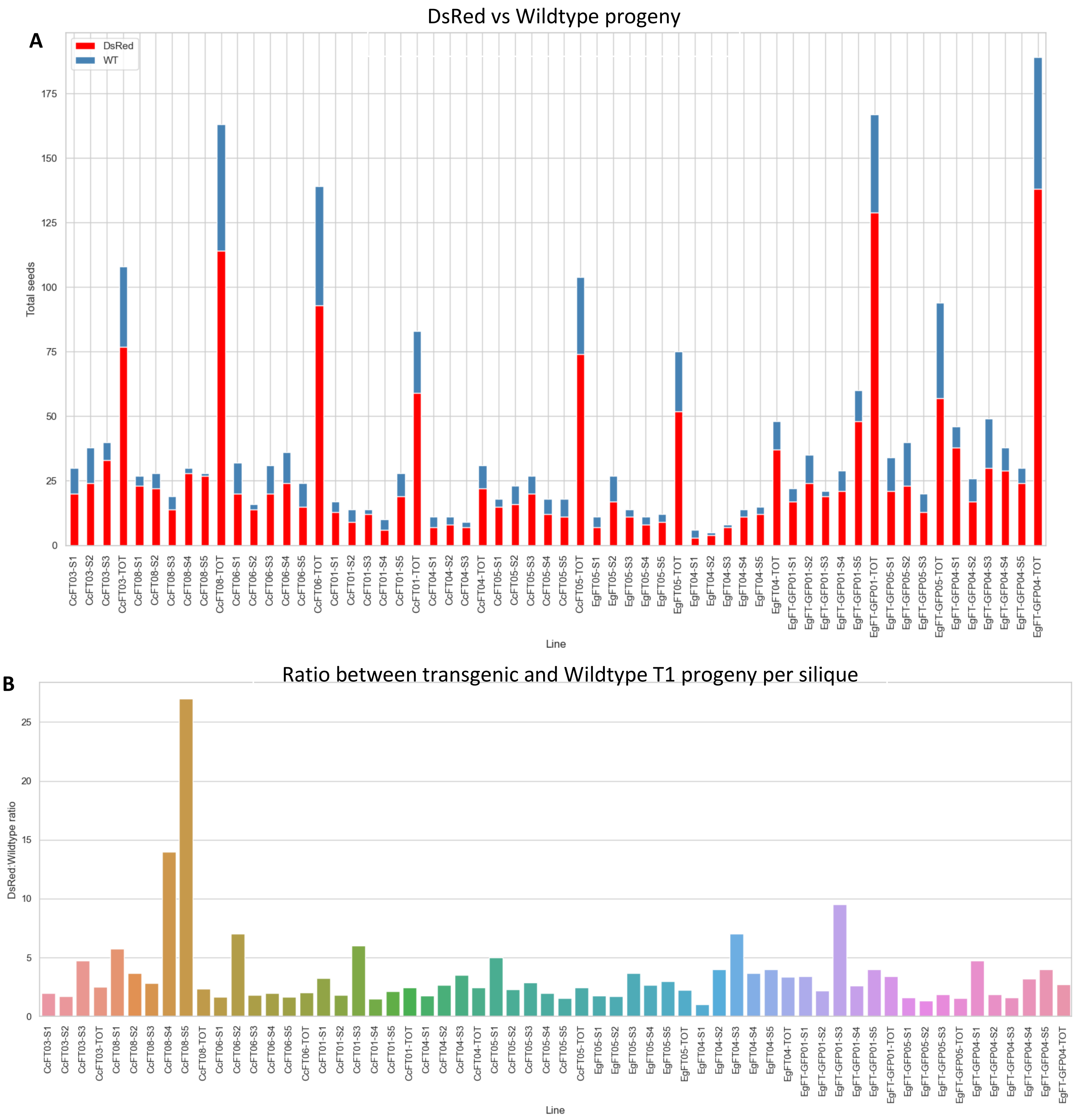

Figure S2. 10 Analysis of T1 progeny. A) Analysis of seeds within siliques of each transgenic line. S# indicates the number of silique analysed. TOT indicates the total number of seeds analysed from each transgenic line respectively. Each stacked bar graph is divided into the numberof dsRed fluorescing seeds and WT seeds. B) The ratio of dsRed to wildtype seeds of each silique and the total of those for each transgenic line respectively. The total from each line evaluated was approximately 3:1 in each transgenic line respectively. This suggests that each construct was inserted once at a single locus, which explains the 3:1 ratio of progeny. The 3:1 ratio represents, homozygous transgenic progeny plus heterozygous transgenic progeny (3), and the homozygous wildtype progeny (1).

Table S2. 2 Statistical tests for comparison of first-generation transgenic lines to wildtype for difference in flowering time and leaf number.

Comparison	Test statistic	P value	Significantly different
			T1 flowering time
dsRed v.s. Wildtype	1.800×10^{1}	1.348×10^{-1}	No
CcFT3 v.s. Wildtype	5.000×10^{-1}	1.672×10^{-4}	Yes
EgFT-GFP v.s. Wildtype	0.000	2.407×10^{-3}	Yes
EgFT v.s. Wildtype	0.000	7.441×10^{-3}	yes
			T1 Leaves
dsRed v.s. Wildtype	1.500×10^{1}	8.071×10^{-2}	No
CcFT3 v.s. Wildtype	0.000	1.520×10^{-4}	Yes
EgFT-GFP v.s. Wildtype	0.000	2.490×10^{-3}	Yes
EgFT v.s. Wildtype	0.000	7.657×10^{-3}	Yes

Table S2. 3 Statistical tests for comparison of second-generation transgenic lines to wildtype for difference in flowering time.

Comparison	Test statistic	P value	Significantly different
		T2 flowering time	
EgFT-GFP v.s. Wildtype	0.000	$1.447\text{x}10^{-10}$	Yes
EgFT v.s. EgFT-GFP	$2.170\text{x}10^{2}$	$1.946\text{x}10^{-15}$	No
EgFT v.s. Wildtype	0.000	$1.992\text{x}10^{-10}$	Yes
CcFT3 v.s. EgFT-GFP	3.500	$8.498\text{x}10^{-9}$	No
CcFT3 v.s. Wildtype	0.000	$1.726\text{x}10^{-6}$	Yes

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Chapter 3

Assessing the effect of paclobutrazol on long-distance graft transmission of the FT protein in *Eucalyptus*

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This research chapter has been prepared in the format required for submission to a peer-reviewed journal (Genetics). I performed all experiments and analyses in this manuscript and prepared the manuscript. Cathleen Ma provided and created the transgenic plant material used in this study in the laboratory of Prof Steven Strauss at Oregon State University. Dr Steven Hussey co-supervised the project and provided technical support and advise on data analysis as well as valuable inputs into the manuscript revision. Prof A.A Myburg was the main supervisor on the project which he conceived as well as providing valuable revisions for this manuscript.

3.1 Abstract

Early floral induction based on ectopic expression of the *Flowering Locus T* (*FT*) gene has been demonstrated in trees. Since the FT protein is known to be graft transmissible, it has been proposed that grafting could be used to induce flowering in non-transgenic scion or rootstock material with FT moving from the transgenic to the non-transgenic part of the plant. Only one study in *Citrus* has achieved this in a tree species. More recently, a study in *Eucalyptus* showed that the application of paclobutrazol promotes flowering of juvenile shoots grafted onto older, already flowering rootstocks. In this study, we aimed to test the possibility that paclobutrazol will also promote flowering of in wildtype *Eucalyptus* material grafted onto transgenic *Eucalyptus* material overexpressing the *Arabidopsis FT* gene. To perform these experiments, we prepared transgenic and wildtype *Eucalyptus* plants for grafting. It was observed that the wildtype *Eucalyptus* material rooted significantly better than the the transgenic AtFT-OX *Eucalyptus*. Wildtype *Eucalyptus* had a rooting efficiency of 95%, while transgenic *Eucalyptus* only had a rooting efficiency of 46%. We conducted a grafting trial consisting of 250 grafts including reciprocal and self grafted controls. Ultimately, these had a survival rate of 55.74% five weeks post graft surgery. Plants treated with paclobutrazol produced a bushy phenotype, in both scion and rootstock, eight weeks post treatment. This was due to the significant shortening of internodal lengths of shoots. No flowering of wildtype material has been observed as of the writing this manuscript. However, the transgenic parts of the plants have flowered profusely. The experiment will still be monitored as it may just be a matter of time before flowering can be seen in wildtype material. We hypothesise that age related cofactors may be absent, which would normally facilitate transport or regulatory action of the FT protein. Another possibility is that the *Arabidopsis* FT protein lacks the homology necessary to interact with *Eucalyptus* cofactors needed for its transport or regulatory function. Thus far, this study has showed that young wildtype *Eucalyptus* shoots grafted onto young transgenic At-OX *Eucalyptus* does not result in early flowering of the wildtype material. Paclobutrazol also does not seem to facilitate flowering of young wildtype material in the same experiment.

3.2 Introduction

Eucalyptus tree species contributes a great deal to the forestry sector world-wide, specifically in Brasil, India, China, Portugal, Spain and South African where its biomass is typically used for charcoal production, and pulp and paper derived products (BRISOLA AND DEMARCO 2011). *Eucalyptus* species have highly advanced breeding programmes with some species, such as *Eucalyptus grandis,* being in their 6th generation of breeding. Cross pollination strategies have a high potential to allow breeders to produce superior trees which have high genetic variation, allowing for greater phenotypic plasticity which is ideal for trees which face highly variable environments throughout their lifetime. However, a major hurdle in the breeding cycle of *Eucalyptus* species is the relatively long time to flower of the plant, in which only vegetative growth occurs and no production of flowers. Earlier flowering species such as *E. grandis* flower uniformly within 2-5 years, while later flowering species such as *E. dunnii* and *E. nitens* can take longer than 10 years to produce flowers uniformly. Thus, when breeders are required to do multiple rounds of crosses followed by field trials for each cross the time becomes considerable to produce a new superior tree.

One way in which researchers have accelerated flowering in plants is by the ectopic expression of the *Flowering Locus T* gene (AN *et al.* 2004; WIGGE *et al.* 2005; LIU *et al.* 2012; XU *et al.* 2012; ZHU *et al.* 2016; CHEN *et al.* 2018; SONG *et al.* 2019; SOARES *et al.* 2020) .The expression pattern of *FT* has been studied and was found to be photo periodically induced mainly within specialised phloem companion cells (AN *et al.* 2004; WIGGE *et al.* 2005; LIU *et al.* 2012; XU *et al.* 2012; ZHU *et al.* 2016; CHEN *et al.* 2018). Ectopic *FT* expression in phloem companion cells is sufficient to initiate early floral development (AN *et al.* 2004; ABE *et al.* 2005). Similar experiments have been conducted in woody plants such as blueberry and even larger woody perennials such as *Jatropha, Citrus* and apple (SONG *et al.* 2013; WENZEL *et al.* 2013; YE *et al.* 2014; SOARES *et al.* 2020). The FT protein moves a relatively long distance from the phloem companion cells in leaf tissue to the shoot apical meristem (SAM), mainly with the help of the *FT-INTERACTING PROTEIN 1* (*FTIP1*) and *SODIUM POTASSIUM ROOT DEFECTIVE 1* (*NaKR1*) proteins (Corbesier *et al.* 2007; Notaguchi *et al.* 2008; Li *et al.* 2011; Liu *et al.* 2012; Zhu *et al.* 2016). Since the FT protein travels through the phloem, it is able to cross a graft junction when expressed in rootstocks, which was first confirmed with ectopic expression and grafting in *Arabidopsis* (An *et al.* 2004; Notaguchi *et al.* 2008; Yoo *et al.* 2013a; Zhu *et al.* 2016).

Grafting is an ancient technique which is still used to generate composite plants with different parts that have desirable traits. An example of this would be a disease resistant rootstock and a high yielding scion, which is often the case with fruit trees. The success of grafting is due to the plant's innate regeneration capabilities. Cytokinin plays a major role in wound induced callus formation. When a plant is wounded, cytokinin biosynthesis genes such as *ISOPENTENYL TRANSFERASE 3* (*IPT3*), *LONELY GUY 1* (*LOG1*), *LOG4*, and *LOG5* are transcriptionally activated, and when these genes are knocked out, defects in wound induced callus formation is evident (Ikeuchi *et al.* 2017). Another set of reprogramming regulator genes known as *WOUND INDUCED DEDIFFERENTIATION* genes (*WIND1, 2, 3 and 4*) are also activated upon wounding (Iwase *et al.* 2011). The cytokinin biosynthesis and *WIND* pathways both facilitate the activation of cytokinin signalling by activating type-*B ARABIDOPSIS RESPONSE REGULATOR 1* (*ARR1*) and *ARR12* which ultimately leads to upregulation of *CYCLIN D3;1* (*CYCD3*;*1*) and re-entry into the cell cycle (Iwase *et al.* 2011; Ikeuchi *et al.* 2017). In the case of grafting, the cells from scion and rootstock both transiently proliferate forming a callus which bridges the gap between scion and rootstock. The callus must then re-differentiate in order to reconnect the plant vasculature. Key genes involved in cell proliferation and re-differentiation have been studied such as *NAC DOMAIN CONTAINING PROTEIN 71* (*ANAC071*), *XYLOGLUCAN ENDOTRANSGLUCOSYLASES/HYDROLASE 19* (*XTH19*) and *XTH20,* and *RELATED TO APETALA2 6L* (*RAP2.6L*) and are affected by auxin and ethylene concentrations (Asahina *et al.* 2011; Pitaksaringkarn *et al.* 2014). Specifically, ectopic expression of *AUXIN RESISTANT 1* (*AXR1*) and *ABERRANT LATERAL ROOT FORMATION 4* (*ALF4*) perturbed the formation and reconnection of the phloem which was also found to reconnect before the xylem (Melnyk *et al.* 2015).

The long-distance movement of the FT protein and the reconnection and continuity of phloem after grafting surgery has led to applications to induce early flowering in wildtype scions. The FT protein, when ectopically expressed, is able to be transported across the graft junction and induce flowering in wildtype tissue (CORBESIER *et al.* 2007; NOTAGUCHI *et al.* 2008). It was realised that this could be used as powerful tool in plants which have a long juvenile phase, where rootstocks/scions which were transgenic and overexpressing a *FT* gene could induce early flowering in wildtype material via grafting. Early flowering rootstocks have been produced in apple, pear, poplar, citrus, *Eucalyptus* and plum and *Jatropha* (ZHANG *et al.* 2010; SRINIVASAN *et al.* 2012a; SONG *et al.* 2013; WENZEL *et al.* 2013; YE *et al.* 2014; FREIMAN *et al.* 2015; KLOCKO *et al.* 2016; VELÁZQUEZ *et al.* 2016; SONG *et al.* 2019; SOARES *et al.* 2020). However, when testing floral induction via grafting onto those rootstocks in poplar, apple, plum and *Eucalyptus* (unpublished) no floral induction was seen in wildtype scions. Only recently, graft transmission and floral induction of wildtype tissue was demonstrated in *Citrus* (SOARES et al. 2020). In *Eucalyptus,* floral induction of juvenile shoots was achieved in a non-transgenic manner by top grafting onto mature, prolifically flowering plants and aided by the application of paclobutrazol (DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022). Paclobutrazol (PBZ), is a chemical which mimics a plant hormone, and primarily inhibits the biosynthesis of gibberellic acid (GA) which leads to symptoms such as shortening of internodal lengths, prolific and earlier flowering (HETHERINGTON *et al.* 1992; MONCUR AND HASAN 1994; HASAN AND REID 1995).

A key gene needed for correct floral development is the *LEAFY* (*LFY)* gene. When *LFY* is mutated and produces a non-functional protein it results in the repeated development of sepals where flowers would normally form, resulting in a sterile plant (SCHULTZ AND HAUGHN 1991). This notion has been used to create genetically contained transgenic plants (ELORRIAGA *et al.* 2021). This could be used in conjunction with graft transmission to completely isolate the transgenic ally flowering plants from the wildtype plant material.

In this study we aimed to determine whether the *AtFT* overexpressing *LFY* knockout (*AtFT-OX lfy)* early flowering *Eucalyptus* lines produced in other studies also exhibited graft transmissible floral induction of wildtype tissue (KLOCKO *et al.* 2016; ELORRIAGA *et al.* 2021). We also aim to determine if the application of paclobutrazol may promote floral induction in the non-transgenic scions (or rootstocks in reciprocal grafts).

3.3 Materials and Methods

3.3.1 Plant material

All experiments were conducted in the *Eucalyptus* SP7 clonal background. *Eucalyptus* wildtype and *FT 4-2 LFY1C/LFY2C, FT 4-2 LFY1C* and *FT 30-3 LFY1C* transgenic lines were all kindly provided by the Strauss laboratory at Oregon State University. The numbers after FT represent the original transgenic event which gave rise to the specific line (KLOCKO *et al.* 2016). LFY refers to the CRISPR knockout event of the *LFY* gene (ELORRIAGA *et al.* 2021). These plants were micro-propagated (4.4 g/L MS, 200 µl/L Biotin, 200 µl/L Calcium, 400 µl/L BAP, 20 µl/L NAA, 25 g/L Sucrose ,3 g/L Gelrite) *in vitro* under sterile conditions. The micro-propagated plantlets were transferred to rooting media (2.2 g/L MS, 2 ml/L NAA, 1 ml/L IBA, 20 g/L Sucrose, 3 g/L Gelrite) to induce root formation and root growth. Subsequently, rooted plantlets were transferred to jiffy pots and placed in artificially lit growth rooms under long day conditions (16 hours light, 8 hours dark), for 3 weeks. Once these plants were acclimatized and had sufficient growth, they were transferred to larger pots and placed in naturally lit phytotrons. The plants were then grown and fertilized until the diameter size of the main stem was large enough (~ 5 mm) to allow for grafting.

3.3.2 Grafting

For our experiments, we opted for mini and macro cleft/wedge grafts. In total we conducted 250 grafts including reciprocal grafts, self-grafted controls and non-grafted controls. The graft junction was first wrapped in budding tape and subsequently the entire scion and a small portion under the graft junction was wrapped in parafilm (Figure 3.2). These were then left until the scion showed signs of shooting.

3.3.3 Paclobutrazol treatment

The rootstock stem diameters were measured, and an average diameter was obtained. A subset of rootstocks with shooting scion material were then chosen for paclobutrazol treatment. Paclobutrazol was mixed with 5 L of water with the following ratio: 1ml of PBZ per 1 cm of rootstock diameter. Of this solution, 180 ml was applied to each plant, which was sufficient for soil saturation. In total, 80 successfully shooting scions were treated with paclobutrazol, and 44 were left untreated.

3.3.4 Statistical analysis

To determine if there was a significant difference in the rooting efficiency between *AtFT* overexpression lines and wildtype *Eucalyptus*, a Fisher's exact test was performed with a threshold (*a*) of 5%. A Mann-Whitney-Wilcoxon two-sided test was performed for each of the internodal measurements to determine whether the PBZ treated plants were growing significantly differently from the non PBZ treated plants

3.4 Results

3.4.1 *Eucalyptus* FT overexpression lines tissue culture

The *AtFT* overexpression plants and the SP7 wildtype plants grew similarly in multiplication media (Figure 1). The *Eucalyptus* SP7 lines had a much higher rooting efficiency than the AtFT overexpression *lfy* (AtFT-OX *lfy*) lines. SP7 wildtypes had approximately 95% rooting efficiency after one month of growing in rooting media while the *AtFT* overexpression lines only had a rooting efficiency of 46% (Table 3.1). A Fisher's exact test yielded a p-value of 2.52×10^{-57}, indicating that there is a highly significant difference between the rooting efficiency of wild-type plants and transgenic plants (Table 3.1). The roots of SP7 wildtype plants in rooting media became long and highly branched within three weeks on rooting media, while the *AtFT* overexpression plants mostly made smaller roots at the same time point of three weeks. This may indicate that the overexpression of the *Arabidopsis FT* or the lfy knockout in *Eucalyptus* is affecting the formation and growth of roots. Another reason might be that the media was optimized for the SP7 clone and that these overexpression lines may need different concentrations of hormones for more efficient growth. Plantlets with roots originating from the stem were then transferred into jiffy pellets and acclimatized in artificially lit growth rooms. The success rate of rooting in jiffy pots varied and seemed to depend on the strength and number of roots which developed in the rooting media (Figure S1). After four weeks of growth in jiffy pots, the plants were transferred into bigger pots and then taken to naturally lit phytotrons (Figure S2).

3.4.2 *Eucalyptus* FT overexpression lines, growth, and grafting

Plants were grown until a stem diameter of at least 5 mm was reached to facilitate grafting. We found that the FT-OX *lfy* lines grew much slower and had a bush-like appearance. Each shoot developed a typical *lfy* mutant flower bud (Figure S3.3) and terminated the growth of the shoot (ELORRIAGA *et al.* 2021). SP7 wild-type plants grew normally and did not develop any signs of flowering (Figure 2S). Once the plants were large enough (approximately 10 months), a grafting trial was done. Cleft grafting was used as the preferred method for *Eucalyptus* grafting. The grafting method is depicted in Figure 3. Grafting was done over two days (8 – 9 June 2022). Approximately one month later, scions started to develop shoots. We recorded the survival and shooting rate of scions. Scions were classified as dead when they became black, or the rootstock died. Twenty-eight days after the grafting was done we recorded a survival rate of 99.57 % and a shooting percentage of 26 % (Figure 4). Ninety-one days post grafting these numbers dropped to 55.74 % and rose to 66.41 % respectively (Figure 4). The survival rate of each scion was also counted and grouped according to the scion genotype (Figure 5). Throughout this period, there were no signs of flowering in any of the grafted wild-type material, whether it was wild-type scion on transgenic rootstocks or the reciprocal. However, AtFT-OX *lfy* material produced floral buds throughout the grafting and scion shooting period (Figure S3.3).

3.4.3 Paclobutrazol application

We applied paclobutrazol to a subset of grafts to determine whether it would promote the flowering of wild-type graft tissue. The paclobutrazol was applied 58 days post grafting (5Th August 2022)), and no signs of flowering have been seen in wild-type tissue to date, more than a month after the application. The transgenic FT-OX *lfy* lines, however, are still producing flowers whether they are rootstocks or scion. One feature of paclobutrazol application is the shortening of the internodal length of plants, thus we measured five internodal lengths of newly growing shoots to determine whether paclobutrazol was having an effect. We found that there is a significant difference between internodal lengths of PBZ treated and non-PBZ treated plant material (Figure 6). Specifically, we found that there is a significant difference between internodes 1 – 4. However, there was no significant difference between the 5th internodes of PBZ treated and PBZ untreated plants, which may be due to the 5th internode already being formed before the PBZ had an effect. The phenotype of the plants was also clear, as PBZ treated plants produced bushy shoots, with short internodes while untreated plants grew normally (Figure 7).

3.5 Discussion

The generation times of *Eucalyptus* species is a prominent hurdle for breeding superior *Eucalyptus* lines. Some *Eucalyptus* species used in industry such as *Eucalyptus grandis* and *E.urophylla,* start flowering after approximately 3 - 5 years, whereas other species such as *E. dunnii* and *E. nitens* that are also used take up to 10 years to flower. Thus, just to obtain pollen and receptive flowers can take up to 10 years for a single breeding cycle, thereafter progeny from cross must be tested and validated which may take a considerable amount of time. Breeding a new superior line will thus take many years if no intervention on generation time is made.

The *Flowering Locus T* (*FT*) gene has been studied extensively and there is a great amount of knowledge regarding the regulation, transport, and effects of FT on the plant. When a plant flowers it has advanced from the juvenile phase to the mature phase of its lifecycle. Once *FT* is expressed under permissive conditions (photoperiod and age), it is first exported out of the phloem companion cells and into the phloem stream, facilitated by *FT-INTERACTING PROTEIN 1 (FTIP1)* and *SODIUM POTASSIUM ROOT DEFECTIVE 1 (NaKR1)* (LIU *et al.* 2012; ZHU *et al.* 2016; CHEN *et al.* 2018). The exact mode of transport when in the phloem stream to the SAM is not yet fully understood. However, a study has shown that FT specifically binds to phosphatidylcholine (PC), a phospholipid present in the plant plasma membrane (NAKAMURA *et al.* 2014). The amount of PC present in the plasma membrane is directly correlated with flowering time and the PC composition showed diurnal oscillation (NAKAMURA *et al.* 2014). We can hypothesize that the FT is baited from the phloem stream by the increased occurrence of PC at the same time at which FT would be at its highest. FT is then transported to the nucleus of the shoot apical meristem (SAM) where it interacts with many other proteins to form the florigen activation complex (FAC), the *Flowering Locus D* gene product (FD) is a crucial part of the FAC, allowing DNA binding and downstream gene activation (TAOKA *et al.* 2011; KAWAMOTO *et al.* 2015; COLLANI *et al.* 2019). Ultimately, FT must move from the phloem companion cells to phloem stream, to the SAM and interact with FD and other proteins for the plant to transition to flowering.

In this study, we used *AtFT-OX lfy* lines, all in the background of the SP7 *Eucalyptus* clone (KLOCKO *et al.* 2016; ELORRIAGA *et al.* 2021). We found that during micropropagation of the *AtFT-OX lfy* lines and wildtype SP7 clones, there was no difference in growth. We then transferred plantlets to media to induce root formation. In this media we found that the *AtFT-OX lfy* lines had a much lower rooting efficiency, at three weeks in rooting media, when compared to the wildtype SP7 plantlets (Tabel 3.1) which had many more roots that were longer and larger in girth. This may be explained by the *AtFT* gene whose expression is driven by the 409S promoter, or may be due to the *lfy* mutation. The ectopic expression may influence the vegetative growth of the newly developing roots. In other studies, it has been shown that the sub-functionalisation of FT homologs plays roles other than floral induction, such as influencing vegetative growth in plants (HSU *et al.* 2011). The ectopic expression of a heterologous FT (the AtFT), may lead to different outcomes than the native *Eucalyptus FT* and its homologs, influencing root development.

After the establishment of roots in tissue culture, the rooted plantlets were transferred to jiffy pots and placed in artificially lit indoor growth rooms to be acclimatised (Figure S3.1). We found that the survival of plantlets at this stage was dependent on the presence of strong roots which were previously developed in tissue culture. After three weeks of growth in the indoor growth rooms, the plants were large enough to be transferred to bigger pots and then placed in naturally lit phytotrons (Figure S3.2). The wildtype SP7 clones grew normally with a large main shoot which grew vertically, while *AtFT-OX lfy* lines grew relatively slowly. The first main shoot of *AtFT-OX lfy* lines started producing *lfy* floral buds within two weeks of transfer to the phytotrons (Figure S3.2) as has been reported before (ELORRIAGA *et al.* 2021). The formation of these floral buds forced the termination of the shoot and subsequently other shoots would form from axial meristematic tissue upstream (towards the roots) of the shoot apex, this would repeat for each new shoot which eventually resulted in a bushy growth phenotype compared to the wildtype plants, which is similar to early flowering transgenic *Citrus* (SOARES et al. 2020). From these results, we can also deduce that the *409S::AtFT-OX* construct was sufficient to cause flowering of younger tissue.

To determine whether the AtFT protein produced would be graft transmissible, we grafted wild-type tissue onto transgenic scions and vice versa. In total, we performed 250 grafts (this includes self-grafted controls, the reciprocals and non-grafted controls). After one-month shoots were visible from successful grafts. However, the survival rate of scions decreased to 55% with a shooting percentage of approximately 66% (Figure 3.3, 3.4). Of all surviving shoots, there was no sign of floral induction in any wild-type tissue (scion or rootstock), but transgenic material continuously produced *lfy* floral buds (Figure S3.3). Similar experiments have been conducted in other species and many also did not achieve graft transmission, nor early flowering of wildtype tissue (ZHANG *et al.* 2010; WENZEL *et al.* 2013; DE OLIVEIRA CASTRO *et al.* 2021). However, graft transmission has been successfully achieved in other species (AN *et al.* 2004; LIFSCHITZ *et al.* 2006; CORBESIER *et al.* 2007; SONG *et al.* 2013; YE *et al.* 2014; FREIMAN *et al.* 2015; VELÁZQUEZ *et al.* 2016; SONG *et al.* 2019; SOARES *et al.* 2020; DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022). It seems that the greatest success of graft transmission came when the native *FT* was used, such as in *Jatropha, Citrus* and blueberry (SONG *et al.* 2013; YE *et al.* 2014; SONG *et al.* 2019; SOARES *et al.* 2020). However, it also seems that in a trial using only wildtype *Eucalyptus* material, a chemical called paclobutrazol aided in the transport of a graft transmissible signal which induced flowering of younger material which normally would take many more years to flower (DE OLIVEIRA CASTRO *et al.* 2021).

Paclobutrazol (PBZ), is a chemical which mimics a plant hormone, and its primary action is the inhibition of gibberellic acid (GA) biosynthesis. It has been known since the 1990s that paclobutrazol helped with the prolific induction of flowering in species that have a longer generation time, even through a graft junction (MONCUR AND HASAN 1994). Since we did not see any floral induction in wild-type tissue in this grafting trial and a previous one conducted at FMG, we decided to apply PBZ to a subset of grafted plants which had scion material which was actively shooting. More than a month after the PBZ treatment we still did not see any signs of floral induction in wildtype material, whether it was wildtype scion or rootstock. This is in contrast to more recently published results which used old flowering rootstocks and young scion material, in one study PBZ was needed to achieve flowering in the younger material while in the other, PBZ enhanced the amount of flowers produced by younger material (DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022). This demonstrates that there is a florigenic signal which is produced in the rootstocks and travels through the graft junction into the younger material, ultimately resulting in early floral induction. We found that there was a significant amount of internodal shortening of the first 4 internodes in PBZ treated plants compared to non PBZ treated plants (Figure 3.5). This internodal shortening is a known symptom of PBZ application (HETHERINGTON *et al.* 1992; HASAN AND REID 1995). However, dissimilar to the previously mentioned studies, we have not yet seen floral induction of wildtype tissue, even though our transgenic lines are overexpressing *AtFT*. We hypothesize that one of the reasons we might not be achieving graft transmissible floral induction is because the *AtFT* being overexpressed lacks the homology needed between the other *Eucalyptus* co-factors which are required to interact with the AtFT protein. This would prevent the export of AtFT via interaction with FTIP1 and NaKR1 and prevent the uploading of AtFT into the SAM nucleus. However, we know that the subsequent interaction with the *Eucalyptus* FD homolog and other components of the *Eucalyptus* FAC with the *Arabidopsis* FT is sufficient to induce floral induction as we see the production of floral buds in transgenic material. Since the *AtFT-OX lfy* lines are driven by the 409S promoter, the AtFT protein has no need for transport to meristematic tissue to induce flowering. Another reason why no flowering is seen in our trial compared to other trials may have to do with the young age of the material we are working with, whereas in other studies the plant material was significantly physiologically older and was actively flowering in an age dependent manner. Thus, we might be missing crucial age dependent co-factors needed for the efficient transport of AtFT from the transgenic material to the young wildtype material.

3.6 Conclusion

In this study we found that the rooting efficiency of *AtFT-OX lfy* lines is lower than wildtype lines. We also showed that the AtFT produced in transgenic lines is sufficient to induce flowering in transgenic material. However, when grafted, we did not see any signs of floral induction in wildtype tissue, whether the wildtype tissue made up the rootstock or the scion. We also found that paclobutrazol application did not assist in the induction of flowering of wild-type tissue. However, we did find that paclobutrazol application significantly shortened the internodal lengths of both rootstock and scion shoots of treated plants. We hypothesize that the AtFT protein lacks the homology required to interact with native *Eucalyptus* co-factors which may be needed for the transport of FT and other interactions needed for floral initiation, and the material we are working with lacks the transcriptional profile of physiologically mature trees.

3.7 Figures and Tables

Table 3.1 Contingency matrix of rooting wildtype (WT) plants and transgenic (TG, AtFT overexpression *lfy* knockout) plants after three weeks of growth in root induction media. A Fisher's exact test yielded a p-value of 2.52×10^{-57} indicating that there is a highly significant difference between the rooting efficiency of wildtype plants and transgenic plants.

Line	Root	No roots	Total
WT	378	22	400
TG	181	219	400
Total	559	241	1600

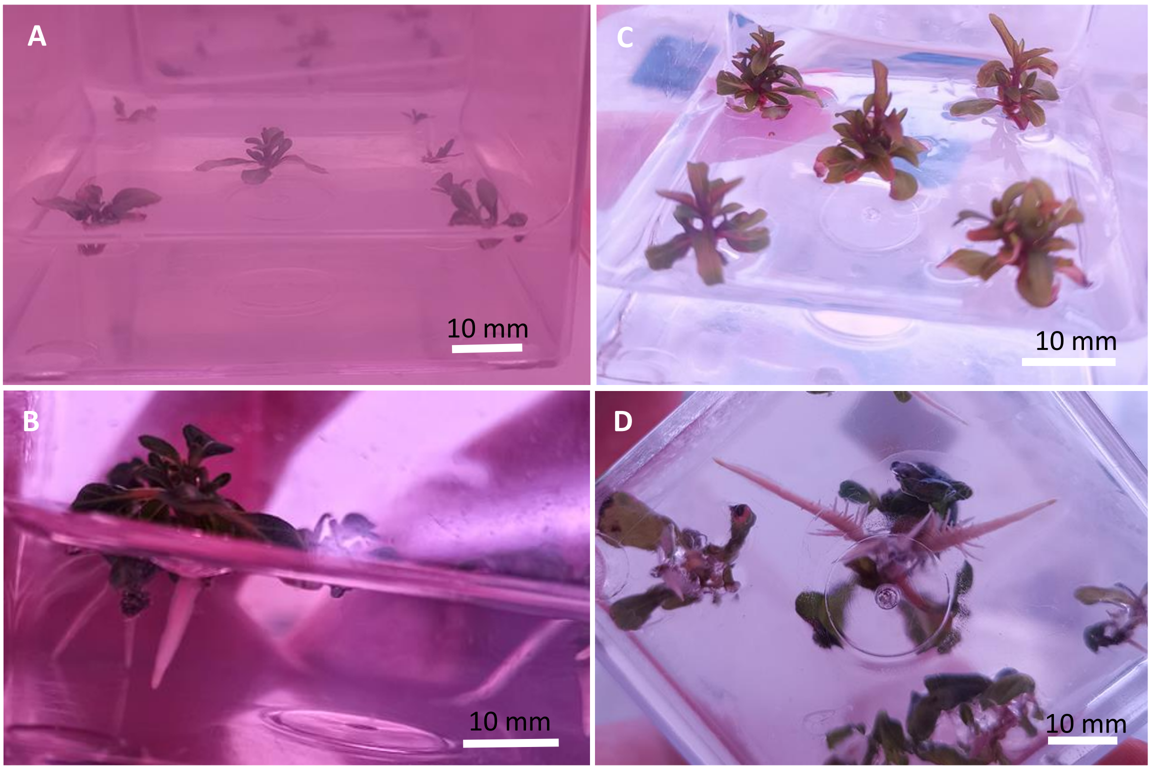

Figure 3.1 Tissue culture and rooting of *AtFT* overexpression *lfy* knockout and SP7 wildtype *Eucalyptus*. A and C show *AtFT* overexpression lines and SP7 wildtype *Eucalyptus* in multiplication media. B and D show *AtFT* overexpression lines and SP7 wildtype *Eucalyptus* plants rooting after three weeks of culturing in root induction media.

Figure 3.2 Cleft grafting of *Eucalyptus* stems. A) A scion is chosen that matches the diameter of the rootstock. B) The scion is cleaned, removing all side branches and leaves. C) The scion is carved with a razor blade to form a wedge shape D). E) The rootstock is cut down the middle to create a slit. F) The previously carved wedge is inserted into the slit of the rootstock. G) The slit and wedge are wrapped tightly with budding tape to ensure that there is no movement of scion and prevent loss of humidity. H) A small portion of the graft junction and the entirety of the scion are then wrapped with one layer of parafilm, to prevent loss of humidity and disease.

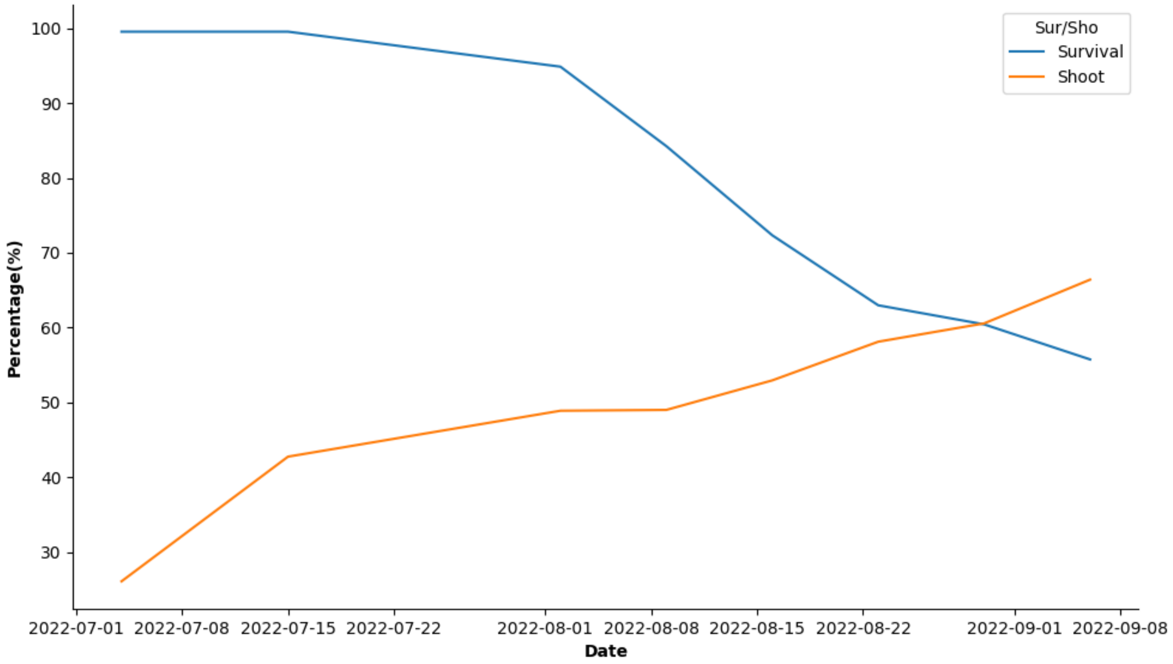

Figure 3.3 Grafting survival and shooting of grafted plants over time. The total number of surviving and shooting grafts were monitored and recorded. X axis shows time points of monitoring grafts. Over time the number of surviving scion material dropped to 55.7%, while the shooting rate increased to 66.4%.

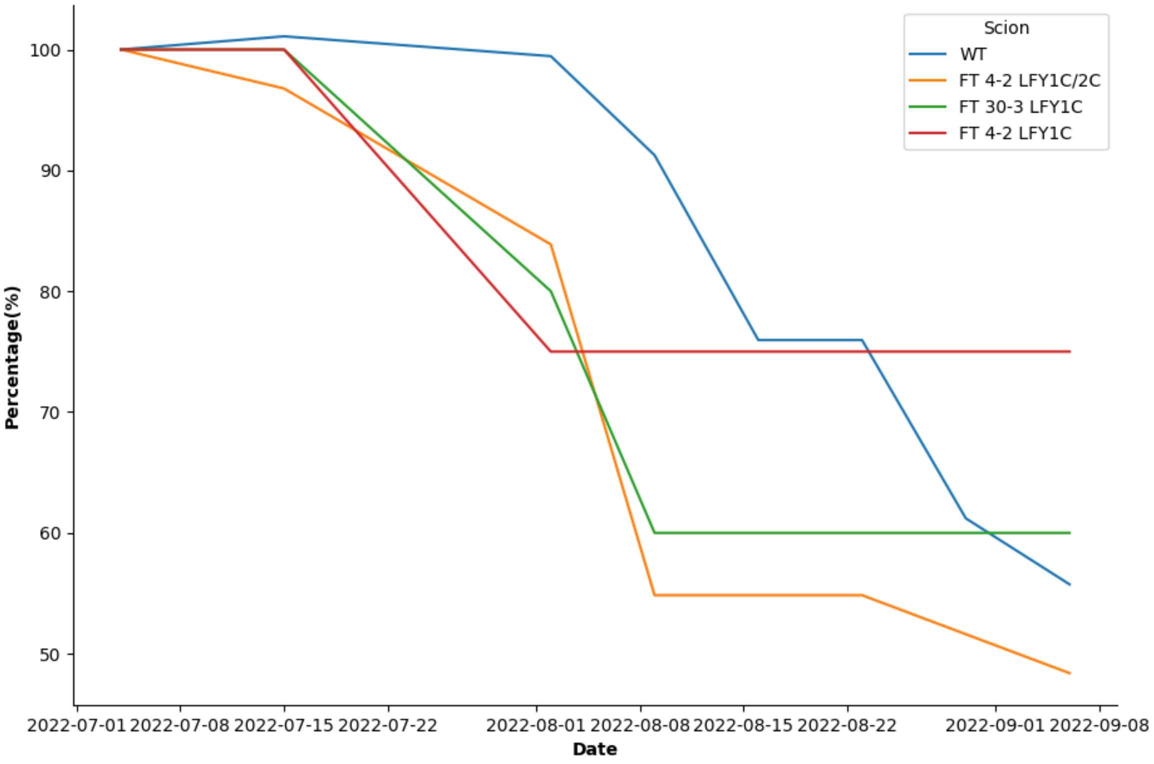

Figure 3.4 Survival of scions by transgenic line. Percentage of scion survival of each line recorded over time. WT = wildtype, each FT line was grouped separately. FT 4-2, FT 30-3 and FT 4-2 refer to the original transformation events (KLOCKO *et al.* 2016). LFY1C/2C, LFY1C refer to the original LFY knockout events (ELORRIAGA *et al.* 2021).

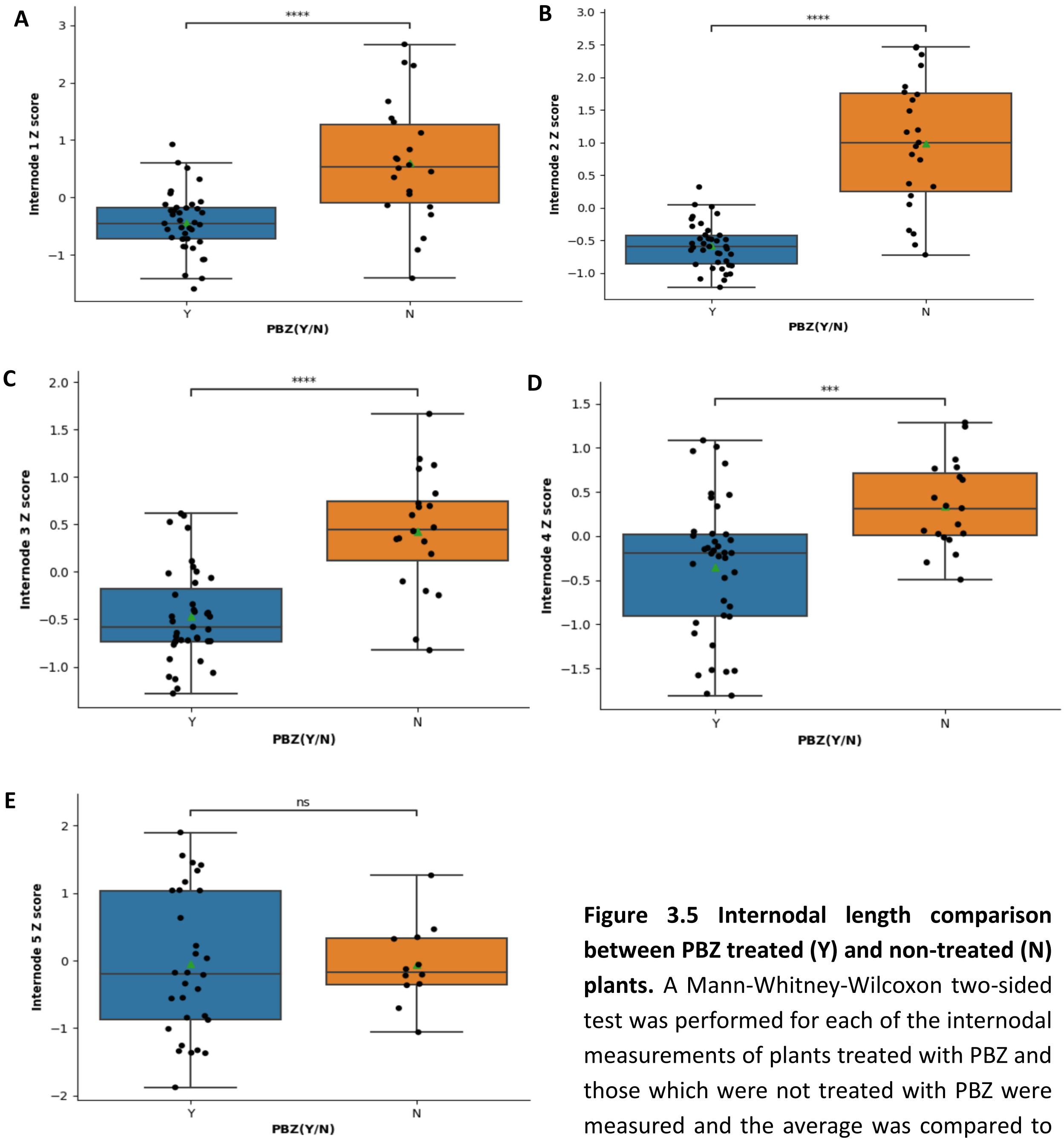

Figure 3.5 Internodal length comparison between PBZ treated (Y) and non-treated (N) plants. A Mann-Whitney-Wilcoxon two-sided test was performed for each of the internodal measurements of plants treated with PBZ and those which were not treated with PBZ were measured and the average was compared to determine whether the PBZ had an effect on the growth of the plants. A) Internode one measurements (n=62). B) Second internodal measurements (n=62). C) Third internodal measurements (n=62). D) Forth internodal measurements (n=60). The average standardized length of treated plants' first, second, third and fourth internodal lengths was significantly shorter on average compared to untreated plants' fourth internodal lengths. E) Fifth internodal measurements. The average (n=43) standardized length of treated plants' fifth internodal lengths was not significantly different from that of the untreated plants' fifth internodal lengths. All test statistics and p values can be found in Table S3.1. P-value annotation: ns (not significant): 5.00e-02 < p <= 1.00e+00, * p <= 5.00e-02, ** p <= 1.00e-02, *** p <= 1.00e-03, **** p <= 1.00e-04.

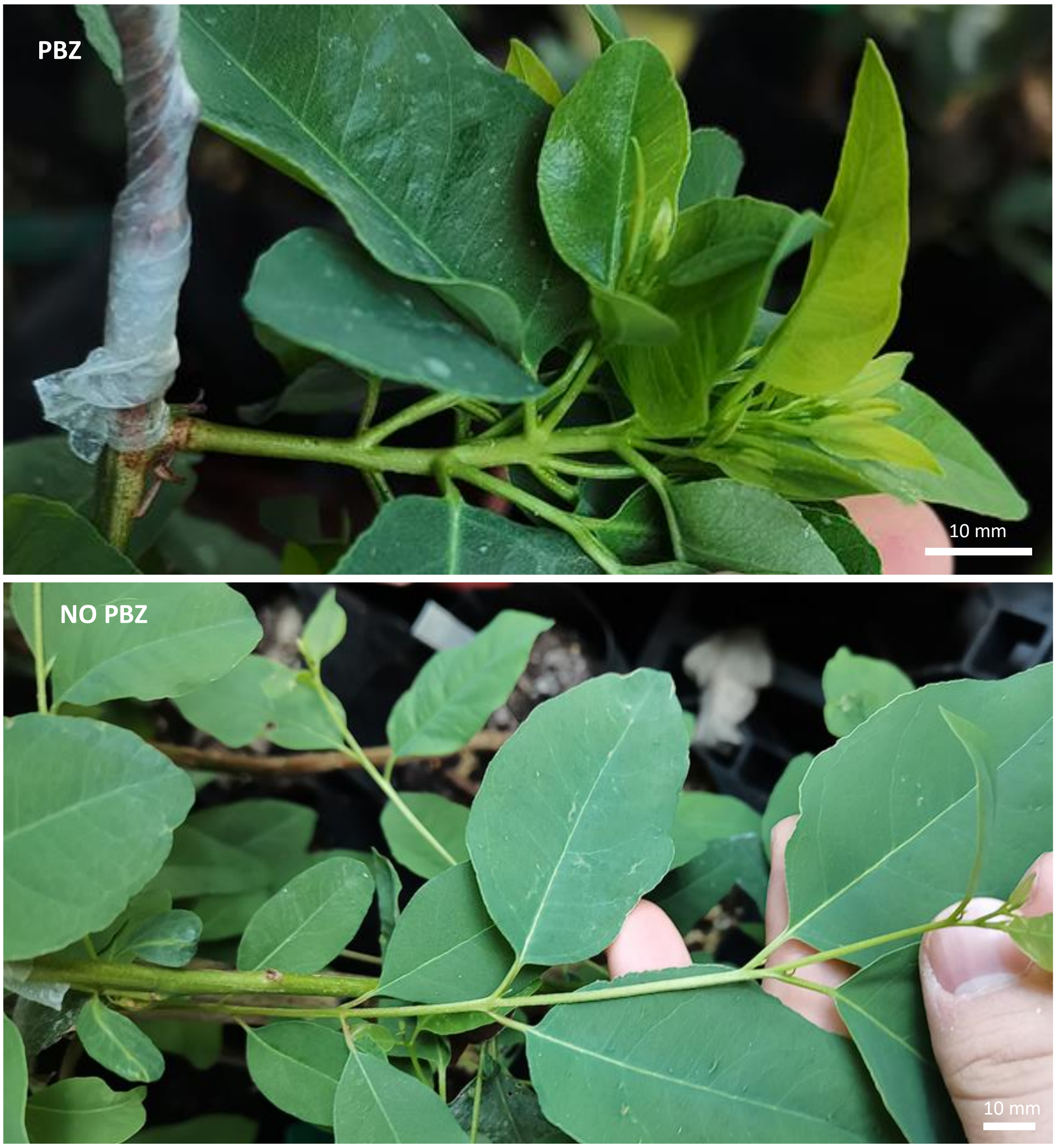

Figure 3.6 Phenotypic differences between PBZ treated and untreated plants. Top panel, an example of bushy, shortened internode phenotype of PBZ treated plants. The bushy phenotype was seen in rootstocks and scions. Bottom panel, normal untreated plant.

3.8 Supplementary tables and figures

Table S3.1 Statistical tests for comparison of internodal lengths of plants treated with and without paclobutrazol.

Internode	Test statistic	P value	Significantly different
1	$1.575x10^2$	$4.694x10^{-5}$	Yes
2	$6.400x10^1$	$2.196x10^{-8}$	Yes
3	$1.110X10^2$	$8.210x10^{-6}$	Yes
4	$1.700x10^2$	$9.214x10^{-4}$	Yes
5	$1.700x10^2$	$7.914x10^{-1}$	No

Figure S3.1 Plantlet acclimation in indoor growth rooms. Left panel, *Arabidopsis* FT overexpression, *LFY* knockout *Eucalyptus* lines producing good root growth in jiffy pellet. Right panel, SP7 wildtype *Eucalyptus* lines in jiffy pellets.

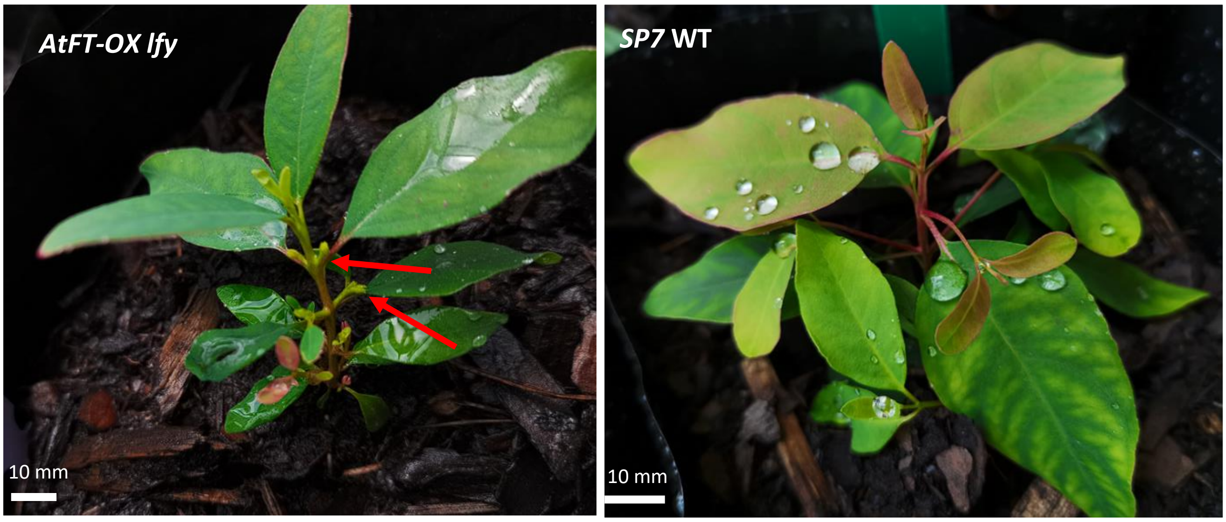

Figure S3. 2 Plantlet acclimation in phytotrons. Left panel, *Arabidopsis* FT overexpression, *LFY* knockout *Eucalyptus* lines, with signs of floral bud production (red arrows). Right panel, SP7 wildtype *Eucalyptus* lines, showing faster growth with no signs of floral induction.

Figure S3.3 Floral buds of *AtFT-OX* and *AtFT-OX lfy Eucalyptus* plants. Left panel depicts the normal *Eucalyptus* floral buds produced by *Arabidopsis FT* overexpression. Right panel depicts floral buds as a result of a *lfy* knockout, the plant is also overexpressing the *Arabidopsis FT* to induce early flowering.

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Chapter 4

Concluding remarks and future prospects

Eucalyptus is one of the most widely cultivated hardwood plantation crops in the forestry industry due to its fast-growth, good wood properties and wide adaptability. Its fast growth rate makes it attractive as a short-rotation crop. In South Africa, *Eucalyptus* plantations are primarily used to derive pulp and paper products and, increasingly, other lignocellulosic biorefinery products. Depending on the species' wood quality, the wood is also used in construction, such as flooring, furniture and mine tunnel supports (FORESTRY SOUTH AFRICA 2022). *Eucalyptus* species that are adapted to different climates is also an advantageous aspect of the genus allowing the cultivation of *Eucalyptus* in different parts of the world, ranging from tropical to cool temperate regions.

Although the genus has many benefits, a challenge from a breeding perspective is that some species are late flowering (sometimes after more than a decade). Some species such as *Eucalyptus grandis* and *E. urophylla* start to flower earlier at approximately three to five years of growth, but there can also be variation among parents which means that the full diversity of pollen may only be present several years after the first genotypes started to flower. There is a notable exception which is *E. occidentalis*, which can produce flowers within thirteen weeks of growth under inductive photoperiodic conditions (BOLOTIN 1975). Flowering time presents a hurdle to breeding programmes increasing the time to develop new elite genotypes. Even where genomic selection approaches can be implemented to predict breeding values at seedling level, the genetic gain can only be realized when the next generation is produced. Early induction of flowering is therefore an important biotechnology target that will help to realize the benefits of genomic selection approaches in forest trees.

In the first chapter of this thesis, we reviewed literature involving the photoperiodic regulation of *FT*, the regulation of *FT* expression in the leaf, the export of FT out of phloem companion cells and into the phloem stream, the unloading of FT and its ability to initiate floral development at the shoot apical meristem and finally biotechnology applications which has been attempted using *FT*. Although there are many studies on FT, the exact mechanism of its transport is not known. However, two major proteins, FTIP1 and NaKR1 are known to play a role (LIU *et al.* 2012; ZHU *et al.* 2016), but has only been investigated in *Arabidopsis,* and still needs to be looked at in large woody trees. Thus, the mechanism of FT transport in woody tree species needs to be investigated. The mechanism of unloading into the SAM is also not fully understood. It is hypothesised that FT binding to phosphatidylcholine may be a route, as well as standard diffusion (NAKAMURA *et al.* 2014), which can be investigated in trees in future studies. The newly discovered temperature regulated release of FT from companion cells (SUSILA *et al.* 2021) must also still be investigated in trees to further understand the regulation of FT and flowering in woody trees. Finally, I discuss how many researchers have used ectopic expression of *FT* to bypass the normal *FT* regulation and thereby achieve early flowering (AN *et al.* 2004; CORBESIER *et al.* 2007; SONG *et al.* 2013; WENZEL *et al.* 2013; YE *et al.* 2014; ADEYEMO *et al.* 2017; SOARES *et al.* 2020). This has also been used in conjunction with grafting to allow for the movement of FT produced in a transgenic manner to wildtype plant tissue and induce non-GMO flowers. This was only recently achieved in larger woody perennials such as *Citrus* (SOARES et al. 2020). From the literature reviewed I conclude that there is great potential in using FT and its graft-transmissible nature to shorten the breeding cycle of forest trees such as *Eucalyptus*.

In Chapter 2 we aimed to identify and functionally analyse the functional FT ortholog in *Eucalyptus*. We identified the most likely ortholog by phylogenetic analysis and 3D protein prediction and comparisons using AlphaFold2. Ultimately, we show that when the *Eucalyptus FT* ortholog is overexpressed it causes the *Arabidopsis* to flower early in a photoperiodic independent manner, similar to previous studies (CORBESIER *et al.* 2007; NOTAGUCHI *et al.* 2008). We also show that the *Eucalyptus* FT is localised to plant vasculature and is also able to cross a graft junction and move long distances, both of these findings are supported by previous literature (CORBESIER *et al.* 2007; CHEN *et al.* 2018). Thus, we hypothesise it is this EgFT protein which we have characterised that is most likely moving through top grafted *Eucalyptus* graft junctions and inducing flowering in juvenile material, shown in recent studies (DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022). Future research should include the investigation of ectopic expression of EgFT in *Eucalyptus*, and its graft transmissible potential.

In Chapter 3 we aimed to determine whether *Eucalyptus* overexpressing the *Arabidopsis FT* would travel into wildtype tissue, whether it is the scion or rootstock. We also wanted to determine whether paclobutrazol had an effect on growth of the grafted plants and if it aided in movement of the FT protein based on the results from other studies that show paclobutrazol shortening the time to flowering and increasing floral output (HASAN AND REID 1995; GARDNER AND BERTLING 2005; DE OLIVEIRA CASTRO *et al.* 2021; CASTRO *et al.* 2022). At the time of writing this thesis I have not recorded any early floral buds on the grafted wildtype *Eucalyptus* shoots. Both scion and rootstock of grafted plants treated with paclobutrazol had significantly shorter internodal lengths compared to those plants which were untreated. However, the plants treated with paclobutrazol showed no signs of early floral induction in the wildtype tissue. The transgenic FT overexpressing *lfy* knockout material continued to profusely produce floral buds similar to the untreated transgenic plants. We hypothesise that the reason for no floral induction in wildtype tissue in our grafting experiments is due to the following:

- First, the *Arabidopsis* FT protein lacks the homology to interact with *Eucalyptus* transport co-factors. This results in the confinement of the *Arabidopsis* FT protein to phloem companion cells and thus cannot be transported into the phloem stream and to the shoot apical meristem.

- Second, the plants may be physiologically too young and lack specific co-factors or other components needed for transport and subsequent floral development. However, it is confirmed that the 409S driven overexpression of the *Arabidopsis FT* in *Eucalyptus* is sufficient to induce early floral development (KLOCKO *et al.* 2016; ELORRIAGA *et al.* 2021), presumably because it is produced in the target cells and does not rely on long-distance transport in those plants.

Looking ahead we propose that the work could be taken further to eventually establish an accelerated breeding platform for *Eucalyptus* (Figure 4.1). Future studies should include *Eucalyptus* ectopically expressing the functional ortholog we have identified in Chapter 2 of this MSc dissertation. Other lines should include FT proteins from other woody perennials such as the *Citrus FT3*, which has been successful in graft transmissible floral induction in *Citrus* (SOARES et al. 2020). These plants can then be evaluated for early floral induction as well as graft transmissible induction of wildtype tissue in *Eucalyptus*. Transgenic rootstocks would preferably include the *lfy* mutation to prevent the formation of transgenic pollen and crossing of wildtype flowers with transgenic flowers. If early flowering of wildtype *Eucalyptus* tissue can be achieved this would allow one to significantly shorten breeding cycles (Figure 4.1). This approach can then also be tested for success in interspecies grafts, as this proposed technology would benefit later flowering species such as *E. dunnii* and *E. nitens* more than *E. grandis* and *E. urophylla*, which the technology is currently being developed in. The selection of progeny could also be assisted by genomic selection for genes (or haplotypes or structural variants) of interest that produce desirable phenotypes. With the significantly shorter breeding cycle, multiple genomic selection rounds could potentially be completed within the same time as a conventional breeding cycle, creating an accelerated, genome-assisted breeding platform, which would allow the creation of elite lines in a much shorter time than with traditional breeding. This would greatly increase the efficiency of breeding for the forestry sector and allow breeders to quickly select for genotypes and thus phenotypic traits of interest. For example, if a new pest or disease begins to damage or destroy plantations, an accelerated breeding strategy would be able to produce trees that are resistant to the pest or disease in a relatively short amount of time. With regards to climate change, specific genotypes could be selected for and bred to adapt with the changing environment. The successful creation and implementation of this technology, potentially using the EgFT we characterise, could create a paradigm shift in the breeding of large woody trees such as *Eucalyptus*.

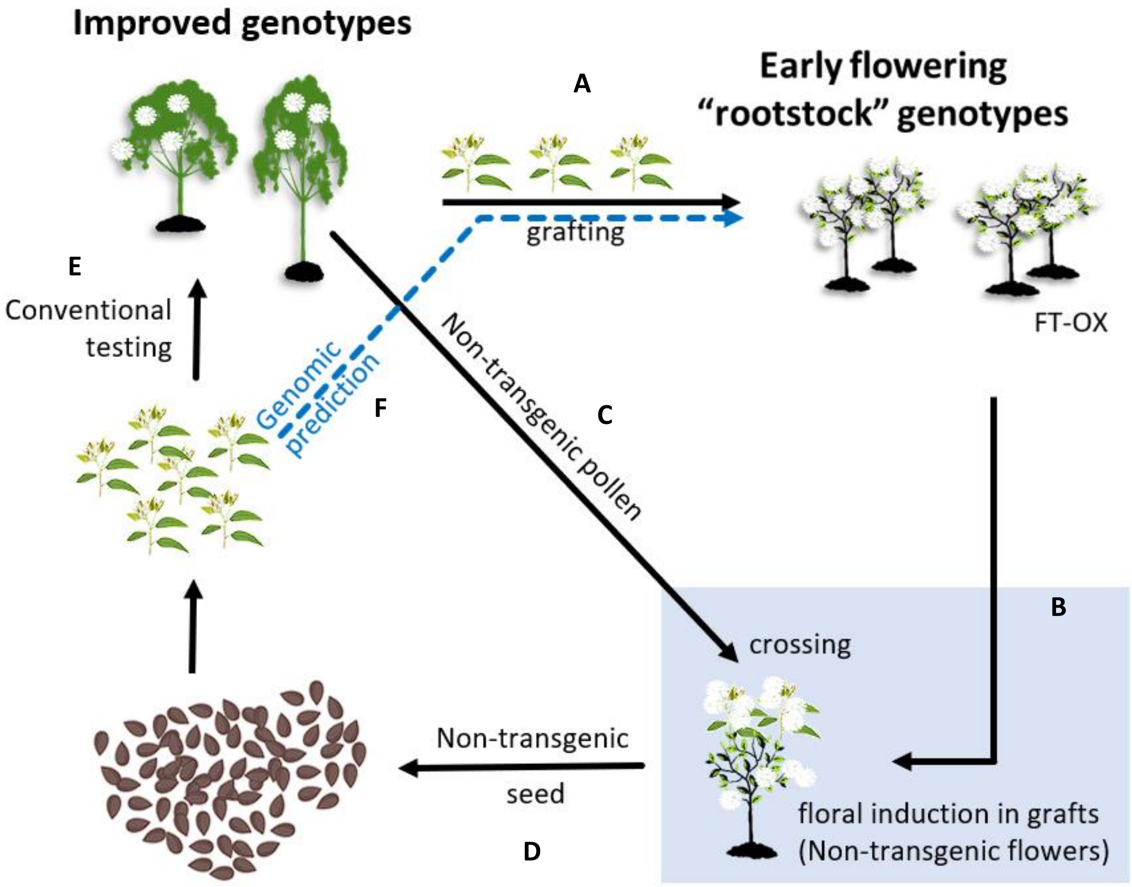

Figure 4.1 Proposed use of FT overexpression combined with grafting to accelerate breeding cycles in *Eucalyptus* trees. A) Current elite trees can be grafted onto transgenic rootstocks which are overexpressing a *FT* gene which can reliably induce early flowering. Rootstocks overexpressing an *FT* gene would preferably include the *lfy* mutation to avoid the production of transgenic pollen and prevent cross-pollination with wildtype flowers. B) Once the elite trees are grafted onto transgenic rootstocks, they will be induced to flower via movement of FT from transgenic rootstocks to wildtype material. C) Elite trees which are flowering early can then be used to make crosses with other elite trees from the field or from other trees which have also been induced to flower early. D) The crosses will result in non-transgenic seed. The seeds can then be germinated and taken through conventional testing (E), or a genomic prediction (F) approach can be used to further accelerate the selection of desirable traits. The progeny can then be taken to the next generation via the same cycle. The proposed breeding approach outlined here would shorten the breeding cycle to approximately 2 to 3 years from seed to seed.

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