Flowering in *Protea*: A Molecular and Physiological Study

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it to any university for a degree.

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Mariette Smart Date

Abstract

Proteas have been extensively cultivated and are grown as floricultural crop plants in many parts of the world, including South Africa. However, the factors that influence the initiation of flowering in *Protea* have not been identified. From data gathered by the *Protea* Atlas Project it is evident that *Protea* spp. have greatly varying flowering times. Furthermore, flowering times between *Protea* spp. and their hybrid cultivars are also very different. Towards a better understanding of the factors involved in floral initiation in this cultivated crop, three aspects of flowering were investigated in this study.

The carbon input into *Protea* inflorescence development was determined by measuring respiration rates and weights of developing structures. By manipulating source-sink ratios in plants, the carbon assimilatory capacities to support inflorescences were investigated in three cultivars and one wild-grown species of *Protea* which develop different sized flowers. As some *Proteas* flower in response to seasonal change, an orthologue of the floral inducer *FLOWERING LOCUS T* (*FT*), *ProteaFT* (*ProFT*), was isolated from 'Carnival' (*P. compacta* x *P. neriifolia*) and its expression pattern followed diurnally and seasonally. Finally, the functions of paralogous genes of *Protea LEAFY* (*ProLFY)* from 'Carnival' displaying sequence similarity to the meristem identity gene *LEAFY* from *Arabidopsis thaliana*, were investigated through heterologous expression studies in *A. thaliana*.

All *Protea* inflorescences were found to be carbon-expensive structures, but cultivated *Proteas* 'Carnival' and 'Ivy' had sufficient carbon assimilatory capacity to support their development. *P. repens,* displaying the wild growth habit of *Proteas*, and the year-round flowering cultivar 'Sylvia' (*P. eximia* x *P. susannae*) were however found to be carbon-limited, as photosynthetic rates of the source leaves were not reduced in response to inflorescence removal. 'Carnival' developed a carbon-expensive inflorescence, but flowering was mostly limited to spring and early summer. *ProFT* expression in 'Carnival' leaves increased during October, spring, when flowering was induced and the diurnal expression profile suggested that it may be up-regulated in response to increasing photoperiod at this time. Signalling pathways under control of the circadian clock may be responsible for the up-regulation of *ProFT* in response to change in day length. Robust circadian rhythms could be extracted from measurements of chlorophyll delayed fluorescence in 'Carnival' and its parental species. Four *ProLFY*

paralogues were isolated from ‘Carnival’ of which one, *ProLFY-WL4*, was able to rescue fertility and restore wild-type flower morphology to *lfy-2* mutants when heterologously expressed in *A. thaliana*. Ectopic expression of two other *ProLFY* paralogues, however, failed to rescue the *lfy-2* mutant flower phenotype and resulted in the development of abnormal-sized leaves.

Results from this study lead to the conclusion that carbon availability may play a role in determining floral competency: the age or developmental stage at which flowers can be borne rather than being directly involved in floral initiation. Seasonally induced flowering may be achieved through a photoperiod sensitive pathway under control of the circadian clock in ‘Carnival’. Studies such as this provide valuable insights into the physiological and genetic limitations of *Protea* flowering that can aid breeders and growers when attempting to manipulate flowering times.

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Publication

The following publication arose from work done during this study:

Smart M, Roden LC. 2010. A small-scale RNA isolation protocol useful for high-throughput extractions from recalcitrant plants. South African Journal of Botany 76: 375-379.

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List of abbreviations

°C	**degrees Celsius**
xg	**centrifugal force**
aa	**amino acid**
AG	**active growing**
ANOVA	**analysis of variance**
BA	**benzyl adenine / 6-benzylaminopurine**
bp	**base pair**
ca.	**'circa' / approximately**
cDNA	**complementary deoxyribonucleic acid**
CFR	**Cape floristic region**
CRE	***cis*-regulatory element**
CO	***CONSTANS***
CO_2	**carbon dioxide**
D	**dormant**
d	**day(s)**
dCAPS	**derived cleaved amplified polymorphic sequence**
DD	**constant dark**
DF	**delayed fluorescence**
DGGE	**denaturing gradient gel electrophoresis**
DNA	**deoxyribonucleic acid**
DW	**dry weight**
eIF4A	***eukaryotic Initiation Factor 4A***
FT	***FLOWERING LOCUS T***
g	**gram(s)**
GA	**gibberellin / gibberellic acid**
gDNA	**genomic deoxyribonucleic acid**
h	**hour(s)**
l	**length**
LSD	**least significant difference**

LD	**long days**
LFY	***LEAFY***
LL	**constant light**
m	**meter(s)**
M	**molar**
min	**minute(s)**
miRNA	**micro ribonucleic acid**
mRNA	**messenger ribonucleic acid**
n	**sample number**
OD	**onset of dormancy**
OG	**onset of growth**
P	**probability**
PCR	**polymerase chain reaction**
ProFT	***Protea FLOWERING LOCUS T***
ProLFY	***Protea LEAFY***
qRT-PCR	**quantitative real-time PCR**
QTL(s)	**quantitative trait locus(loci)**
r	**correlative value**
RAE	**relative amplitude error**
RIL	**recombinant inbred line**
RNA	**ribonucleic acid**
s	**second(s)**
SAM	**shoot apical meristem**
SD	**short days**
SDev	**standard deviation**
SE	**standard error**
semi-qPCR	**semi-quantitative PCR**
smRNA	**small RNA**
sp.	**species**
spp.	**several species**
TAIL PCR	**thermal asymmetric interlaced PCR**
TSS	**transcription start site**

UTR	**untranslated region**
v/v	**volume per volume**
vol	**volume**
w	**width**
w/v	**weight per volume**
WL	**wavy leaf**
ZT	**zeitgeber time**

Generally used terminology and nomenclature

Generally accepted gene names, symbols and genetic terminology were used in this thesis.

Organisms were referred to firstly by their common names, if these exist, and then their scientific names are given in parentheses and italicised. Names given to commercially developed hybrids are given in quotation marks and not italicised e.g. 'Carnival'.

Gene names are given in upper case letters and italicised e.g. *LEAFY* abbreviated to *LFY*. The abbreviations are defined in text upon first use in each chapter. In the case of mutated genes these are written as lower case letters e.g. *leafy* abbreviated to *lfy*. When referring to protein products, names are given in upper case letters but not italicised e.g. LEAFY / LFY. Mutant proteins are given in lower case and not italicised e.g. leafy / lfy.

To prevent confusion between proteins and genes from different species the prefixes *At*, for *Arabidopsis thaliana*, and *Pro*, for *Protea*, were included preceding the protein or gene name in chapter 4.

Definitions of terminology used:

Orthologous genes: Genes that show sequence similarity to previously identified genes, but were identified from different species.

Paralogous genes: Multiple genes with sequence similarity present within the same species' genome.

Protea spp.: *Protea* species from the *Protea* genus.

Proteas: Used to refer to both *Protea* spp. and hybrids of species.

CHAPTER 1: INTRODUCTION

1.1. Proteaceae and their cultivation

The Proteaceae comprises 1400 species in over 60 genera occurring exclusively on the southern continents: Australia; Africa; Central and South America; New Guinea and surrounding islands; New Caledonia; Madagascar; Southwest Asia and New Zealand (Rebelo, 2001). This distribution suggests a Gondwanan origin (Brits, 1984; Rebelo, 2001), although oceanic dispersal has also been implicated (Barker *et al.*, 2007). The species-rich Cape Floristic Region (CFR) of South Africa (SA) is home to 330 Proteaceae species representing 14 genera (Rebelo, 2001). Proteaceae, named after the mythological Greek god Proteus who could change shape at will, is a fitting name for such a large family diverse in floral shapes and sizes (Fig. 1.1 A-F; Rebelo, 2001).

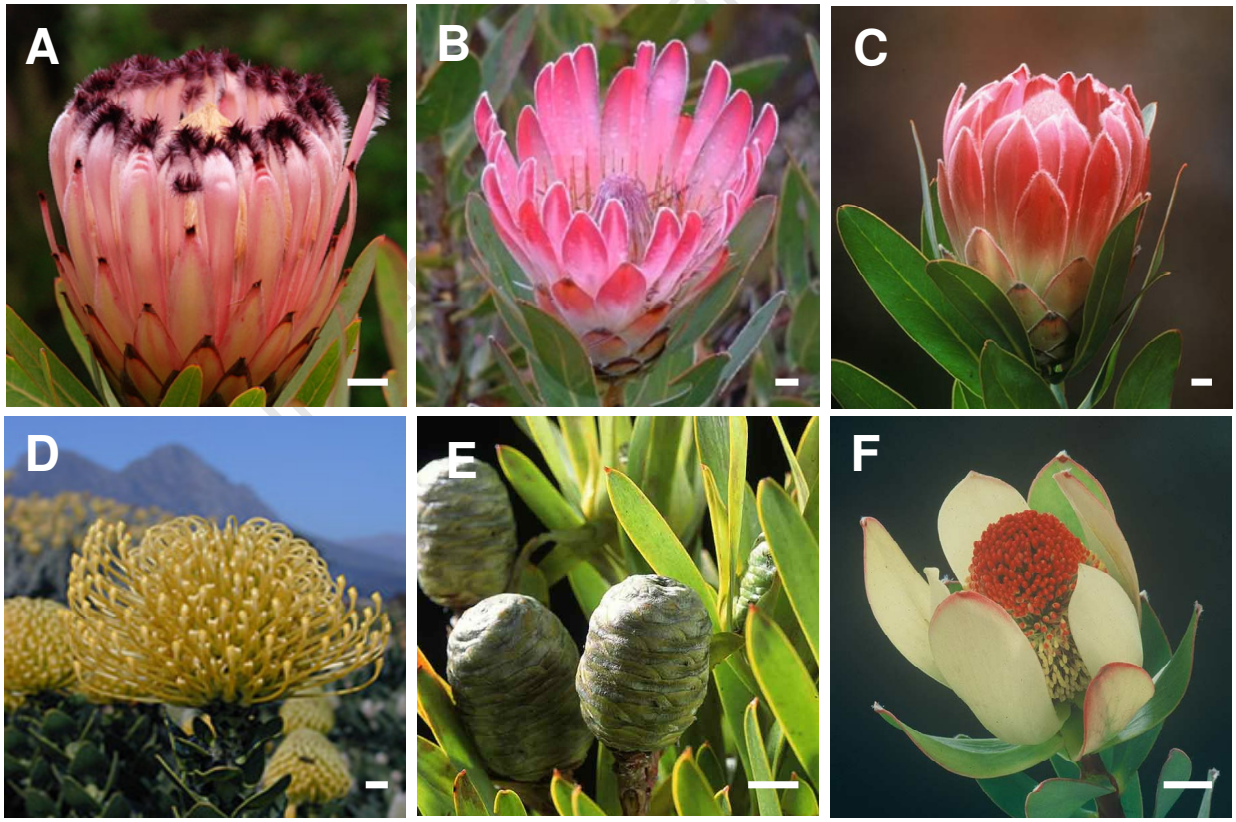


Figure 1.1. Floral diversity between Proteaceae species commonly used in floriculture. **A**. *Protea neriifolia*.. **B**. *Protea compacta*. **C**. 'Carnival' (*P. compacta* x *P. neriifolia*). **D**. *Leucospermum* 'High Gold' (*Ls. cordifolium* x *Ls. patersonii*) **E**. *Leucadendron meridianum* (♂). **F**. *Leucadendron discolor* (♀).Scale bars indicate 10 mm.

The large, solitary, terminal flowers of certain genera and their colourful displays have made them ideal candidates for cultivation as cut flowers. *Protea* (Fig. 1.1 A-C), *Leucospermum* (Fig. 1.1 D), *Serruria* and the dioecious *Leucadendron* (Fig. 1.1 E(♀) and F(♂)) genera have been cultivated in SA and consequently hybridised to produce new cultivars. Other Proteaceae genera native to Australia such as *Banksia, Grevillea* and *Telopea* are also cultivated, mostly there but also in SA. The area under Proteaceae cultivation in SA was estimated at 1000 ha in 2008 with 1100 ha forecasted for 2010 (Dorrington, 2008). The industry is worth a gross value of $40 million per annum (Dorrington, 2008), but has a much greater potential value. Industry expansion is mostly hampered by the seasonality of peak flower production. For many cultivars this does not fall within the optimal marketing period, September to February, corresponding to European winters and religious holidays (Gerber *et al.*, 2001c). 70% of Proteaceae flowers produced in SA are sold to Western Europe, with the rest (30%) divided between the United Kingdom, Middle and Far East, Eastern Europe and the United States (Dorrington, 2008). *Protea* is by far the most well known and sought after of the cultivated genera.

1.1.1. Protea *growth habit*

Protea spp. exhibit diverse growth habits ranging from upright to low-lying, sprawling shrubs (Rebelo, 2001), depending on their pollination syndrome. Bird pollinated flowerheads are carried in an 'upright' position, whereas rodent pollinated *Proteas* carry flowerheads at the ground-level. Wild-growing *Proteas* display a sympodial, branched growth habit (Kruger, 1979). Single growth flushes on mature plants terminate in developing inflorescences and the subsequent loss of apical dominance leads to the development of side shoots from the axillary meristems subtending the inflorescence. These side shoots, once mature, carry the following season's inflorescences. Commercially grown cultivars do not display this growth habit because of pruning, harvesting and 'de-budding' (removal of axillary meristems) that create a more attractive product and shift flowering time (Gerber *et al.*, 1995; Gerber *et al.*, 2001c; Greenfield *et al.*, 1994). Shoot growth in *Protea* cultivars occurs in successive growth or seasonal flushes. These flushes are pre-formed in the apical meristem during the elongation of the previous flush (Gerber *et al.*, 2001a). Some cultivars such as 'Carnival' (Fig. 1.1 C), a commercially developed hybrid of *P. compacta* and *P. neriifolia* (Fig. 1.1 A and B),

arrest growth during the winter season and enter a period of dormancy. During early spring, growth resumes with the formation of a new seasonal (spring) growth flush.

1.1.2. Flowering of Protea

Protea do not develop single flowers, but rather up to 240 individual flowers (florets; Gerber *et al*., 2001a) comprised of a perianth, style and pollen presenter grouped together on a receptacle (Fig. 1.2 A; Rebelo, 2001). Up to 100 colourful involucral bracts surround these florets (Fig. 1.2 B) and are often white, yellow, orange or red to attract birds, such as the Cape Sugarbird (*Promerops cafer*), for pollination. Florets have a superior ovary containing one ovule at the base (Fig. 1.2 B; Rebelo, 2001). These bird-pollinated species also produce large amounts of 'sugary' nectar from four nectaries situated at the base of the ovary (Fig. 1.2 B; Rebelo, 2001). *Protea* florets are hairy and have a silvery-white to light pink colour and silky appearance as can be seen on Fig. 1.1 A-C. Average inflorescence width can range from 22.5 mm for *P. punctata*, the smallest flowered sp., to 210 mm for *P. cynaroides*, the large 'King' *Protea* (Rebelo, 2001).

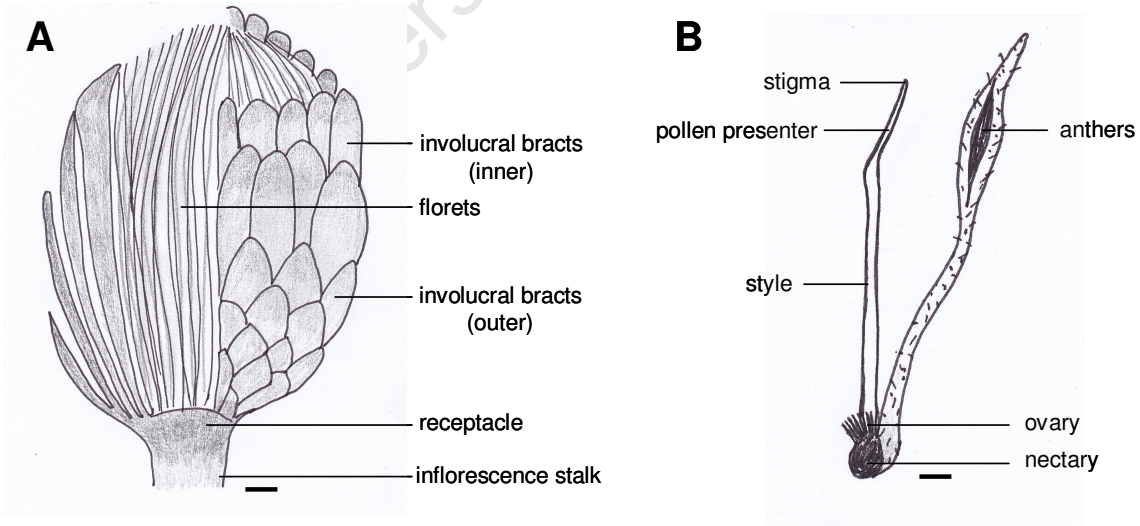


Figure 1.2. Morphologic characteristics of a *Protea* inflorescence and floret. A. Diagrammatic representation of *Protea* inflorescence (adapted from data within Rebelo, 2001). Scale bar indicates 10 mm. **B.** Single *Protea* floret (flower). Scale bar indicates 1 mm.

Flowering times of *Protea* spp. and hybrids differ significantly, with some flowering in spring to autumn, some late winter to early summer, and others throughout the year (Gerber *et al.*, 2001c). Most *Proteas*, however, will flower at the same or similar time in successive years (Gerber *et al.*, 2001a). Very little is known about floral initiation and development in these plants and therefore manipulation of flowering times to secure a continuous supply of cut flowers, as can be achieved for other species such as chrysanthemum (*C. morifolium*; Langton, 1978) and globe daisy (*Globularia sarcophylla*; Katz *et al.*, 2003), is not yet possible. Efforts to manipulate flowering times of *Protea* have been focused on pruning and the application of the cytokinin 6-benzyladenine (BA; reviewed below).

'Carnival' (*P. compacta* x *P. neriifolia*) is routinely pruned for biennial bearing which synchronises shoot development to ensure maximum flower production (Gerber *et al.*, 1995). This involves removal of all shoots during the winter to leave only a 15 to 20 cm portion (bearer) that will carry the new flushes that will develop from axillary meristems in the spring. If subtended by three previously-formed growth flushes (Greenfield *et al.*, 1994), these shoots will flower on the spring flush two years after pruning (Gerber *et al.*, 2001a). 'Carnival' (Fig. 1.1 C) flowers in the summer months January to May, whereas 'Sylvia' (*P. eximia* x *P. susannae*) flowers throughout the year (Gerber *et al.*, 2001a). Pruning of 'Sylvia', as described for 'Carnival', performed at 4-weekly intervals beginning late September (early summer) and ending in early May (autumn) successfully shifted peak flowering periods (Gerber *et al.*, 2001a). However, flowering was always more readily initiated on the spring flush. Hoffman *et al.* (2009) reported that topical treatment of 'Carnival' meristems in autumn with a 500 ppm BA solution after the extension of the autumn flush shifted the start of flower production from January to November. BA-treated shoots did not enter a winter dormant state as observed for untreated shoots, but rather developed another growth flush. Flowering was subsequently initiated on the 'out-of-season' flush during the winter months. This resulted in flower harvest between November and January, within the peak European marketing time.

1.2. Requirements of flowering

Proteaceae develop especially large inflorescences (Fig. 1.1 A-C; Coetzee and Littlejohn, 2001; Rebelo, 2001) that require sufficient resources such as carbon,

nutrients and water (Bazzaz and Carlson, 1979) to ensure reproductive success. To ensure an adequate supply of the above-mentioned resources, flower initiation needs to be tightly regulated to coincide with periods wherein these are plentiful and post-initiation conditions will be favourable for pollination, seed set and dispersal. Some plants induce flowering in response to short days, some do not depend on photoperiod to induce flowering (i.e. day neutral plants) while others flower in response to an increase in photoperiod as experienced at the onset of spring. Seasonally-induced flowering is dependent on the circadian clock to ensure its correct timing. A limited amount of literature is available on the resource requirements and floral initiation of *Protea*, therefore most of the work reviewed here is from model annuals and woody perennial trees.

1.2.1. Annual versus perennial growth and flowering

The annual herbaceous plant, *Arabidopsis thaliana* (*A. thaliana*), has been extensively used for molecular genetics and physiological studies alike. Its short life-cycle, ease with which transgenic plants can be produced and the fact that the genome was fully sequenced more than 10 years ago makes it the ideal model plant (Somerville and Koornneef, 2002; Amasino, 2010). However, even in extensively studied model plants a large number of proteins with unknown functions are still being identified. Many years of research, mostly on *A. thaliana*, snapdragon (*Antirrhinum majus*), morning glory (*Ipomoea nil*) and rice (*Oryza sativa*) have been focused on the pathways involved in the agriculturally important event, flowering. These annual 'model' plants include monocotyledonous and dicotyledonous plants as well as long day (LD) and short day (SD) responsive plants. The perennial-plant gap has recently been partially filled with the completion of the genome sequencing of the long-lived, woody tree *Populus trichocarpa* (Jansson and Douglas, 2007). Research on woody perennials is important for forestry and farmed crops such as fruits and nuts that are produced on woody trees. Woody perennials differ from their annual counterparts in many ways, the definitive difference being their long life-spans and the maintenance of some meristems in a vegetative state while developing others florally in a process known as polycarpy. This allows perennial plants to complete several cycles of vegetative and reproductive growth during their life-spans. Understanding the mechanisms behind these periodic growth habits and the extended juvenile periods experienced before long-lived

perennials are responsive to floral stimuli are of agricultural importance to growers and breeders (Wilkie *et al*., 2008; Bangerth, 2009).

Following embryonic development, during which plants utilise stored reserves from seeds for growth, three ontogenetic states can be discerned for flowering plants namely; the juvenile vegetative, adult vegetative and adult reproductive phases (Greenwood, 1987; Poethig, 1990). These age-related phases are often linked to changes in morphology, physiology and biochemical characteristics which may include changes in: leaf shape, size and pattern of cellular differentiation (heteroblasty); whole plant branching pattern; disease and pest resistance; capacity for adventitious root development; and finally reproductive competency (Gatsuk *et al*., 1980; Poethig, 2010). The juvenile phase is associated with the 'incompetence' of the shoot apical meristem (SAM) to respond to floral inductive conditions; while during the adult phase the SAM is 'competent' and floral transition can occur under inductive conditions. Phase changes do not occur abruptly and may lead to stages where there is a mix of morphological characteristics, termed intermediacy (Poethig, 1990). The most obviously example of this is the transition from an adult vegetative to a reproductive meristem and the development of flowers. Organs in the first two floral whorls, sepals and petals, have a vegetative appearance and only organs comprising the inner two whorls, stamens and carpels, have specific reproductive function. The division of transitional states into three distinct phases is therefore a gross oversimplification of a very complex process that requires the action of multiple pathways acting in parallel and series to facility phase transition (Poethig, 1990).

Interactions between early acting genes versus those that control downstream processes are required to facilitate successful phase transition (Lawson and Poethig, 1995). In the case of vegetative to reproductive development, early acting genes would be those that respond to floral inductive conditions such as photoperiod, vernalisation or phytohormonal signals (discussed in sections 1.3.1 through 1.3.4); whereas late acting genes would be the meristem and floral-organ identity genes (discussed in section 1.4). The transition between juvenile and adult vegetative phases is less studied, however is of great importance in perennial systems where this transition is required before plants will become competent to flower and consequently produce fruit and seeds. The floral

inhibitor gene, *TERMINAL FLOWERING LOCUS1* (*TFL1*), has been implicated in the control of this transition in perennial plants (Bergonzi and Albani, 2011).

TFL1 acts in the shoot apical meristem (SAM) of *A. thaliana* and is responsible for maintaining a vegetative state (Shannon and Meeks-Wagner, 1993; Bradley *et al.*, 1997; Ratcliffe *et al.*, 1998). Studies of *TFL1* orthologues from woody perennial plants suggest that they fulfil a similar role in these systems and may be responsible for maintaining the vegetative state of juvenile SAMs (Bergonzi and Albani, 2011). Similarly, recent work by Wang R *et al*. (2011) on a vernalisation sensitive perennial species, *Arabis alpine* (*A. alpine*) closely related to *A. thaliana*, has shown that *AaTFL1* is responsible for maintaining juvenility in young meristems. When young plants were exposed to an inductive vernalisation treatment, *AaTFL1* mRNA levels did not decrease as observed for older plants. *AaLFY*, an orthologue of the floral meristem identity gene *LEAFY* (*LFY*; discussed in section 1.3.6.1), was not expressed in the meristems of younger plant whereas vernalisation lead to an up-regulation of *AaLFY* in older plants. The authors propose that *AaTFL1* blocks flowering in the meristems of young plants exposed to inductive floral conditions. This 'repression' decreases as the meristem ages thus allowing flowering to occur at an age-dependent gradient along the plant, thus ensuring some meristem are available for the next season's flowering. Similarly, high levels of *CsTFL* present in stem tissue of juvenile orange (*Citrus sinensis*) trees prevented flowering, while in adult trees low *CsTFL* levels in stem tissue during floral inductive conditions allowed floral development (Pillitteri *et al.*, 2004). Strong down-regulation of *PopCEN1*, the *TFL1* orthologue in *Populus trichocarpa*, by interfering RNA (RNAi) resulted in a reduction of the juvenile phase in *P. trichocarpa* plants from four to two years (Mohamed *et al.*, 2010). *P. trichocarpa* expressing *35S::PopCEN1* did not flower during the five years of study. This suggests that *TFL1* orthologues in woody perennial trees may be involved in maintaining juvenility. *TFL1* may act redundantly with other genes involved in maintaining the *A. thaliana* meristem in a vegetative state such as: *FLOWERING LOCUS C* (*FLC*; Helliway *et al.*, 2006; Searle *et al.*, 2006) and *SHORT VEGETATIVE PHASE* (*SVP*; Hartmann *et al.*, 2000). Studies of *FLC* (*PtFLC*) and *SVP* (*PtSVP*) orthologues identified from trifoliate orange (*Poncirus trifoliate*) suggest possible involvement in juvenility. Different alternatively spliced variants of *PtFLC* were identified from juvenile and mature buds suggesting that the regulation of *PtFLC* transcript levels through alternative splicing may be involved in this phase

transition (Zhang *et al*., 2009). *PtSVP* was highly expressed in juvenile shoots and *35S::PtSVP* expression in *A. thaliana* plants caused delayed flowering and the appearance of juvenile characteristics during the adult phase (Li *et al*., 2010).

Small RNAs (smRNAs), specifically the microRNA (miRNA) miR156, and plant-specific transcription factor family SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) have also been implicated in juvenile to adult vegetative transition (Wu and Poethig, 2006). Ten of the 16 known *SPL* genes in Arabidopsis contain target sites for miR156 binding (Rhoades *et al*., 2002). During the juvenile phase miR156 levels are high and act to bind *SPL* transcripts and thus repress translation of SPL transcription factors. However, during juvenile to adult phase transition the levels of miR156 decreases to allow an increase in SPL proteins in the shoot apex (Wu and Poethig, 2006). Many of these SPL transcription factors have been directly linked to early flowering events (Schwab *et al*., 2006). SPL3 transcriptionally activates expression of *FUL* (*FRUITFULL*), *AP1* (*APETALLA1*) and *LFY* (Yamaguchi, 2009) involved in cellular differentiation and meristem identity, while SPL9 is known to up-regulate expression of *FUL*, *SOC1* and *AGL42* (Wang *et al*., 2009) involved in the floral transition (Fig. 1.3). Another miRNA, miR172, highly expressed in the adult phase is involved in the further initiation of flowering. It is expressed to high levels in adult leaves where it leads to a reduction in AP2-like proteins such as the floral repressors APETALA2 (AP2; Chen, 2004) TARGET OF EAT1-3 (TOE1-3; Aukerman and Sakai, 2003), SCHLAFMÜTZE (SMZ) and SCHNARCHZAPFEN (SNZ; Schmid *et al*., 2003). These proteins repress the expression of *FLOWERING LOCUS T* (Aukerman and Sakai, 2003; Mathieu *et al*., 2009) the floral integrator discussed in section 1.3.5. In summary, an age dependent decline in miR156 allows the up-regulation of flowering related genes which together with an increase in miR172, responsible for down-regulating the repressors of FT, facilitates the transition from juvenile to adult development permissive for flowering. Evidence that miR156 may also be involved in juvenile to adult transition of perennial plants were derived from the ectopic over-expression of *Corngrass 1*, the maize miR156, in switchgrass (*Panicum virgatum*; Chuck *et al*., 2011). Constitutive expression of *Cg1* resulted in the arrest of transgenic switchgrass plants in a juvenile state. These results suggest that the role of miR156 in phase transition is conserved across annuals and perennials.

The first evidence that a similar mode of action may be prevalent in woody perennial plants came from a recent study by Wang J-W *et al*. (2011). Expression levels of miR156 and miR172 were measured in woody trees for which the juvenile and adult characteristics have been well defined. miRNA levels for two *Acacia* species, *A. confuse* and *A. colei*, *Eucalyptus* (*E. globulus*), English ivy (*Hedera helix*) and sawtooth oak (*Quercus acutissima*) correlated well with juvenile-to-adult phase transition based on shoot and leaf development. miR156 was highly expressed in juvenile leaves, while miR172 had the opposite expression pattern. The expression of miR156 targets, homologues of A*. thaliana AtSPL3* and *AtSPL9* in *E. globulus*, were also higher in adult leaves which correlated well with the lowered miR156 levels in these tissues. In the same study, further evidence for the involvement of miR156 in juvenile-to-adult transition in woody trees was gained from *35S::MIR156* transformed hybrid poplar (*Populus* x *canadensis*; *P. deltoids* x *P. nigra*). Transformants displayed lengthened juvenile phases and anatomical leaf characteristics corresponding to the juvenile phase. The length of juvenility is especially important for perennial plants as a lengthened juvenile phase results in increased leaf and root biomass (Jones, 1999); thus ensuring the availability of sufficient reserves, such as carbon and nutrients, during flowering and for continued cycles of growth and reproduction following flowering.

1.2.2. Carbohydrates and flowering

Carbohydrates are required for the formation of floral structures, pollen germination and pollen-tube growth (Stadler *et al.*, 1999). Developing inflorescences also expend carbon for 'maintenance respiration', protein turnover and metabolism (Penning de Vries, 1975; McDermitt and Loomis, 1981; Ploschuk and Hall, 1997). Some flowers excrete nectar as an attractant for pollinators and these require even greater amounts of carbon to produce the sugar-rich compounds (Southwick, 1984; Thomson *et al.*, 1989). In *Protea*, 'Carnival' shoots defoliated 40 days before spring bud-break did not develop any inflorescences, suggesting that the presence of leaves was essential for floral initiation (Gerber *et al.*, 2002). The spring growth flushes developed on these 'leaf-less' shoots were shorter and had fewer leaves than those of un-manipulated shoots. Similar results were also found for the cultivar 'Lady Di' (*P. compacta* x *P. magnifica*; Gerber *et al.*, 2001b). Studies in flowering rose plants (*Rosa hybrida* L., cv. Dallas) showed that the leaves closest to the developing flower had the highest photosynthetic rates and were thought to contribute the most carbon to flower development (Matloobi *et al.*, 2008).

However, the utilisation of stored reserves for the development of sink tissues has been reported under unfavourable conditions for orchid flowers (*Oncidium* Gower Ramsey; Wang C-Y *et al*., 2008), peach fruit (*Prunus persica*; Lopez *et al*., 2007) and grape berries (*Vitis vinefera*; Bennet *et al*., 2005). Some Proteaceae, such as *P. cynaroides,* are known to store carbohydrates in large lignotubers, which are utilized for resprouting following the destruction of above ground tissue by fire (Stock *et al*., 1992; Rebelo, 2001). It is possible that these storage carbohydrates may also supplement recently fixed carbon during flowering. Current photosynthate provided by photosynthetically active source leaves is, however, likely to be the most important for inflorescence development. In many species an insufficient supply of photosynthate leads to inflorescence or fruit abortion (Day *et al*., 1994; Marcelis *et al*., 2004; Rajala *et al*., 2009; Hiyane *et al*., 2010, Ruan *et al*., 2010).

The photosynthetic capacities of leaves are partially determined by their size and shape (allometry), as these determine surface area available for sunlight interception. As photosynthesis provides the metabolites for the construction and maintenance of new cells and tissues, and leaf size determines total carbon assimilation, it can be assumed that the number and size of source leaves will dictate the number and size of sink tissues it can support (Niklas, 1994). A number of studies have observed correlations between the size or number of leaves and those of either flowers or fruit. For example, in apple (*Malus x domestica*) the number of leaves was strongly correlated with the number of flowers on 1-year old shoots (Lauri *et al*., 1996). Leaf number was also correlated with the number of fruit that developed. Species across the *Puya* genus (Bromeliaceae) showed a significant correlation between inflorescence and leaf length (Hornung-Leoni and Sosa, 2006). Within *Protea* significant allometric relationships have also been demonstrated between leaf and inflorescence mass for a range of species (Midgley and Bond, 1989; Le Maitre and Midley, 1991). An allometric study by Weiner *et al.* (2009) reviewing the relationship between vegetative and reproductive biomass in a number of herbaceous plants showed statistically significant correlations between these two characteristics for many of the species studied. Some species showed higher degrees of plasticity around the general trend, suggesting that plants may differ in their response to photosynthate availability and that other environmental pressures may also be influencing vegetative and reproductive characteristics. These authors proposed that plants may fit one of the following reproductive strategies: 1) plants following the simple

linear reproductive to vegetative biomass relationship, such as herbaceous annuals, will start reproduction as soon as they reach the minimum size required, or 2) plants will invest more resources into growth and only flower when they reach a specific size or age, a strategy which fits the perennial growth habit. The photosynthetic capacity of plants depends on the surface area and rate of photosynthesis of source leaves, which has been found to be controlled by sink activity in a number of plants (see below for examples).

Source-sink relationships in plants are dynamic and influenced by environmental factors and the developmental stages of plant organs (Stephenson, 1981). Many plants will coordinate source supply and sink demand as required (Hofmeyr and Cornish-Bowden, 2000). The photosynthetic rates of source leaves from sweet pepper (*Capsicum annuum*) could adjust their photosynthetic rates to match the carbon demand of flowers, or fruit, during reproduction (González-Real *et al.*, 2009). Leaves subtending rapidly growing fruit with high carbon requirements had higher photosynthetic rates than leaves subtending fruit at other development stages. Interestingly, leaves subtending flowers had higher photosynthetic rates than those supporting fully developed fruit, suggesting that flower development in pepper surpasses the carbon demand of fruit after the initial high growth rates experienced during early development. This indicates that the photosynthetic rates of source leaves have a high degree of plasticity and can fluctuate as the requirement for carbon changes during sink development. Changes in the photosynthetic rates of leaves are often utilised to investigate source-sink relationships by manipulation of source- or sink-strength (Herold, 1980; Obeso, 2002). Sink-strength may be altered by flower/fruit removal or the girdling of stems, whereas source-strength is often altered by defoliation or shading. For instance, artificially decreased source capacity lead to an increase in the photosynthetic rates of un-manipulated leaves of sugarcane (McCormick *et al.*, 2006) and citrus (Iglesias *et al.*, 2002), whereas partial defoliation of the *Protea* hybrid, 'Lady Di' (*P. magnifica* x *P. compacta*) resulted in delayed flowering (Gerber *et al.* 2001b). Reducing sink strength by girdling, defruiting or sucrose supplementation resulted in reduced photosynthetic rates in source leaves of citrus (Iglesias *et al.*, 2002), sweet pepper (*Capsicum annuum* L.; Hall and Milthorpe, 1978) and spinach (*Spinacia oleracea* L.; Krapp and Stitt, 1995).

These studies are useful when studying the carbon demand of developing inflorescences, source leaf capabilities and identifying source- and sink-limited systems. This may be important for assessing production or possible manipulation thereof in a horticultural setting. Changes in the photosynthetic rates of source leaves or the biomass of sink tissue after source-sink manipulation may provide evidence of the carbon status of a plant system. For example, variation in source-sink ratios of *Chrysanthemum* hybrids by flower-bud removal, removal of axillary shoots and varying daily light periods had a significant effect on flower size measured as dry weight (Carvalho *et al.*, 2006). Removal of all competing sinks (axillary shoots and other flowers) leaving only a single flower on a plant, resulted in the development of flowers that were 2.4-times heavier than those of the control plants which supported up to 12 flowers simultaneously. In the same study, increased photoperiod subsequent to floral induction under SD conditions, resulted in the development of heavier flowers presumably linked to increased source activity under longer day-lengths. Similar increases in reproductive output, total soluble solids and fresh weights of fruits, were also found after sink reduction through the partial removal of a proportion of flowers from fruit trees (Walsh *et al.*, 2007). In cloudberry (*Rubus chamaemous*), ramets showed no photosynthetic response when developing berries or foliage from ramets were removed, although smaller berries developed on defoliated ramets (Gauci *et al.*, 2009). $^{14}CO_2$ labelling indicated that ramets carrying developing berries were the sole source of carbon supply to the berries, and that they also translocated carbon to the rhizome. The authors suggest that the lack of increase in photosynthetic rates after defoliation may mean that leaves are already photosynthesising at maximum capacity. These systems are examples of source-limited plants, as any available carbon will be utilised for sink growth and development. In a sink-limited system, as suggested for girdled fruit-bearing branches of coffee (*Coffea arabica*), removal of fruit resulted in a reduction in the photosynthetic rates of leaves on the supporting branch (Franck *et al.*, 2006). Girdling of the stem/branch prevented possible translocation of carbon to other sinks, thus localising the response to the unit studied.

A role for sugar signalling in the balance of carbon supply in plants has been suggested by Smith and Stitt (2007) and a similar signalling mechanism may be utilised by plants to measure carbon status prior to initiating flowering. Preceding floral initiation the sugar concentration in the phloem has been shown to increase (Corbesier *et al.*, 2002). An

earlier study by King and Zeevaart (1973) also showed increased movement of assimilates in the phloem upon floral induction in *Perilla crispa*. *A. thaliana* seedlings grown under dark conditions could initiate flowering after formation of a certain number of leaves when grown on media containing sucrose (Roldán *et al.*, 1999), whereas those not supplemented with sugar remained vegetative. The authors suggested that sugar-induced flowering in the dark was initiated through a GA (gibberellin) dependent pathway (discussed in section 1.3.) as GA-deficient or insensitive mutants did not flower in the dark. Similarly, increased sugar efflux from leaves into sink tissue of potato (*Solanum tuberosum*) resulted in early flowering and tuberisation (Chincinska *et al.*, 2008). The sugar status of a plant is measured through the hexokinase glucose sensor (recently reviewed by Smeekens *et al.*, 2010). Hexokinase is the enzyme responsible for the conversion of glucose to glucose 6-phosphate and has been shown to be involved in a complex crosstalk with hormone signalling. *A. thaliana hxk1* mutants, deficient in hexokinase, showed reduced vegetative growth, delayed flowering and altered sensitivity to hormones such as auxins and cytokinins (Moore *et al.*, 2003). The transport between source and sink tissue is regulated by hexose transporters. Recently, a hexose transporter was identified localised to the vacuolar membranes of *A. thaliana* floral organs (Aluri and Büttner, 2007). *Atvgt1* mutants with defective AtVGT1 function displayed delayed flowering and the seeds had low germination efficiencies. Sugars therefore do not only play an important part in plant development as nutrients for growth, but also as signalling molecules that influence floral initiation. A strong interaction between nutritional status and carbohydrate assimilation exists in plants. Limited nutrient availability has been found to negatively affect photosynthetic rates as well as vegetative and reproductive growth of various plant species (Milewski, 1983; Karlsson, 1994; Kanai *et al.*, 2007). This may be especially relevant to members of the Proteaceae which mostly grow in nutrient-poor soils.

1.2.3. Nutrient requirements for flowering and seed filling

Proteaceae generally inhabit soils with limited nutrients such as nitrogen and phosphate (Cowling and Holmes, 1992; Groom and Lamont, 2010) and will allocate up to 40% of annual phosphate uptake to seeds (Kuo *et al.*, 1982; Witkowsky and Lamont, 1996; Groom and Lamont, 2010). Although nutrient availability may be limited to Proteaceae plants, they develop some of the most costly seeds within the angiosperms, both in total weight and nutrient content (Stock *et al.*, 1990; Henery and Westoby, 2001). Many

authors suggest that nutrients rather than carbon are the limiting resources for *Protea* growth, development and reproductive success (Johnson, 1992; Stock *et al.*, 1992; Witkowsky and Lamont, 1996).

Seeds from Proteaceae plants grown in nutrient-rich soils have a lower nutrient content compared to seeds of plants grown in nutrient-poorer soils (Esler *et al.*, 1989; Stock *et al.*, 1990). This is probably because seedlings germinating in nutrient rich soils, close to their parental plants, will be able to access nutrients from the environment and therefore require fewer stored reserves. Under nutrient-enriched conditions two *Hakea* species (Proteaceae) showed increased plant dry mass and stem height compared to plants grown under nutrient-poor conditions (Knox and Clarke, 2005). Furthermore, under nutrient-limiting conditions plants accumulated more starch in their tissues compared to nutrient-enriched conditions. Trade-offs in resource allocation under suboptimal nutritional conditions may lead to carbon-enriched situations (Milewsky, 1983), especially for Proteaceae species growing in Mediterranean-type infertile soils. To ensure that flowering is initiated under favourable conditions for photosynthesis and nutrient uptake, plants utilise environmental cues such as day-length which is perceived through the circadian clock. Links between the circadian clock and nutritional status through nitrogen availability (Gutiérrez *et al.*, 2008) and plant metabolism through sucrose signalling (Dalchau *et al* 2011) has previously been proposed, indicating cross-talk between these pathways and circadian timing.

1.2.3. Perception of environmental changes

Circadian clocks are endogenous timekeepers present in most living organisms. These clocks facilitate the anticipation of predictable environmental changes such as the onset of day or night and the changing of seasons, thus allowing the correct physiological and developmental adjustments to occur. In plants, the circadian clock is responsible for regulating many of the developmental changes associated with seasonal change. Circadian rhythms, generated by the circadian clock, oscillate with an approx. 24 h period (McClung, 2006). These circadian rhythms, or endogenous oscillators, are entrained by environmental cues (so called ‘zeitgebers’, meaning ‘time givers’) such as light/dark (photoperiod) or temperature cycles (Somers *et al.*, 1998; Salomé and McClung, 2005). The key characteristic of circadian clocks is that they are self-sustained, meaning that they will persist under constant conditions such as constant

light (LL) or dark (DD) (Wang and Tobin, 1998; Tóth *et al*., 2001). Another defining feature is the ability of the clock to reset itself when given different stimuli (Edwards *et al*., 2010). In *A. thaliana*, the plant clock has been well studied at the molecular level and the current understanding is that rhythms are generated by the interlocked transcriptional and translational feedback loops between genes and proteins of the core oscillator.

The gene products of *CIRCADIAN AND CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), that encode for MYB transcription factors, together with a member of the pseudo-response regulator (PRR) family, TIMING OF CAB 1 (TOC1), were identified as components of the plant circadian clock core oscillator (Millar *et al*., 1995; Schaffer *et al*., 1998; Wang and Tobin, 1998; Strayer *et al*., 2000; Makino *et al*., 2002; Más *et al*., 2003). These findings were based on the abolishment (arrhythmia) or alteration of the circadian rhythm when these genes were mutated, silenced or over-expressed. The current consensus is that CCA1 and LHY act redundantly (Alabadi *et al*., 2001; Mizoguchi *et al*., 2002), to bind the *TOC1* promoter and repress its expression during the early morning (Alabadi *et al*., 2001). During the day, CCA1 and LHY levels decrease through transcriptional repression of *CCA1* and *LHY* by PRR5, 7 and 9 (Nikamichi *et al*., 2010). This results in increased *TOC1* levels with a peak in expression during the evening. TOC1 physically associates with *CCA1* and *LHY* promoters to activate their expression (Pruneda-Paz *et al*., 2009), thus completing the loop. The oscillations generated by the circadian clock directly regulate the expression of many clock-controlled genes involved in diverse physiological processes. However, it is through circadian regulation of GIGANTEA (GI) and its interaction with *CONSTANS* (*CO*) that the photoperiodic induction of flowering is achieved (Park *et al*., 1999; Mizoguchi *et al*., 2005). Photoperiodic flowering will be discussed in more detail in later sections.

A robust and accurate circadian clock was found to enhance plant fitness in *A. thaliana* (Green *et al*., 2002; Micheal *et al*., 2003; Dodd *et al*., 2005). Measurement of clock function in agriculturally important crop plants could therefore provide information on the 'fitness' traits of these species (Gould *et al*., 2009). Clock function is measured under LL or DD conditions in the absence of zeitgebers. Rhythmic leaf movements and hypocotyl elongation were some of the earliest circadian outputs observed and measured in plants

(reviewed in McClung, 2006). These measurements have been used in numerous *A. thaliana* studies to investigate clock function (for example Schaffer *et al.*, 1998; Park *et al.*, 1999). More accurate and robust rhythms are however generated by measuring the transcript levels of clock genes (as seen in Park *et al.*, 1999). Gene expression may be investigated in two ways: 1) by periodic sampling of tissue for RNA extraction and subsequent expression analyses by real-time PCR; or 2) using promoters from clock genes to drive expression of reporter genes such as Firefly *luciferase* (*luc*; Millar *et al.*, 1995). Both these methods require a molecular knowledge of the circadian clock in the study organism and the latter also requires a stable transformation system. These are luxuries mainly limited to studies of model plants and make these methods unsuitable for assaying clock function across various species.

A recent study by Gould *et al.* (2009) identified measuring levels of delayed fluorescence (DF) as an accurate means to study circadian rhythms in plants. Delayed, or chlorophyll, fluorescence is emitted from chlorophyll *a* in the red and far-red spectra and is produced during the initial stages of photosynthesis (Pedrós *et al.*, 2008). DF was found to be under robust circadian control in *A. thaliana* and results were comparable to those generated by *CHLOROPHYLL A/B BINDING PROTEIN2::LUC* (*CAB2::LUC*) expression studies (Gould *et al.*, 2009). DF was also successfully used to measure clock function in non-model plants such as barley (*Hordeum vulgare*), maize (*Zea mays*), lettuce (*Lactuca sativa*), *Capsella bursa-pastoris* and *Kalanchoë fedtschenkoi* (Gould *et al.*, 2009). As the clock is pivotal in the measurement of day-length changes associated with specific seasons, measuring the functionality of circadian clocks is also important for flowering studies.

1.3. Floral inductive pathways: the link between stimulus perception and flower formation

A combination of external and endogenous factors, their perception and relay of information through various floral pathways to the SAM are required for the initiation of flowering. Our current understanding of the genetic control of flowering has been compiled predominantly from the model plant systems discussed in 1.2.1. The following sections will briefly review our current understanding of the four most prominent flowering pathways as well as mention alternative roles for some of the key regulatory genes in woody perennial species.

Flowering in *A. thaliana* is induced through the integration of environmental and endogenous signals from the flowering pathways. Environmental signals such as inductive photoperiod or prolonged cold exposure influence flowering through the photoperiodic and vernalisation pathways; whereas endogenous signals are relayed through either the autonomous or gibberellin (GA) pathways. The integral role of the floral inducer, *FLOWERING LOCUS T* (*FT*), is discussed in section 1.3.5. *FT* has been identified as the point of convergence for the floral inductive pathways, with the exception of the GA pathway. An integrative overview of the flowering pathways and their interaction is shown in Fig. 1.3.

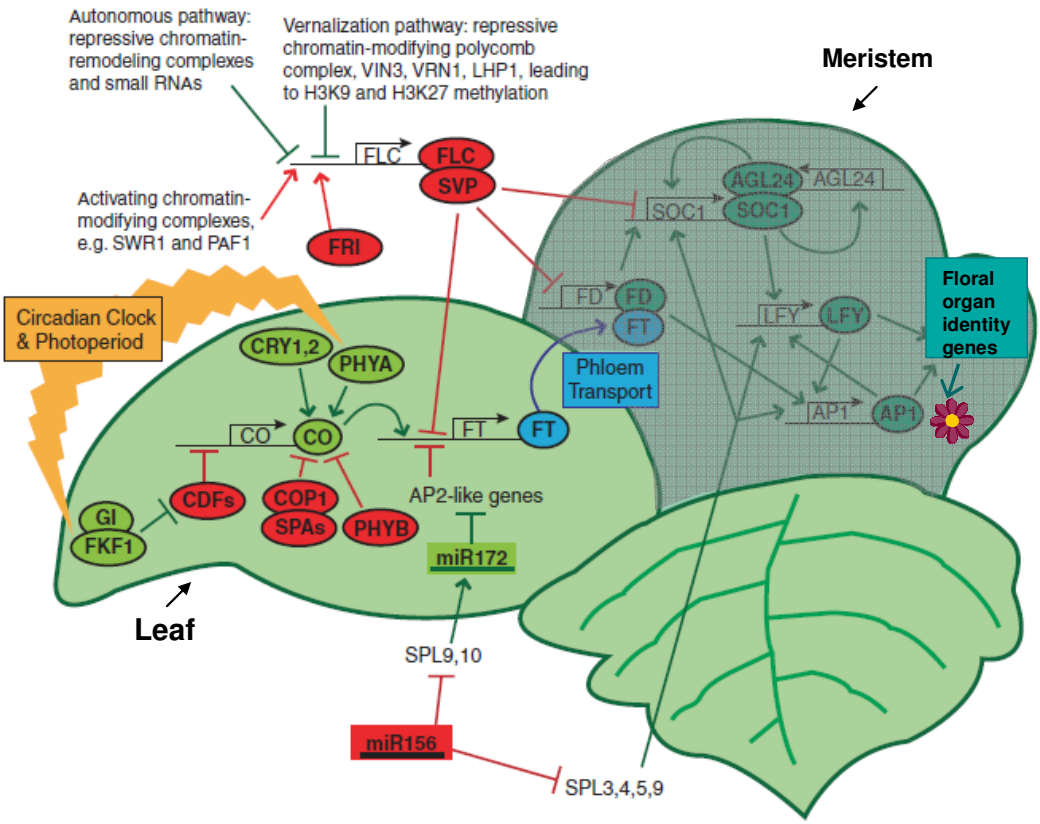


Figure 1.3. An integrated view of the floral inductive pathways. The various pathways and their interacting genes and proteins are as discussed in the text. Green arrows indicate promotion whereas red lines with perpendicular bars indicate repression. Repressors of flowering are indicated in red, activators in green. Proteins are indicated by abbreviated names in circular shapes; gene transcripts are indicated by lines and arrows with abbreviated names (taken from Amasino, 2010). The full names of genes and proteins are given in text when discussed in appropriate sections.

1.3.1. Vernalisation

Vernalisation is a period of prolonged cold exposure (10°C to -1°C) that is required for the meristems of some plants to become competent to respond to floral inductive conditions (Simpson and Dean, 2002). The main purpose of a vernalisation requirement is to inhibit floral induction during the autumn period (Amasino, 2010), thus preventing flower development during the winter months when low temperatures may damage floral tissue and most pollinators are absent. Photosynthetic rates may also be low during the winter due to low temperatures and light intensities resulting in reduced carbon available for flower development. Unlike photoperiod which is perceived in the leaves, vernalisation is perceived directly by the SAM. The site of perception was established by grafting and localised chilling experiments (Chouard, 1960; Bernier *et al.*, 1981). The vernalised state can be stable in SAM cells as a 'memory of winter' for a lengthy period before responding to a floral inductive cue (Sung and Amasino, 2006). This is achieved by the stable epigenetic suppression of the floral inhibitor *FLOWERING LOCUS C* (*FLC*).

The requirement for vernalisation has been linked to the *FRIGIDA* (*FRI*) allele (Gazzani *et al.*, 2003). FRI represses flowering, until plants are exposed to a sufficient cold period, by maintaining high mRNA levels of the floral suppressor *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 2000). FLC represses flowering through the down-regulation of the floral activators *SOC1*, *FD* and *FT*, which is achieved through the direct binding of FLC to the promoters of *SOC1* and *FD* and to a region in the first intron of *FT*, thus preventing transcriptional activation of these genes and subsequent floral transition (Fig. 1.2; Helliwell *et al.*, 2006; Searle *et al.*, 2006). To down-regulate *FLC* expression after exposure to a sufficient cold period, a complex regulatory process is employed. After an initial exposure to cold temperatures, the expression of a non-coding anti-sense RNA, *COOLAIR*, located in the 3' region downstream of *FLC* increases (Swiezewski *et al.*, 2009). As the exposure to cold temperatures continues, *COOLAIR* transcript keeps increasing until it reaches a peak at which stage *FLC* mRNA levels start decreasing. At this time the expression of another non-coding RNA, expressed in the sense orientation, starts increasing. *COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*) is also located at the *FLC* locus and recruits the polycomb repressive complex 2 (PRC2) proteins to the FLC locus (Heo and Sung, 2011). The PRC2 complex is composed of the following proteins: CURLY LEAF, SWINGER and VERNALIZATION2 (VRN2).

PRC2 together with VERNALIZATION INSENSITIVE 3 (VIN3) which is expressed under cold conditions (Sung and Amasino, 2004) are responsible for the stable repression of *FLC* (De Lucia *et al.*, 2008; Wood *et al.*, 2006). Epigenetic repression of *FLC* is achieved through the trimethylation of H3 K27 histones (Bastow *et al.*, 2004), thus allowing flowering to occur (Fig. 1.3; He *et al.*, 2004; Sung and Amasino, 2004).

1.3.2. Autonomous

Autonomous flowering refers to floral induction in the absence of environmental cues. Mutations in the genes involved in this pathway cause delayed flowering under both LD and SD (Simpson and Dean, 2002). Together these genes act to suppress *FLC* expression through RNA binding/processing and chromatin-remodelling events (Fig. 1.3; Kim *et al.*, 2009; Michaels, 2009) independently from the vernalisation pathway. RNA-binding domains and homology to RNA-processing factors have been identified in the following autonomous genes: *FCA*; *FPA*; *FLOWERING LOCUS K* and *FY* (Feng *et al.*, 2011; Herr *et al.*, 2006; Liu *et al.*, 2007). Other autonomous pathway genes are predicted to act as histone methyltransferases or demethylases. These include *FLOWERING LOCUS D*, *RELATIVE OF EARLY FLOWERING 6* and several members of the *PROTEIN ARGININE METHYLTRANSFERASE* family (Liu *et al.*, 2007). *FLC* expression is regulated through a complex interaction that involves non-coding RNA transcripts and chromatin modifications (reviewed by De Lucia and Dean, 2010).

Another autonomous pathway has recently been identified and includes the miRNAs, miR156 and miR172, which are also involved in the transition from a juvenile to an adult phase and were discussed in detail in section 1.2.1. Expression of miR156 delays flowering and lengthens the juvenile phase of *A. thaliana* and maize (*Zea mays*) through repression of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes (Fig. 1.3; Wu and Poethig, 2006; Wang J-W *et al.*, 2008; Wang J-W *et al.*, 2009). Conversely, miR172 expression was found to promote flowering by repressing the *AP2*-like genes that represses *FT* expression (Fornara and Coupland, 2009).

Temperature-sensitive genes have also been shown to influence *A. thaliana* flowering time. High temperatures induce flowering through *FT* regulation by FLOWERING LOCUS M (FLM; Balasubramanian *et al.*, 2006). Floral induction by FLM and vernalisation respond to extreme temperature fluctuations whereas small fluctuations are perceived by the thermo-sensory pathway which also influences flowering through

FT. *SHORT VEGETATIVE PHASE* (*SVP*) encodes an *FT* repressor (Fig. 1.2; Hartmann *et al*., 2000; Lee *et al*., 2007) that is under clock-dependent regulation by GI (Yoshida *et al*., 2009). At elevated ambient temperatures *SVP* expression levels reduce, thus allowing *FT* expression. The *PHY* (Franklin *et al*., 2003; Halliday *et al*., 2003) and *CRY* (Blázques *et al*., 2003) photoreceptors have also been implicated in temperature sensing.

1.3.3. Gibberellins (GAs)

The GA-dependent pathway acts on genes downstream of *FT* in the SAM. GAs responsible for induction of flowering in *A. thaliana* are produced in the leaves under SD conditions and transported to the SAM where they up-regulate *LFY* (Blázques *et al*., 1998; Eriksson *et al*., 2006) and *SOC1* (Bernier and Perilleuz, 2005) thus promoting flowering. The induction of flowering by GAs has been best demonstrated in the grass *Lolium temulentum* where GA_5 has been shown to be the intermediate signal between LD induced flowering in the leaves and the initiation of flowering in the shoot apex. Following an increase in GA_5 levels in the SAM other GA levels, GA_1 and GA_4, increase. These are presumed to be later acting in the floral response (King *et al*., 2001; King and Evans, 2005; King *et al*., 2006). GA dependent floral inductive signalling also requires the action of GA specific MYB (GAMYB) transcription factors (Gocal *et al*., 1999; Gocal *et al*., 2001). The classic phytohormones however, do not seem to have consistent responses across the plant kingdom. Treatments including the exogenous application of various GA functional-analogues or GA biosynthesis inhibitors and the measurement of endogenous GA concentrations in fruit trees have provided evidence that GAs act as floral repressors in these trees (see references below). In cherry (*Prunus avium*) the concentration of various GAs were lower in the shoots of flowering plants compared to vegetative shoots and seeds (Blake *et al*., 2000). While in mango (*Mangifera indica*) trees, the application of a GA biosynthesis inhibitor (paclobutrazol) by soil drench resulted in the acceleration of flowering time and increased the number of flowers produced (Blaikie *et al*., 2004). The exogenous applications of GA_3 and GA_{4+7} to apple also resulted in the delayed transition of vegetative to reproductive development (Bertelsen *et al*., 2002). As many different biosynthetically active GAs are produced in plants, experiments where GAs are exogenously applied often result in highly variable results. These variations may be a consequence of the specific GA applied and the concentration as well as the location of application (see issues discussed by Bangerth,

2009). Together these results suggest a floral inhibitory role for GA in fruit trees, although the molecular target and mode of action are still unknown.

1.3.4. Photoperiod

Floral induction in response to seasonal change is regulated by the circadian clock in angiosperms. Some plants flower in response to SD, such as rice and *Ipomoea nil*; some in response to LD such as *A. thaliana*; and others such as tomato (*Solanum lycopersicon*) flower irrespective of day-length, and are thus 'day neutral'. The *CONSTANS* (*CO*) gene is critical in photoperiodic sensing (Putterill *et al.*, 1995); linking day-length measurement by the circadian clock with up-regulation of the floral inducer *FT* (Samach *et al.*, 2000; Wigge *et al.*, 2005). The coincidence of *CO* mRNA with light (Fig. 1.4; Roden *et al.*, 2002; Yanovsky and Kay, 2002; Song *et al.*, 2010) allows the stabilisation of CO protein through complex interactions with inducers and repressors. In LD plants this leads to *FT* induction (Fig. 1.4), whereas in rice, a SD plant, the light-stabilised orthologous CO protein, Hd1, acts to repress expression of the *FT* orthologue *Hd3a* (Hayama *et al.*, 2003), thus inhibiting floral induction under non-inductive LD conditions.

The expression of *CO* is controlled by the circadian clock, through the action of GIGANTEA (GI), such that *CO* levels peak during the night phase under SDs (Fig. 1.4 and Fig. 1.5; Yanovsky and Kay, 2002; Imaizumi *et al.*, 2003). CO protein is however rapidly degraded in darkness by CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1; Jang *et al.*, 2008; Liu *et al.*, 2008) and SUPPRESSOR OF PHYA proteins (SPA; Fig. 1.5; Laubinger *et al.*, 2006). The *CO* mRNA peak in LD is broader as a result of the breakdown of a *CO* transcriptional suppressor, CYCLING DOF FACTOR (CDF) by the ubiquitin ligase FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN1 (FKF1; Imaizumi *et al.*, 2003; Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). This allows translation of *CO*, with CO protein stability in the late afternoon further enhanced by phytochrome A and cryptochrome 2 which repress CO degradation (Yanovsky and Kay, 2003; Valverde *et al.*, 2004). Active and stable CO activates *FT* expression through promoter binding (Adrian *et al.*, 2010; Tiwari *et al.*, 2010) possibly in complex with a haem activator protein (HAP; Wenkel *et al.*, 2006). See Fig. 1.5 for a diagrammatic representation of the control of *CO* mRNA and protein levels (Lagercrantz, 2009).

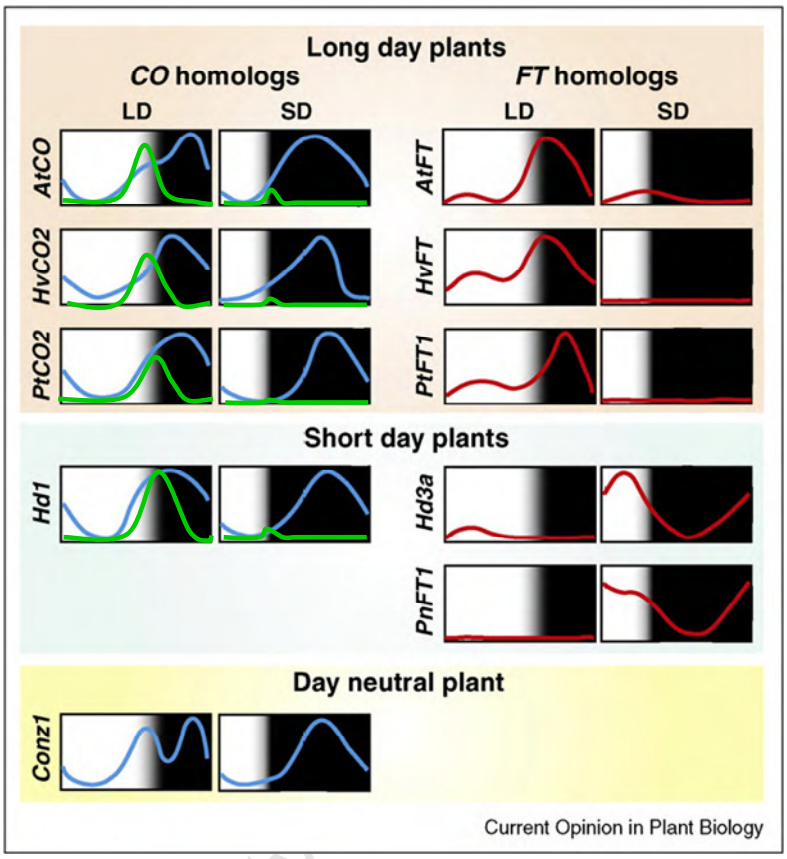


Figure 1.4. *CO* and *FT* expression under different photoperiods for plants with different flowering strategies. The long day (LD) plants are: *Arabidopsis thaliana*, barley (*Hordeum vulgare*) and poplar (*Populus tricocarpa*). Rice (*Oryza sativa*) and Pharbitis (*Ipomoea nil*) are used as examples of short day (SD) plants and maize (*Zea mays*) a day neutral plant. *CO* mRNA expression (blue line) is similar under LD and SD, but CO protein (green line) is only present when the mRNA expression peaks coincide with light. In LD plants this results in an up-regulation of *FT*, while in SD plants CO suppresses *FT* expression. White and black areas indicate day and night. Where profiles are omitted the expression is currently unknown. Adapted from Song *et al.* (2010).

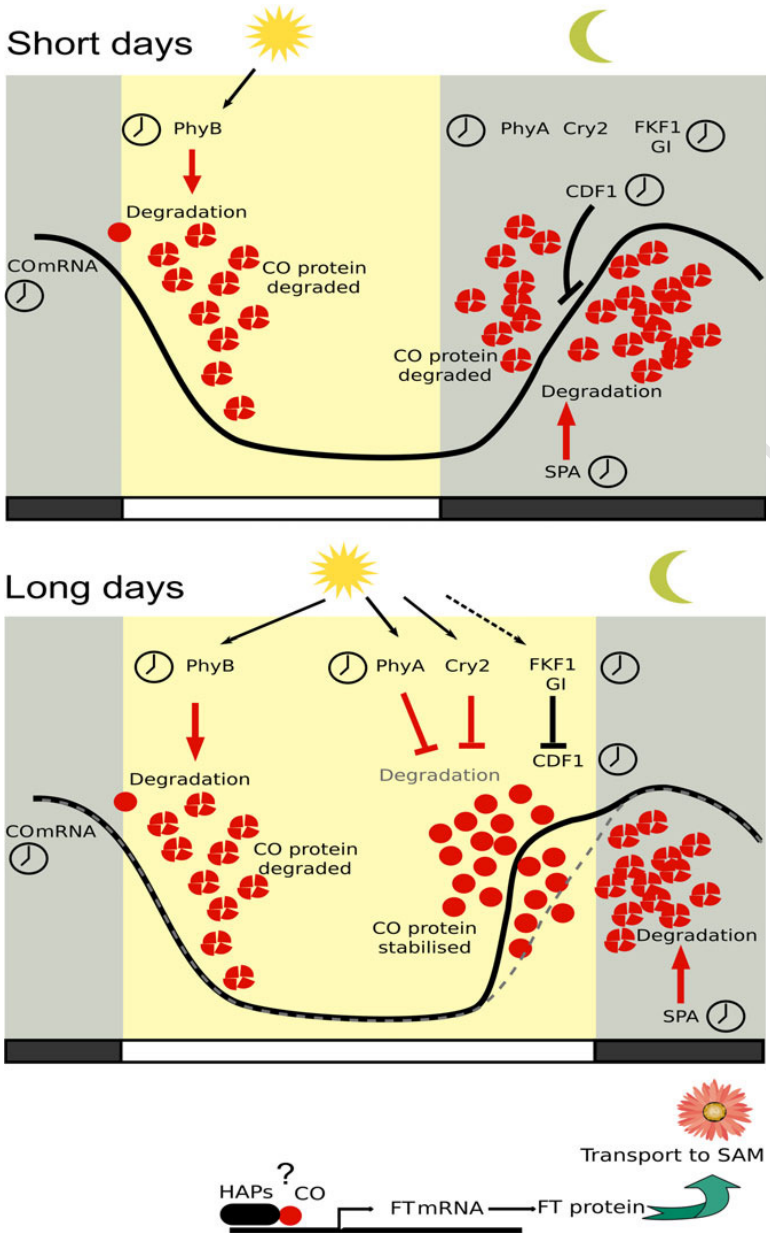


Figure 1.5. Regulation of the *CONSTANS* gene at both the mRNA and protein level. Black lines represent oscillations in *CO* mRNA, whereas CO protein is represented by red spheres (intact protein), or red split spheres (degraded protein). In short days *CO* mRNA is mainly expressed in darkness and the resulting protein is degraded partly through the action of *SUPPRESSOR OF PHYA* (*SPA1, SPA3* and *SPA4*). Morning produced protein is also degraded by the action of PhyB. In long days, the repression of *CO* expression by *CYCLING DOF FACTOR1* (*CDF1*) is released by FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN1 (FKF1) and GIGANTEA (GI), resulting in elevated *CO* expression in the afternoon. CO protein is stabilized in light through the action of PhyA and Cry2. Stable CO binds to the *FT* promoter, possibly in complex with haemactivator protein (HAP). FT protein is transported through the phloem to the SAM to induce flowering. Clock symbols indicate genes controlled by the circadian clock. Taken from Lagercrantz (2009).

A number of *CO* orthologues have been identified in woody perennials. In grapevine (*Vitis vinifera*; Almada *et al.*, 2009), *VvCO* and *VvCOL1*, and Norway spruce (*Picea abies*; Holefors *et al.*, 2009), *PaCOL1* and *PaCOL2*, showed oscillating diurnal profiles under LD and SD and roles in both flowering and growth regulation were proposed for these *CO* orthologues.

1.3.5. FT the mobile 'florigen', floral integrator and regulator of growth and development

The FT peptide was recently identified as the mobile 'florigen', the elusive graft transmissible floral inducer (Corbesier *et al.*, 2007). It has been sought since the 1930s when it was first proposed that a single signal could be responsible for relaying the floral stimulus from the site of perception, leaves, to the SAM (Chailakhyan, 1936). Evidence for the translocation of FT protein in the phloem was provided by FT:Green Fluorescent Protein (GFP) fusion studies (Corbesier *et al.*, 2007). *A. thaliana ft-7* plants transformed with *SUC2::FT:GFP* constructs displayed early flowering and the chimeric FT:GFP protein was present in the SAM, whereas *FT:GFP* mRNA was absent. This suggested movement of FT from the leaf to the SAM presumably through the phloem. Phloem transport of the orthologous FT protein, Hd3a, in rice was also demonstrated by Hd3a:GFP localisation studies (Tamaki *et al.*, 2007). The isolation of three FT-like proteins from rice phloem sap also suggested that in rice FT orthologues are translocated via the phloem (Aki *et al.*, 2008). Leaf-to-SAM FT translocation has also been demonstrated in *Cucurbits maxima* (Lin *et al.*, 2007) and tomato (*Solanum lycopersicon*; Lifschitz and Eshed, 2006; Shalit *et al.*, 2009). *SFT*, the tomato *FT* orthologue, induced early flowering in graft-receptor plants when a leaf from a *35S::SFT* plant was used as graft donor. Careful semi-quantitative PCR on apices of graft receptor plants could not detect the presence of *SFT* transcript, indicating that the movement of the SFT peptide was responsible for the early flowering phenotype (Lifschitz *et al.*, 2006).

FT acts as part of a protein complex with FLOWERING LOCUS D (FD), a bZIP transcription factor, in the SAM to up-regulate the meristem identity genes (Fig. 1.3; Abe *et al.*, 2005). FT has also been suggested to directly up-regulate the meristem identity genes, *APETALA 1* (*AP1*) and *FUL* by binding to their promoter regions and activating their transcription (Abe *et al.*, 2005; Wigge *et al.*, 2005). Another possible target of the FT/FD complex is the floral integrator *SOC1* as *SOC1* expression is delayed in *ft* and *fd*

mutants (Yoo *et al.*, 2005). These FT targets together with LFY and AGAMOUS-LIKE24 (AGL24; Lee *et al.*, 2008) are responsible for initiating the expression of the floral organ identity genes (Fig. 1.3; section 1.4.1).

1.3.5.1. FT forms part of the FT/TFL, PEBP, family of developmental regulators

FT encodes a 19.8 kDa protein (Giakountis and Coupland, 2008) with high homology to the phosphatidylethanolamine-binding proteins (PEBP; also known as Raf1 kinase inhibitor proteins) found in a wide range of species from bacteria to animals and plants. PEBP peptides are MAP-kinase (mitogen-activated protein kinase) inhibitors involved in signalling cascades that influence a broad range of developmental processes in animals (Banfield and Brady, 2000; Vallée *et al.*, 2003). Similarly, in plants PEBP-like proteins are present in tissues undergoing physiological changes such as growth arrest, dormancy and meristematic phase transitions (Karlgren *et al.*, 2011) suggesting that they may be involved in these processes.

FT is a member of the *FT/TFL*-like family comprised of six genes*: FT, TERMINAL FLOWERING LOCUS* (*TFL*)*, ARABIDOPSIS THALIANA CENTRORADIALIS* (*ACT*)*, BROTHER OF FT* (*BFT*)*, MOTHER OF FT AND TFL1* (*MFT*) and *TWIN SISTER OF FT*(*TSF*). These genes encode either floral promoters or repressors in *A. thaliana*. Based on sequence homology and the functions of their protein products, these genes have been divided into three subfamilies: *TFL*-like, *MFT*-like and *FT*-like (Kobayashi *et al.*, 1999). Proteins acting as either floral activators or repressors are distinguishable by conserved amino acid residues Tyr85 and Gln140 in activators compared to His88 and Asp144 in repressors (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006).

TFL, *BFT* and *ACT* form the so-called '*TFL*-like' clade. BFT and TFL act as floral repressors by inhibiting floral transition in the meristematic region of *A. thaliana* (Kobayashi *et al.*, 1999; Hanano and Goto, 2011), while the function of ACT is still largely unknown. TFL1 interacts with FD in the SAM inhibiting transcription of FD target genes, thus repressing flowering (Hanano and Goto, 2011). The floral repressive function of TFL is also conserved in other plants. Recently, continuous flowering phenotypes in rose (*Rosa* spp.) and strawberry (*Fragaria vesca*) have been linked to diminished TFL function (Iwata *et al.*, 2012). In these plants and tomato, *TFL* orthologues are also involved in regulating shoot architecture together with *FT* (Iwata *et*

al., 2012; Shalit *et al*., 2009). The ancient ancestral clade, *MFT*-like, is involved in reproduction of moss (*Physcomitrella patens*; Hedman *et al*., 2009), and in angiosperms has been implicated in floral induction (Yoo *et al*., 2004; Carmona *et al*., 2007), seed development (Danilevskaya *et al*., 2008) and germination (Nakamura *et al*., 2011). The *FT*-like clade comprises the floral inductive members *TSF* and *FT*, which act redundantly but have different spatial expression profiles (Yamaguchi *et al*., 2005).

FT/TFL families have also been identified in woody perennial trees such as apple (*Malus x domestica*; Kotoda *et al*., 2003; Kotoda and Wada, 2005; Kotoda *et al*., 2006; Mimida *et al*., 2009; Kotoda *et al*., 2010), grapevine (*Vitis vinifera*; Carmona *et al*., 2007) and poplar (*Populus nigra*; Igasaki *et al*., 2008). However, the number of *PEBP*-like genes found in these families is mostly greater than the six present in *A. thaliana*. Expression patterns and copy number of *PEBP*-genes in perennials, displaying sympodial growth, suggest that they fulfil an additional role in controlling the growth forms of these plants (Lifschitz and Eshed, 2006; Lifschitz *et al*., 2006; Ruonala *et al*., 2008; Shalit *et al*., 2009).

1.3.5.2. Spatial and temporal expression of FT

In *A. thaliana*, *FT* expression is exclusively confined to the vasculature (Michaels *et al*., 2005; Yamaguchi *et al*., 2005) and up-regulated when increased day lengths are experienced. However, due to the low expression levels of *FT,* detection of endogenous transcript by *in situ* hybridisation is notoriously difficult. Therefore, most information on spatial and temporal expression patterns of *FT* are from reporter gene or PCR studies (Takada and Goto, 2003; Turck *et al*., 2008). In transgenic *A. thaliana* plants transformed with a Pro_{FT}::*β-glucuronidase* (*GUS*) construct, *GUS* expression was detected in the leaf vasculature but not in leaf primordia, SAMs, hypocotyls or roots (Kobayashi *et al*., 1999; Takada and Goto, 2003). However, later during plant development expression could be detected in the inflorescence vasculature, flowers and siliques. Further evidence for the exclusion of *FT* transcript from the SAM in wild type *A. thaliana* plants was from a study by An *et al*., 2004. *FT* expression driven from SAM specific promoters could induce early flowering and complement *co* mutants, whereas expression of *CO* under SAM specific promoters was unable to induce early flowering. As CO up-regulates *FT* expression in *A. thaliana*, the inability of *CO* expression in the

SAM to induce early flowering indicates the absence, or repression, of *FT* expression in the SAM.

Chromatin modifications have also been shown to influence *FT* expression in *A. thaliana*. *TERMINAL FLOWER2* (*TFL2*), encoding an epigenetic repressor, and *EARLY BOLTING IN SHORT DAYS* (*EBS*), which encodes a putative chromatin remodelling factor, have been proposed to reduce expression levels of *FT* and prevent the early induction of *FT* by activators (Gomez-Mena *et al*., 2001; Pineiro *et al*., 2003). More recently, a plant-specific histone H3 lysine 4 demethylase, PKDM7B, has also been shown to mediate de-methylation of *FT* and *TSF* chromatin (Yang *et al*., 2010). *A. thaliana* mutant *pkdm7b* plants flowered significantly earlier than wild type plants, but otherwise developed normally. This suggested that PKDM7B specifically acts to repress flowering through chromatin mediated gene repression of *FT* and *TSF* in the vasculature where the spatial expression of *PKDM7B* also overlapped with that of *FT* and *TSF*. Results such as these suggest that the epigenetic regulation of *FT* expression may be an important level of control which ensures the correct temporal expression of this developmentally important gene. Although it has not been investigated to date, a similar level of control may exist in woody perennial plants especially as a much broader role in growth and development has been suggested for *FT* orthologues in these plants.

1.3.5.3. FT *plays a role in supporting the perennial growth habit*

In most transgenic studies, overexpression of *FT* orthologues either in annual model or woody perennial plants resulted in altered growth phenotypes. Tobacco (*Nicotiana tabacum*) and *A. thaliana* transgenic plants ectopically expressing *PaFT*, an *FT* orthologue from London plane (*P. acerifolia*) displayed reduced apical dominance and the formation of flowers on the terminal position (Zhang *et al*., 2011). In poplar, a woody perennial tree, expression of *FT* orthologues is excluded from the terminal meristem which remains vegetative during the tree's lifespan (Hsu *et al*., 2006). Ectopic expression of FT orthologues in these meristems resulted in the formation of inflorescences at the terminal position of the shoot and inhibited any new growth (Hsu *et al*., 2011; Shen *et al*., 2012). Similar results were obtained when *FT* orthologues were ectopically expressed in apple (Katoda *et al*., 2010). Presumably, the suppression of *FT* orthologues and their downstream target genes in some meristems would be essential to maintain an indeterminate state in some meristems to support perennial growth. Hsu

et al. (2006) suggested that expression of *FT* orthologues may normally be suppressed at the terminal position, possibly through chromatin mediated repression as suggested for *FT* in *A. thaliana* (Gomez-Mena *et al*., 2001; Pineiro *et al*., 2003; Yang *et al*., 2010) or by the action of the floral inhibitor FLC (Hanano and Goto, 2011). In *A. thaliana*, the down-regulation of direct target genes of FT resulted in the development of perennial characteristics never documented before for this model annual (Melzer *et al*., 2008). *A. thaliana soc1 ful* double mutant displayed multiple growth cycles with a markedly increased life span, a scrub-like phenotype and secondary growth with the development of woody stems. Loss of FT function also resulted in an abnormal growth architecture as a consequence of indeterminacy at the apical meristem resulting in plants growing up to 1 m tall with reduced side branching (Melzer *et al*., 2008).

Tomato (*Solanum lycopersicon*) plants are fast-growing, day-neutral perennials with regular and predictable developmental patterns which make them excellent systems for investigating how vegetative and reproductive cycling is controlled. (Lifschitz and Eshed, 2006). Altered expression levels of the *FT* and *TFL1* tomato orthologues, *SINGLE FLOWER TRUSS* (*SFT*) and *SELF-PRUNING* (*SP*), in two different tomato cultivars Money Maker (MM) and VFNT (resistant to verticillium wilt, fusarium wilt, nematodes, and tobacco mosaic virus) affected shoot architecture (Shalit *et al*., 2009). The authors showed that high levels of SFT protein resulted in the termination of sympodial growth by inhibiting the further vegetative development of meristems, but that in wild type plants SFT levels are regulated by SP. Earlier studies by Lifschitz *et al*. (2006) also suggested a role for *SFT* in tomato growth architecture. Mutant *sft* tomato plants developed indeterminate inflorescences and apical dominance was maintained in the absence of functional SFT. Whereas *35S::SFT* transgenic plants developed less complex leaves, shorter internodes and thinner stems with arrested apical growth. Roles for orthologous *TFL* genes in growth architecture of continuous flowering populations of rose and strawberry has also been suggested (Iwata *et al*., 2012). Rose and strawberry plants carrying functional *TFL* genes display seasonal flowering and growth patterns. An indeterminate growth form is maintained as the primary shoot remains vegetative and flowering occurs on axillary shoots. In continuous flowering plants, however, all shoots are terminated in an inflorescence. This suggests that *FT*-like together with *TFL*-like genes form a regulatory hierarchy affecting diverse growth processes that results in a level of plasticity around growth forms. These results suggest

that together with activating flowering, FT also regulates the fate of meristems and affect growth form.

1.3.5.4. FT *orthologues in woody perennials*

FT orthologues have been identified from a number of woody perennial species (Table 1.2) and more recently orthologues from both litchi (*Lichi chinensis*; *FT1* and *FT2*; Ding *et al*., 2011; unpublished) and pear (*Pyrus pyrifolia*; *PpFT1atw* and *PpFT2atw*; Moriguchi *et al*., 2011, unpublished) have been submitted to GenBank (accessed 30 December 2011). When ectopically expressed in *A. thaliana*, many of the woody perennial *FT* orthologues have shown floral promoting effects (Hisada *et al*., 1997; Böhlenius *et al*., 2006; Hsu *et al*., 2006; Sreekantan and Thomas, 2006; Hou and Yang, 2009; Zhang *et al*., 2011). In woody perennial plants the constitutive expression of *FT* orthologues, under control of the *Cauliflower Mosaic Virus 35S* promoter, resulted in a shorter juvenile phase and early flowering in trifoliate orange (*Poncirus trifoliate*; Endo *et al*., 2005) and poplar *(P. trichocarpa*; Böhlenius *et al*., 2006). Similarly, early flowering was achieved in apple with ectopic expression of the *FT* orthologue, *MdFT1*, through *35S::MdFT1* and *SUC2::MdFT1* constructs (Tränkner *et al*., 2010). As the *SUCROSE TRANSPORTER2* promoter (*SUC2*) is only active in phloem companion cells, these results suggest that orthologous FT peptides in woody perennial plants may also function as floral activators translocated from the leaf to the SAM. However, due to the differences between annual and perennial growth habits and length of juvenile phases it is difficult to match developmental stages and compare *FT* expression patterns between annuals and perennials. Some perennial plants also have lengthy floral development periods: two seasons for *Platanus acerifolia* (Zhang *et al*., 2011) and three to five months for *Protea* (Gerber *et al*., 2001a), adding to the complexity of comparing expression data between different woody perennial species.

Table 1.2. FLOWERING LOCUS T (FT) orthologous genes identified from woody perennial species.

Plant species and cultivar	Common name	Gene name	Sites of expression	References
Citrus unshiu Marc.	Satsuma mandarin	*CiFT1*	mature fruit	Nishikawa *et al*., 2007
		CiFT2	mature fruit	
		CiFT3	stem and leaves	
Fortunella crassifolia Swingle x *F. margarita* Swingle cult. 'Puchimaru'	Kumquart	*CiFT*	stems and leaves	Nishikawa *et al*., 2011
Malus x *domestica* Borkh. cult. 'Pinova'	Apple	*MdFT1*	leaves and apical buds	Tränkner *et al*., 2010; Kotoda *et al*., 2010
		MdFT2	floral buds and young fruit	
Picea abies L. Karst.	Norway spruce	*PaFT2*	low levels in needles and buds	Gyllenstrand *et al*., 2007
		PaFT4	needles and buds	
Platanus acerifolia Willd	London plane	*PaFT1*	young inflorescences	Zhang *et al*., 2011
		PaFT2	young inflorescences	
Populus deltoides	Poplar	*PdFT1*	dormant buds	Hsu *et al*., 2006 and 2011
		PdFT2	mature leaves and inflorescence meristems	
Populus nigra var. *italica* Koehne	Poplar	*PnFT1*	all organs, summer leaves and capsules	Igasaki *et al*., 2008
		PnFT2	summer leaves and capsule	
		PnFT3	stems and winter buds	
		PnFT4	stems and winter buds	
Populus simonii var. Carrière	Poplar	*PsFT1*	not investigated	Shen *et al*., 2012
		PsFT2	not investigated	
Populus trichocarpa	Poplar	*PtFT1*	inflorescence meristems and leaves	Böhlenius *et al*., 2006
		PtFT2	not investigated	Zhang *et al*., 2010
Rhododendron x *pulchrum* cult. 'Oomurasaki'	Azalea	*RpFT*	floral buds (other sites not investigated)	Cheon *et al*., 2011
Vitus vinifera L.	Grapevine	*VvFT*	inflorescence meristems, flowers (stamens, ovary and ovule), berries	Skreekantan and Thomas, 2006

Of all woody perennial genera, the *FT* orthologues from poplar (*Populus*) species have been the most extensively studied. Two *FT*-like genes have been identified and characterised from the genomes of *P. deltoides* (Hsu *et al*., 2006), *P. trichocarpa* (Zhang *et al*., 2010) and *P. simonii* (Shen *et al*., 2012); while four have been isolated from *P. nigra* (var. *italica* Koehne; Igasaki *et al*., 2008). Although these paralogous proteins share greater than 90% homology, they are functionally diverged with distinct temporal and spatial expression patterns proposed to coordinate repeated cycles of vegetative and reproductive development in poplar (Hsu *et al*., 2011). Ectopic expression of *PtFT1*, in *P. trichocarpa* (Böhlenius *et al.,* 2006; Zhang *et al*., 2010), and *PdFT1*, in *P. deltoides* (Hsu *et al*., 2011) resulted in early flowering of poplar plants, suggesting that these orthologues are involved in floral induction. Further evidence that *PdFT1* is involved in the floral induction of *P. deltoides* is that expression is also up-regulated in leaf, stem and meristematic tissue (reproductive and vegetative) after a cold treatment, required for floral induction; while micro-array analyses showed the altered expression of 18 genes related to reproductive development in Pro_{HSP}*::PdFT1* plants after exposed to cyclic heat treatments (Hsu *et al*., 2011). The ectopic expression of *PdFT2*, the other *FT* orthologue in *P. deltoides*, did not result in early flowering; however, plants continued vegetative growth under SD after control and Pro_{HSP}*::PdFT1* plants had ceased growth (Hsu *et al*., 2011). Expression of *PdFT2* in axillary buds and leaves of *P. deltoides* declined rapidly when plants were exposed to cold temperatures, suggesting that *PdFT2* needs to be down-regulated to ensure onset of dormancy. Two *FT* orthologues of *P. nigra PnFT1* and *PnFT2* which are highly expressed in summer leaves have been proposed to play a role in the floral induction of these plants, while *PnFT3* and *PnFT4,* the other two orthologues present in *P. nigra,* were expressed in lateral buds during winter (Igasaki *et al*., 2008). Böhlenius *et al.* (2006) demonstrated that *FT* together with *CO* orthologues in *P. trichocarpa* played roles in the seasonal growth cessation of these woody trees. Findings from poplar studies, suggested that *FT* orthologues are involved in relaying information about seasonal change to the apical meristem not only to initiate flowering, but also to facilitate periodic growth cycles. This is proposed to be the primary and ancestral function of *FT*-like genes.

At more extreme latitudes, i.e. close to the poles, seasonal change is marked by more rapid day-length changes compared to latitudes closer to the equator. Different conditions may signal the onset of dormancy and bud burst for plant species inhabiting

these areas as suggested for the gymnosperm *Picea abies* (Gyllenstrand *et al*., 2007). *P. abies* express two *FT*-like genes and the expression levels of one of these, *PaFT4*, has been correlated to rhythmic growth of this tree (Gyllenstrand *et al*., 2007). The expression of this gene was investigated in seedlings from two populations, from either Romania or the Arctic Circle, showing highly diverged bud set responses under specific photoperiodic conditions. *PaFT4* expression levels in needles and the buds of adult trees correlated to bud set under SD conditions for the two different populations, and were also affected by photoperiod and light quality. The expression of *PaFT4* showed a clear diurnal pattern, suggesting that its control may be *CO* mediated as in *P. trichocarpa* (Böhlenius *et al*., 2006).

FT orthologues have also been identified from horticulturally important fruit trees, and expression profiles suggest they fulfil roles in floral induction, fruit development and tree architecture in many of these species. Three *FT* orthologues, *CiFT1-3*, were isolated from Satsuma mandarin tree tissue and the expression of *CiFT3* correlated well with that of a floral inducer. *CiFT3* levels were low in the summer months and increased in both stem and leaf tissue in response to low temperatures during winter, inductive for flowering in citrus (García-Luís *et al*., 1992; Nishikawa *et al*., 2007). Whereas, expression levels of the other two orthologues, *CiFT1* and *CiFT2,* were high in seeds, flowers and during the late stages of fruit development (Nishikawa *et al*., 2007), suggesting they may fulfil an alternative function in Satsuma. Two *FT* ortholgues are expressed in cultivated apple (*Malus x domestica*) and these show different temporal and spatial expression patterns (Kotoda *et al*., 2010). *MdFT1* showed high levels of expression in apical buds and was also expressed in leaves. The seasonal pattern of *MdFT1* expression in apical buds of fruit bearing shoots correlated well with the initiation of flowering in summer. Expression of *MdFT2* was limited to floral buds, reproductive organs and young fruit. Similar to the spatial expression of *MdFT2* in apple, *VvFT* the *FT* orthologue in grape (*Vitis vinifera*) showed high levels of expression in young berries, developing tendrils, flowers and developing ovules (Sreekantan and Thomas 2006). *In situ* hybridisation showed these transcripts localised to the basal parts of petals, which could indicate vascular expression. Similarly, in the buds of azalea (*Rhododendron x pulchrum*) *RpFT* expression in meristems increased to peak during flower bud formation suggesting that *Pp*FT may fulfil a role in the formation of floral organs in these ornamental plants (Cheon *et al*., 2011). This is similar to the expression

pattern of *FT* in *A. thaliana* during inflorescence development when *FT* transcript can be detected in the vasculature of inflorescences and high expression levels are detected in developing siliques (Kobayashi *et al*., 1999; Takada and Goto, 2003).These results may suggest an additional, as yet undescribed, role for *FT* genes in later stages of flowering which may include fruit and seed development.

Recently, two *FT*-like genes from London plane (*Platanus acerifolia*), *PaFT1* and *PaFT2*, were identified (Zhang *et al*., 2011). At the nucleotide level *PaFT1* and *PaFT2* shared >99% similarity, with the presence of two SNPs, but the translated amino acid sequences were 100% identical. Numerous alternative spliced forms of *PaFT1* and *PaFT2* with different temporal and spatial expression patterns were detected and their abundance was both tissue-specific and developmentally regulated, correlating to the developmental stages of London plane flower development. Alternative splicing of *FT* transcripts have also been reported for maize (*Zea mays*; Danilevskaya *et al*., 2008) and sunflower (*Helianthus annuus*; Blackman *et al*., 2010). Peptides arising from alternative splicing forms may show functional divergence from the archetypical FT as suggested in sunflower (Blackman *et al*., 2010).

1.4. Convergence of floral pathways: Meristem identity and floral organ formation

Conditions suitable for floral induction, such as those discussed above, result in the up-regulation of the meristem identity genes *LFY* and *AP1* (Weigel *et al*., 1992; Weigel and Nilsson, 1995; Mandel and Yanofsky, 1995). In turn, *LFY* and *AP1* initiate the cascade of events required for floral organ development by regulating expression of the homeotic, or floral organ identity, genes.

1.4.1. Meristem identity genes

LFY expression has been shown to precede *AP1* expression during floral transition (Blàzques *et al*., 1997; Hempel *et al*., 1997). In turn, LFY up-regulates *AP1* by interacting with a binding site in the *AP1* promoter region (Benlloch *et al*., 2011; Hamès *et al*., 2008; Parcy *et al*., 1998). However, *AP1* can be up-regulated independently of LFY, as loss-of-function *lfy* mutants will develop some floral meristems and the *lfy* phenotype is enhanced when combined with an *ap1* mutation (Irish and Sussex, 1990). LFY independent up-regulation of *AP1* has been suggested to be mediated through the FT/FD complex (Abe *et al*., 2005; Wigge *et al*., 2005), and as no functional FD binding

site has been found on the *AP1* promoter this activation is assumed to be indirect (Benlloch *et al*., 2011). In *A. thaliana*, strong levels of *LFY* expression are specific to floral primordia (Weigel *et al*., 1992), although low levels of expression have been reported in vegetative meristems and leaves (Blázques *et al*., 1997). *AP1* expression, on the other hand, is restricted to young florally-determined meristems (Mandel *et al*., 1992) where it regulates specific homeotic genes in floral organ patterning (Ng and Yanofsky, 2001; Weigel and Meyerowitz, 1993; discussed in 1.4.2 and depicted in Fig. 1.6). *AP1* also acts as a homeotic gene itself, displaying A functionality required for sepal and petal development (Mandel *et al*., 1992; Fig. 1.6; discussed in 1.4.2). LFY interacts with a number of homeotic genes to control their expression and therefore is directly involved in the determination of floral architecture.

In *A. thaliana*, *LFY* expression increases in the SAM during vegetative development until it reaches a threshold level at which flowering is initiated (Blázquez *et al*., 1997; Hamès *et al*., 2008). Floral induction by LFY is thereafter achieved through the up-regulation of homeotic genes and by repressing expression of floral repressors such as *EMBRYONIC FLOWER1* (Chen *et al*., 1997, Chou *et al*., 2001) and *TFL1* (Liljegren *et al*., 1999; Parcy *et al*., 2002; Ratcliffe *et al*., 1998). The transcriptional activation of specific LFY targets also require the actions of WUSCHEL (WUS; Lohmann *et al*., 2001) and UNIDENTIFIED FLORAL ORGAN (UFO; Lee *et al*., 1997; Chae *et al*., 2008) which function as co-regulators of LFY in *A. thaliana* (Chae *et al*., 2008; Parcy *et al*., 1998). Known LFY targets include: *CAULIFLOWER* (*CAL*) a paralogue of *AP1* with redundant A functionality (William *et al*., 2004); *AGAMOUS* (*AG*) a C-function gene responsible for the formation of stamens and carpels (Lohmann *et al*., 2001; Hong *et al*., 2003; William *et al*., 2004); *APETALA3* (*AP3*) a B-function gene involved in petal and stamen development (Sessions *et al*., 2000; Lamb *et al*., 2002) and the E-function genes *SEPALLATA2* (*SEP2*) and *SEP3* (Schmid *et al*., 2003; Wagner *et al*., 2004; William *et al*., 2004). The E-function in floral organ development is a recent addition to the ABCD model (Rijpkema *et al*., 2010), and these SEP proteins are proposed to function as co-regulators in the formation of protein complexes between different homeotic genes (Immink *et al*., 2009; Melzer *et al*., 2009). These interactions emphasise the pivotal role of LFY in the control of floral architecture.

As *LFY* up-regulation is one of the earliest events in floral transition, the function of this gene in both annual and perennial systems and its involvement in this agriculturally important event has been well studied. The first *LFY*-like gene was identified from snapdragon (*Antirrhinum majus;* Coen *et al*., 1990). *FLORICAULA* (*FLO*) was found to be highly expressed in early inflorescence development, while snapdragon plants expressing a mutant *flo* gene which encoded a truncated protein lacking the C-terminal region never produced flowers. Another *flo* mutant produced fewer floral primordia compared to the five produced by wild type plants. Together, these findings suggested that *FLO* acted as a switch between vegetative to reproductive development and was also involved in the floral patterning of snapdragon. At that time no DNA or protein sequences with homology to *FLO* were available in databases and little was known about the transition of meristems from vegetative to reproductive development. Two years later Weigel *et al*. (1992) isolated the *LFY* gene from *A. thaliana*. LFY showed 70% homology to FLO on a deduced amino acid level and similarly to *FLO*, *LFY* was expressed during early inflorescence development. However, loss of *LFY* function did not result in the complete conversion of floral meristems to inflorescences in *A. thaliana*. This suggested that although *FLO* and *LFY* were both involved in the transition from vegetative to reproductive development, different spatial and temporal expression and downstream targets of *LFY*-like genes may be responsible for different floral forms. Since the discovery of the early *LFY* genes, *LFY* homologues have been identified from a number of plants including annuals and perennials. However, *LFY* is still the best studied in the model annual *A. thaliana*.

LFY encodes a plant specific transcription factor (Weigel *et al*., 1992; Weigel and Nilsson, 1995) and is present in all land plants which evolved within the last 400 million years (Maizel *et al*., 2005). LFY-like proteins contain two highly conserved domains in the N- and C-terminal regions (Maizel *et al*., 2005). The DNA binding domain, responsible for transcriptional activation or repression of target genes, is present in the C-terminal region (Hamès *et al*., 2008). Mutations in the DNA binding domain abolish or reduce LFY activity and these mutants display abnormal floral morphologies with the ectopic appearance of some floral organs, conversion of flowers to 'leaf-like' structures and reduced fertility (Weigel *et al*., 1992). These effects are presumably because the mutant protein, lfy, is unable to correctly regulate downstream homeotic genes that control floral organ formation. LFY is present as a monomer in the absence of DNA,

however, binds DNA as a homodimer (Hamès *et al*., 2008; Siriwardana and Lamb, 2012). The N-terminal region of LFY, even though not directly involved in DNA binding, appears to facilitate this binding. This was shown by the diminished capacity of lfy-22, which has an amino acid substitution in the N-term region (G70D), to bind to the *AP1* promoter (Maizel *et al*., 2005). A recent study by Siriwardana and Lamb (2012) confirmed the function of the N-terminal region in facilitating LFY-LFY dimerization. Deletions in the N-terminal sequence affected the strength of the homodimer formation and also its ability to transcriptionally regulate target genes by DNA binding.

The expression patterns of orthologous *LFY* and *AP1* genes in woody perennial trees, suggest they also fulfil roles in meristem identity. One of the first *LFY* orthologues isolated and studied from a woody perennial tree was *ELF1* from *Eucalyptus globulus* (Southerton *et al*., 1998). *ELF1* was expressed in young floral buds, young leaves and leaf primordia but was absent from mature leaves, roots, stems and mature floral buds. *EgLFY*, a *LFY* orthologue isolated from *E. grandis*, was similarly expressed in leaf primordia and floral meristems (Dornelas *et al*., 2004). Orthologous *AP1* genes have also been identified from *Eucalyptus*, and Kyozuka *et al*. (1997) reported the presence of two paralogues, *EAP1* and *EAP2*, in *E. globulus*. Both of these genes were predominantly expressed in floral buds suggesting they fulfil a role in meristem identity. In *E. occidentalis*, expression of an *AP1* orthologue (*EOAP1*) was also identified as a good marker of floral transition (Jaya *et al*., 2010 and 2011). In the woody perennial *Sophora tetraptera*, the expression of *LFY* and *AP1* orthologues, *StLFY* and *StAP1*, were up-regulated during early flower development and showed low levels of expression in inflorescence and vegetative meristems (Song *et al*., 2008). Later expression also correlated with the up-regulation of downstream homeotic genes *StPI* and *StAG*, *PISTILLATA* (*PI*) and *AG* orthologues. Expression of the *LFY* orthologue *CfLFY,* in the tropical cedar tree (*Cedrela fissilis*) was also restricted to developing floral organs and meristems undergoing vegetative to reproductive phase transitions (Dornelas and Rodriguez, 2006). *LFY* orthologues have also been identified from poplar species such as *P. trichocarpa* and *P. tomentosa*. In *P. trichocarpa, PTLF* is expressed in floral and vegetative meristems, bracts and young leaves (Rottmann *et al*., 2000). While in other poplar species, *P. tomentosa, PtLFY* was expressed in male and female floral buds. Low levels of *PtLFY* expression was observed in vegetative tissues such as stems and vegetative buds, however, high levels of *PtLFY* transcript were detected in

roots (An *et al*., 2011). Expression a *LFY* orthologue, *AFL2*, in root tissue of apple was also reported (Wada *et al* 2002). The possible function of *LFY* orthologues in roots is unknown.

In plants which have lengthy floral developmental periods that may span two seasons such as kiwifruit (*Actinidia deliciosa*) and the Pōhutukawa tree (*Metrosideros excelsa*), the expression patterns of *LFY* and *AP1* orthologues are often bimodal, i.e. show two peaks (Walton *et al*., 2001; Sreekantan *et al*., 2004). The first of these peaks corresponded to the period of floral initiation preceding winter dormancy and the second to floral development during spring. *In situ* hybridization in kiwifruit showed expression of the *LFY* orthologue, *AFL*, localised to the leaf primordia and excluded from the apical meristem, while expression of *AAP1,* the kiwifruit *AP1* orthologue, was localised to primordia destined for petal development (Walton *et al*., 2001). Together, these results suggested that these orthologues fulfil similar roles in floral determination and development as suggested for *LFY* and *AP1* in *A. thaliana* (Mandel *et al*., 1992; Weigel *et al*., 1992; Weigel and Meyerowitz, 1994; Blázques *et al*., 1997) even though floral development is interrupted by a dormant period in kiwifruit and Pōhutukawa.

In ancient gymnosperm species such as pine (*Pinus radiata*) two *LFY* paralogues, *PRFLL* and *NEEDLY* (*NLY*) are expressed. These genes show some divergence in spatial and temporal expression, with *PRFLL* expressed primarily in male (Mellerowich *et al*., 1998) and *NLY* mostly in female cones (Mouradov *et al*., 1997). These findings suggested that although both paralogues are expressed in vegetative meristems some divergence of functionality may have occurred between these two paralogues as they each have additional roles in the development of preferably male or female reproductive structures. However, after the identification and expression analyses of orthologous *LFY* and *NLY* genes from other gymnosperms such as *Picea abies* (Carlsbecker *et al*., 2004) and later *Podocarpus reichei* and *Taxus globosa* (Vázques-Lobo *et al*.,2007) this theory was mostly disregarded as in these studies expression of *LFY* and *NLY* orthologues could be detected in both female and male structures. The functions of orthologous *LFY* genes from some gymnosperm species such as: *P. radiata NLY* (Mouradov *et al*., 1998), *Gnetum parvifolium LFY* (Shindo *et al*., 2001) and *Pinus caribaea LFY* (Dornelas and Rodriguez, 2005) seem to be similar to those of angiosperms based on their ability to complement *A. thaliana lfy* mutants. Maizel *et al*. (2005) found that the ability of

WeINDLY, *NLY* orthologue from *Welwitchia* and those of even more basal eudicot species such as the fern (*Ceratopteris richardii*) *LFY* orthologue, *CrLFY2*, only partially complemented the *lfy* mutation in *A. thaliana*. While the moss (*Physcomitrella patens subsp. patens*) *LFY* genes, *PpLFY1* and *PpLFY2*, were inactive in *A. thaliana* (Himi *et al*., 2001). Phylogenetic analyses of these genes suggested that the duplication of these genes occurred before gymnosperm-angiosperm divergence (Vázques-Lobo *et al*., 2007).

A study of *LFY* orthologous genes in apple indicated the presences of three genomic copies of the gene (Wada *et al*., 2007). All three copies: *AFL1*, *ALF1a* and *AFL2* were expressed, and *ALF1* and *ALF2* showed different spatial and temporal expression patterns when GUS activity was assayed in transgenic Pro_{ALF1}*::GUS* and Pro_{ALF2}*::GUS A. thaliana* plants. *ALF1* expression was restricted to the stipules of rosette and cauline leaves, whereas *AFL2* was expressed in young floral buds and leaf stipules. The nucleotide sequence of *ALF1a* was identical to *ALF1*, except for the insertion of a 790 bp transposable element, abundant in the genome of domesticated apple, within the first intron. An earlier study by Wada *et al*. (2002) found that in apple, *AFL1* expression was limited to florally determined meristems, whereas *AFL2* showed a much broader expression pattern including sepals, stamens, carpels, floral and vegetative meristems and roots (Wada *et al*., 2002). A study by Esumi *et al*. (2005) identified *LFY* orthologues from six members of the Maloid subfamily of Rosaceae, of which apple is a member. These species included: Japanese pear (*Pyrus pyrifolia*), European pear (*Pyrus communis*), quince (*Cydonia oblonga*), Chinese quince (*Chaenomeles sinensis*), loquat (*Eriobotrya japonica*) and apple. All of these species expressed two copies of *LFY*, *LFY-1* and *LFY-2*. Deduced amino acid sequences of genes belonging to these two groups, LFY-1 and LFY-2*,* resolved into two distinct phylogenetic clades and sequence analysis revealed a number of amino acid substitutions unique to each group. *LFY-1* and *LFY-2* paralogues showed slight differences in spatial and temporal expression patterns in tree tissue, although both paralogues were expressed in floral buds suggesting that they may both be involved in floral development. The presence of two *LFY* copies in the Maloideae may be due to the polyploid origin of this family (Evans and Campbell, 2002). Expression data from the study by Esumi *et al*. (2005) for *AFL1* in apple was in contradiction to those previously reported by Wada *et al*. (2002). Wada *et al*. (2002) reported *ALF1* expression only in florally determined meristems, whereas Esumi *et al*.

(2005) found *AFL1* expression in all tissue types. These differences may be due to the fact that two different apple cultivars and transcript detection methods were used in the studies. Wada *et al*. (2002) measured *ALF1* expression by Northern analysis in the 'Jonathan' cultivar, while Esumi *et al*., (2005) performed RT-PCR to detect the *ALF1* transcript in the 'Fuji' cultivar.

LFY genes are present at low copy numbers in most plant genomes (Moyroud *et al*., 2009). Maintaining a low *LFY* copy number may be important to ensure conserved *LFY* function due to its importance in correct floral formation critical for successful reproduction. However, multiple copies of orthologous *LFY* genes are expressed in some species; two copies are expressed in gymnosperms (*Pinus radiata*; Mellerowicz *et al*., 1998; Mouradov *et al*., 1998), tobacco (*Nicotiana tabacum*; Kelly *et al*., 1995), maize (*Zea mays*; Bomblies *et al*., 2003) and various Maloid species (Esumi *et al*. 2005), while three copies are present in apple (Wada *et al*., 2007). In some species the second *LFY* copy has been reduced to a pseudogenes such as *ELF2* in *E. globulus* (Southerton *et al*., 1998) and as suggested for the second copy of *StLFY* in *S. tetraptera* (Song *et al*., 2008). The multicopy nature of *LFY* in tobacco is a consequence of it being the allotetrapoid progeny of *N. sylvesfris* and *N. tomentosiformis* (Kelly *et al*., 1995). Southern analyses on the DNA from the parental species suggest that tobacco maintained a copy of *LFY* from each of the parental species. Results from loss of function mutations in maize, suggest that the two *LFY* copies, *ZFL1* and *ZFL2*, are involved in meristem identity and inflorescence architecture of this monocotyledonous species (Bomblies *et al*., 2003). Multiple *LFY* copies have also been identified in various Brassicaceae such as *Jonopsidium acaule* (Shu *et al*., 2000) and *Idahoa scapigera* (Sliwinski *et al*., 2007) and the heterologous expression of these genes in *A. thaliana* suggest they play a role in floral architecture.

The crucial role of *LFY* in both floral initiation and control of floral architecture has been well characterised through transgenic studies. Orthologous *LFY* genes from many plants have been identified and heterologously expressed in *A. thaliana* or over-expressed *in planta* to determine functional equivalence to the meristem identity gene, initially aimed at creating 'fast flowering' plants (Meeks-Wagner, 1996; Smyth, 1996; reviewed in Martín-Trillo and Martínez-Zapater, 2002). In poplar (*P. trichocarpa*) early flowering was successfully achieved by constitutive expression of *AtLFY* (*A. thaliana*

LFY) and the poplar orthologue, *PTLF* (Weigel and Nilsson, 1995; Rottmann *et al.*, 2000). Constitutive expression of *AtLFY* in citrus (*Citrus sinensis*; Peña *et al.*, 2001) and rice (*Oryza sativa*; He *et al.*, 2000) also successfully caused precocious flowering. This suggests that *LFY* orthologues are highly conserved between various angiosperms and also fulfil roles as meristem identity genes in woody perennial species. However, these transgenics often showed abnormal floral and vegetative phenotypes (He *et al.*, 2000; Rottmann *et al.*, 2000; Peña *et al.*, 2001; Zhang *et al.*, 2008; Flachowsky *et al.*, 2010). Floral abnormalities were also observed in *A. thaliana* plants transformed with constitutively expressed *LFY* orthologues from other species (Mouradov *et al.*, 1998; Wada *et al.*, 2002).

Although, the primary role of *LFY* in angiosperms appears to be in the floral transition of the meristem, an additional role for *LFY* in controlling leaf shape has also been identified in the Fabaceae species. Pea plants have compound leaves with many leaflets and tendrils combined together to form a typical dissected leaf. Plants deficient in UNIFOLIATA (UNI) activity, the *LFY* ortholgoue in pea, produce no tendrils and simple leaves in place of compound leaves (Hofer *et al.*, 1997). Similarly, during grapevine tendril development the *LFY* orthologue *VFL* was highly expressed suggesting it may be involved in the development of these structures (Carmona *et al.*, 2002). The involvement of *LFY* orthologues in leaf development has also been suggested in *lfy* mutants from *Lotus japonica* (Dong *et al.*, 2005), *Medicago trunculata* (Wang H *et al.*, 2008) and to a lesser extent tomato (*Lycopersicon esculentum*), a member of the Solanaceae family (Molinero-Rosales *et al.*, 1999). *UNI* is expressed at the leaf margin during leaf development and is suggested to maintain cells in an indeterminate state (Hofer *et al.*, 1997). A role in regulating vegetative outgrowths from the inflorescence meristem of rice has also been demonstrated for the *LFY* orthologue, *RFL* (Rao *et al.*, 2008). Even in *A. thaliana*, the possibility that *LFY* may have a function in leaf development cannot be ruled out, as *LFY* is expressed in newly emerging leaf primordia (Blázques *et al.*, 1997). Moyroud *et al.* (2010) suggest that *LFY* function in early leaf development may be due to its capacity to stimulate meristematic growth, an additional function to conferring floral identity to meristems.

1.4.2. Homeotic genes

The meristem identity genes, *LFY* and *AP1* have been well studied in a number of woody perennial plants, however the expression of floral homeotic genes have been less characterised in these systems. Where they have been analysed, however, function and interactions of homeotic genes appear to be well conserved across species and also between annuals and woody perennials.

Homeotic genes encode transcription factors that are responsible for the formation of floral parts. These genes control the development of the floral whorls, i.e. sepals, petals, stamens and carpels contained within a typical angiosperm flower. Correct organ formation ensures reproductive success and is tightly controlled both spatially and temporally. Homeotic genes mostly encode for MADS-box transcription factors (Shore and Sharrocks, 1995; Theissen and Saedler, 1995). These proteins have very conserved N-terminal regions important for DNA binding and interactions with other proteins. A number of recent reviews have dealt with the complex interactions between these so-called ABCDE-function proteins (Causier *et al*., 2010; Ito, 2010; Rijpkema *et al.* 2010) and the *A. thaliana* example is briefly described below (and depicted in Fig. 1.6).

Sepal development requires the action of both A- and E-function genes, whereas petals require A-, B- and E-function genes, stamens B-, C- and E-function genes and carpels C- and E-function genes. *AP1* and *AP2* constitute the A-function genes (Mandel *et al*., 1992), *PI* and *AP3* the B-function (Jack *et al*., 1992), *AGAMOUS* (*AG*) the C-function (Yanofsky *et al*., 1990) and the *SEPALLATA* genes: *SEP1*; *SEP2*; *SEP3* and *SEP4* the E-function genes (Honma and Goto, 2001; Ditta *et al*., 2004). The D-function gene, *SEEDSTICK*, is involved in ovule development (Pinyopich *et al*., 2003; Fig. 1.6). E-function genes are redundantly active in all the floral whorls and have been implicated in facilitating protein-protein interactions between the homeotic genes.

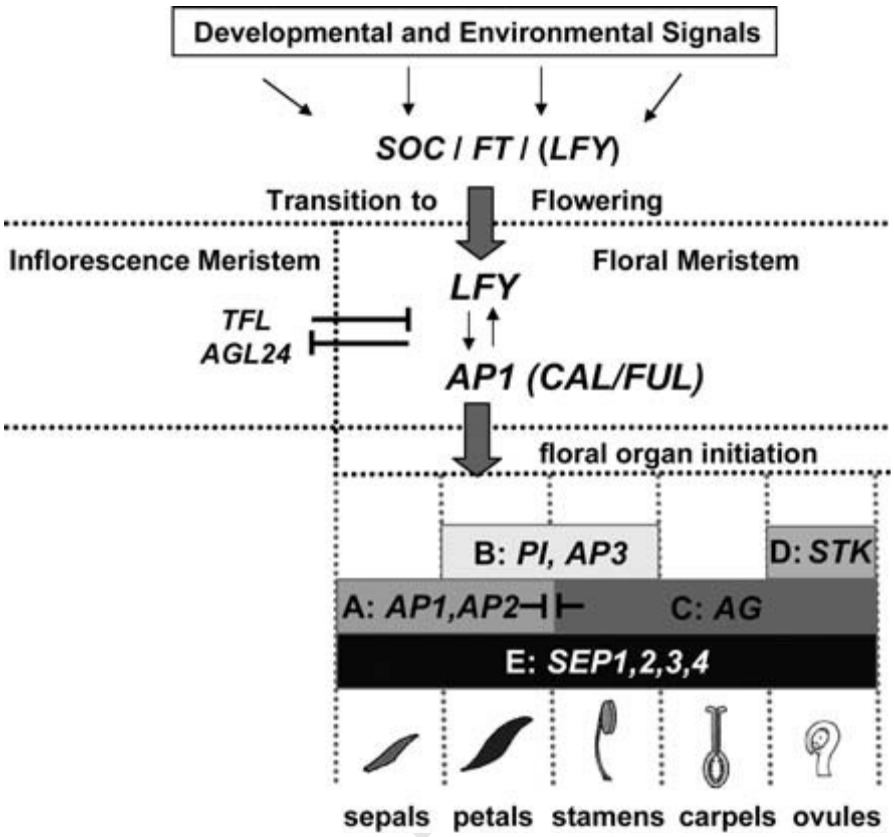


Figure 1.6. Regulatory pathway of floral induction and floral organ formation in *A. thaliana*. Letters (A, B, C, D and E) refer to specific functions of genes in the formation of different floral organs. Full names of genes abbreviated on figure are given in text at first mention. Taken from Bemer and Angenent (2010).

The large floral diversity displayed within the angiosperms is presumably the result of differences in interactions between meristem identity and homeotic genes resulting in altered temporal and spatial expression of homeotic genes. The presence of additional genes may also contribute to the floral diversity. In rose (*Rosa* x *hybrida*), two *AP1*-like orthologues, *RhAP1-1* and *RhAP1-2*, are exclusively expressed in sepals and petals, while the *FUL* orthologue (*RhFUL*) is expressed in sepals and leaves (Mibus *et al*., 2011). Although AP1 function for the two AP1-like proteins seem to fit the ABCDE model, *FUL* expression was also detected in vegetative tissue of rose. Vegetative expression of *FUL* has not been reported for *A. thaliana*, but the *FUL* orthologue from

Betula penula (*BpMADS4*) is also expressed in vegetative tissue (Elo *et al*., 2001 and 2007). Other homeotic genes from rose such as *RhB*, *RhC1* and *RhC2*, the orthologues of *PI* and two *AG* genes, were consistent with their roles in B- and C-function in the ABCDE model of floral organ development.

A large number of homeotic genes from grape have been isolated and their expression during flower and fruit development studied. The A-function gene orthologues of *FUL* and *AP1*, *VFUL1-L* and *VAP1*, showed some similarities in their expression patterns to those suggested from *A. thaliana* studies (Calonje *et al*., 2004). *VFUL1-L* expression was restricted to the centre of young florally determined meristems and was later observed in the carpel region. This pattern of expression was largely similar to that of *FUL* from *A. thaliana*, suggesting it plays a role in floral transition and development of grapevine. During early floral transition, high levels of *VAP1* expression were consistent with that of a meristem identity gene. However, during floral organ development *VAP1* was expressed in primordia destined for petal, stamen and carpel development and excluded from sepals, thus diverging from the traditional A-functionality. Analysis of three B-function genes: *VvPI, VvAP3* and *TM6*, showed that the expression of *VvPI* and *VvAP3* was specific to petals and stamens consistent with their roles as B-function genes (De Martino *et al*., 2006; Rijpkema *et al*., 2006). *TM6*, a gene from a subgroup of *AP3* absent from *A. thaliana*, was expressed throughout the plant with high levels of expression in flowers and berries, suggesting that it fulfils a broad role in flower and fruit development (Poupin *et al*., 2007). Some C-function genes have also been isolated from grapevine. *VvMADS1* showed sequence homology to *AG* and *SHATTERPROOF* (*SHP1* and *SHP2*), which functions to aid seed dispersal upon fruit maturation by regulating the opening of siliques in *A. thaliana* (Liljegren *et al*., 2000). In grape *VvMADS1* was expressed at the later stages of flower development and also throughout berry development (Boss *et al*., 2001). Ectopic expression of *VvMADS1* in tobacco resulted in the altered morphologies of the first two floral whorls. This phenotype is similar to that observed for *AG* overexpression in *A. thaliana*, as *AG* is normally excluded from these two whorls through down-regulation by the *AP*-like genes (Mizukami and Ma, 1992). The expression of four other C-function genes: *VvMADS2* and *VvMADS4* with homology to *SEP* genes; *VvMADS3* with homology to *AGAMOUS-LIKE6* and *13* (*AGL6* and *AGL13*) and *VvMADS5* homologous to *AGL11*, also suggested they fulfil broad roles in flower and fruit development (Boss *et al*., 2002).

Interestingly, *VvMADS5* expression was restricted to the carpels of female flowers and may suggest it is involved in ovule development, whereas the other genes were expressed in the inner whorls of both male and female flowers suggesting they fulfil the function of C class genes in grapevine.

1.5. Understanding flowering in *Protea*

Currently, little is known about the molecular mechanisms of flowering in *Protea*. However, the identification and study of key floral inducers in other species have provided insights into the genetic control of their flowering response. Towards creating transgenic plants with altered flowering times, and in elucidating the function of these genes in agriculturally important species, this information has been used for the genetic manipulation of plants. Currently, however, there is no transformation system available for *Protea* and therefore transgenic work in this floricultural crop is not possible as yet.

Protea plants are cultivated mostly in the Western Cape of South Africa, but less extensive cultivation is also practiced in the Eastern Cape and Gauteng regions. There is variation in the flowering times of wild-growing, widely distributed species, such as *P. repens*, *P. neriifolia* and *P. cynaroides* (Rebelo, 2001), between the eastern and western occurring plants. An early study on the cultivation of *P. neriifolia* in Eastern Gauteng, a summer rainfall region, compared shoot growth and flowering seasonality to that in its natural habitat, the Western Cape winter rainfall region (Heinsohn and Pammenter, 1988). The study found that *P. neriifolia*'s periods of active growth and flowering differed between these regions. Flowering occurred during late summer to autumn in the summer rainfall area compared to autumn and early winter in the Western Cape (Kruger, 1981). *P. neriifolia* is widely distributed across the Cape Floristic Region and different peaks in flowering have also been reported between eastern and western stands (Rebelo, 2001).

Why is there such a great variation of flowering times across the genus? Why do some commercially developed hybrids have different flowering times to either of the parental species? Flowering is controlled by a number of interacting factors. Firstly, the genetic make-up of each species may have differing flowering related alleles as is suggested by their different flowering times. Hybridisation to create cultivars for commercialisation may create hybrids with a combination of parental alleles that therefore display different

flowering responses. Similar segregation of flowering traits has been suggested with the hybridisation of two different accessions of *A. thaliana*, one naturally occurring on the Cape Verde Islands (Cvi) and another from Northern Europe namely Landsberg *erecta* (L*er*; Alonso-Blanco *et al.*, 1998). Secondly, many resources are required for successful reproduction such as: nutrients for seed filling; water for sustained photosynthesis and phloem transport; and carbon for structural development and metabolic activity. The flowering times of *Proteas* may therefore be determined by any of these factors.

Very little literature is available that deals with floral induction in *Protea*. This study aimed to unravel some of the complexities involved in floral initiation and flowering of *Protea*. To achieve this, a combination of physiological and molecular techniques was employed.

1.6. Hypotheses of this study

1) Protea *inflorescences develop very large inflorescences that require large amounts of photosynthate for structural and metabolic maintenance. The timing of flowering in* Protea *could therefore be dependent on the photosynthetic capacity of source leaves and environmental conditions that would support the continuous supply of carbon throughout inflorescence development.*

To test this hypothesis, both structural and metabolic carbon requirements of various sized *Protea* inflorescences were measured. The ability of source leaves to support these developing structures was assessed by source-sink manipulation. The findings of these experiments are discussed in chapter 2.

2) *Seasonal flowering of* Protea *is likely to be under control of the CO/FT module as described for other woody plants that flower in response to seasonal change. This control would hinge on the presence of a functional circadian clock to allow accurate measure of photoperiod and temperature changes.*

As molecular studies in *Protea* have not previously been performed, the optimisation of various techniques was required. To investigate gene expression, an RNA extraction protocol was developed that allowed the high-throughput isolation of high quality RNA suitable for reverse transcriptase PCR and quantitative real-time PCR (published as

Smart and Roden, 2010). The functioning of the circadian clock in *Protea* was investigated by measuring DF, which is under circadian regulation. An *FT* orthologue was isolated from *Protea* cultivar 'Carnival' and *ProFT* diurnal expression profiles followed over a number of months, corresponding to various seasons. These results are discussed in chapter 3.

3) ProLFY *may be involved in floral transition and patterning of* Protea *inflorescences similar to the roles of* LFY *orthologues in other species. As* LFY *orthologues are often present as multiple copies in woody perennials, and polyploid plants, multiple* ProLFY *orthologues may be present in hybrid cultivars such as 'Carnival'.*

LFY orthologues were identified from 'Carnival' and the biological function of these was investigated by heterologous expression in *A. thaliana*. These results are discussed in chapter 4.

CHAPTER 2: How costly is flowering? Source-sink alteration affects photosynthesis and inflorescence development of commercially grown *Protea*

2.1. Abstract

Protea inflorescences incur high carbon costs because of their large size and lengthy developmental period. To investigate whether the metabolic cost of inflorescence development could be a determinant of the flowering time of *Proteas*, respiration rates were determined for inflorescences from three *Protea* hybrids, 'Carnival' (*P. compacta x P. neriifolia*), 'Sylvia' (*P. eximia* x *P. susannae*) and 'Ivy' (*P. lacticolor* x *P. mundii*), and from a wild grown species, *P. repens*. 'Carnival' and 'Sylvia' developed the most carbon-expensive inflorescences, requiring *ca*. 315 and 221 g carbon for respiration and structural development, whereas *P. repens* and 'Ivy' only required 15 and 8 g carbon respectively. To investigate the carbon status of *Protea* plants, the photosynthetic response to altered source and sink ratios by either inflorescence removal or defoliation was measured. Removal of inflorescences led to a reduction in the photosynthetic rates of the leaves on the flushes immediately subtending the inflorescences of 'Carnival' and 'Ivy', but similar responses were not observed in 'Sylvia' or *P. repens*. Partial defoliation of 'Carnival' inflorescence-bearing shoots did not result in any significant change in the photosynthetic rates of the remaining leaves or in the relative inflorescence volume of developing inflorescences. However, severe defoliation of 'Sylvia' shoots carrying inflorescences developing between late autumn and early winter led to an increase in the photosynthetic rates of the remaining leaves, and these plants developed inflorescences with reduced relative volumes. Despite this, the inflorescences were not aborted even after complete defoliation. Based on these results, inflorescence development in 'Sylvia' and *P. repens* plants is source-limited. For 'Sylvia', this may be a consequence of year-round development of carbon 'expensive' inflorescences. Whereas, the growth habit of *P. repens* requires the development of inflorescences photosynthetically supported by leaves from only one growth flush. These data do not suggest that the flowering times of these cultivars are determined by carbon availability, although the carbon cost of inflorescence development is high. Other resources such as nutrients are also required for inflorescence development and seed filling; flowering time may therefore be dependent on a combination of factors.

2.2. Introduction

Mature, wild-grown *Proteas* display a sympodial, branched growth habit (Kruger, 1979; Fig. 2.1) with single growth flushes terminating in inflorescences and the loss of apical dominance, leading to the development of side shoots from axillary meristems subtending the inflorescence. These side shoots will carry the following season's inflorescences. Commercial cultivars do not display this growth habit because of cultivation, pruning and harvesting practices to increase stem length, production and shift flowering times (Greenfield *et al.*, 1994; Gerber *et al.*, 1995; Gerber *et al.*, 2001c). This leads to the development of inflorescences on stems with two to four seasonal growth flushes, depending on the cultivar and the growth conditions (Gerber *et al.*, 2001a).

Many Proteaceae develop especially large inflorescences consisting of numerous spirally arranged flowers enclosed in colourful involucral bracts (Rebelo, 2001). Wild *Protea* species flower mostly in spring and summer with only a few flowering in autumn (Rebelo, 2001). Commercially cultivated hybrids often display much broader flowering times than their parental species. Gerber *et al.* (2001a) categorized the flowering times of these cultivated *Proteas* into: 1) summer to autumn, 2) late winter to early summer and 3) all-year-round flowering. The latter two categories may require inflorescence development under sub-optimal conditions for carbon acquisition in winter, associated with low light levels. Flower initiation in angiosperms is controlled by both intrinsic and environmental factors (Bernier, 1988). As the flowering times of *Protea* spp. and their hybrids vary greatly, it is unlikely that a single stimulus is responsible for floral initiation across the genus.

When assessing the resource requirements for reproduction, two factors need to be considered. Firstly, which resources may be constraining for the study species i.e. depending on the environmental conditions and ability of the plant to acquire these resources; and secondly the period for which resources would be required, i.e. floral longevity (Ashman and Schoen, 1996). To ensure reproductive success, adequate water and carbon for maintenance and structural development as well as nutrients and minerals for seed filling are essential (Bazzaz and Carlson, 1979). The resource costs involved in developing and supporting *Protea* inflorescences are expected to be especially high because of their large size (Coetzee and Littlejohn, 2001; Rebelo, 2001),

copious nectar production (Nicolson and Van Wyk, 1998; Rebelo, 2001; Nicolson, 2002) and extreme longevity (up to 100 d for some cultivars; Gerber *et al.*, 2001a). Timing of inflorescence induction may therefore be linked to the availability of resources, particularly photosynthate.

Photosynthate availability may be a constraint on the induction of flowering in Proteaceae, resulting in the timing of flowering coinciding with environmental conditions that ensure an adequate photosynthetic supply to developing inflorescences. Apart from environmental constraints, the availability of photosynthate is also likely to be determined by the photosynthetic capacity of the subtending shoot to supply the inflorescence. To test these hypotheses, the consequences of inflorescence removal on the photosynthetic rates of subtending shoots were investigated. Shoots were also partially or completely defoliated to determine the developmental penalties for inflorescences. The rates of inflorescence respiration were compared to the photosynthetic activity of the subtending shoots to determine whether the supply was adequate to meet the inflorescence demand. For all the cultivars studied, except the year-round flowering 'Sylvia', shoots had excess photosynthetic capacity to support flowering. This is partly because cultivated Proteaceae often have several growth flushes subtending the same inflorescence, unlike the situation in wild-grown *Protea* spp that only have one flush per inflorescence.

2.3. Materials and methods

2.3.1. Plant material, sampling and site description

Protea plants are composed of a large number of branched structures that carry many flowering (Fig. 2.1) and vegetative shoots. *P. repens* plants used in this study grew wild and displayed the natural growth habit of *Protea* spp. (Fig. 2.1). After floral initiation, apical dominance is lost, allowing the development of the subtending axillary buds. Commercially grown *Proteas*, such as the hybrids 'Carnival', 'Sylvia' and 'Ivy', are pruned and the auxiliary buds removed resulting lengthened shoots (Fig. 2.1) which are commercially more desirable as cut-flowers. The lengthened shoots are comprised of a number of seasonal flushes, numbered from the oldest to the youngest for this study (Fig. 2.1). In cultivation, 'Carnival' predominantly develops inflorescences on shoots carrying four seasonal flushes; 'Sylvia' develops inflorescences on either three- or four-flush shoots; and 'Ivy' develops inflorescences on two-flush shoots.

Measurements were made on plants grown in commercial plantations on the farm 'Protea Heights' (33°54'S; 18°48'E), Stellenbosch, South Africa; between November 2007 (mid-summer) and June 2008 (early winter). The cultivars 'Sylvia' (hybrid of *P. eximia* x *P. sussanae*), 'Carnival' (hybrid of *P. compacta x P. neriifolia*) and 'Ivy' (*P. lacticolor* x *P. mundii*) were grown in rows spaced 1 m apart with 4 m between rows. 'Carnival' was pruned for biennial bearing (Gerber *et al.*, 1995). Shoots of similar size and developmental stage were selected from independent plants (n=5) to serve as replicates for each experiment.

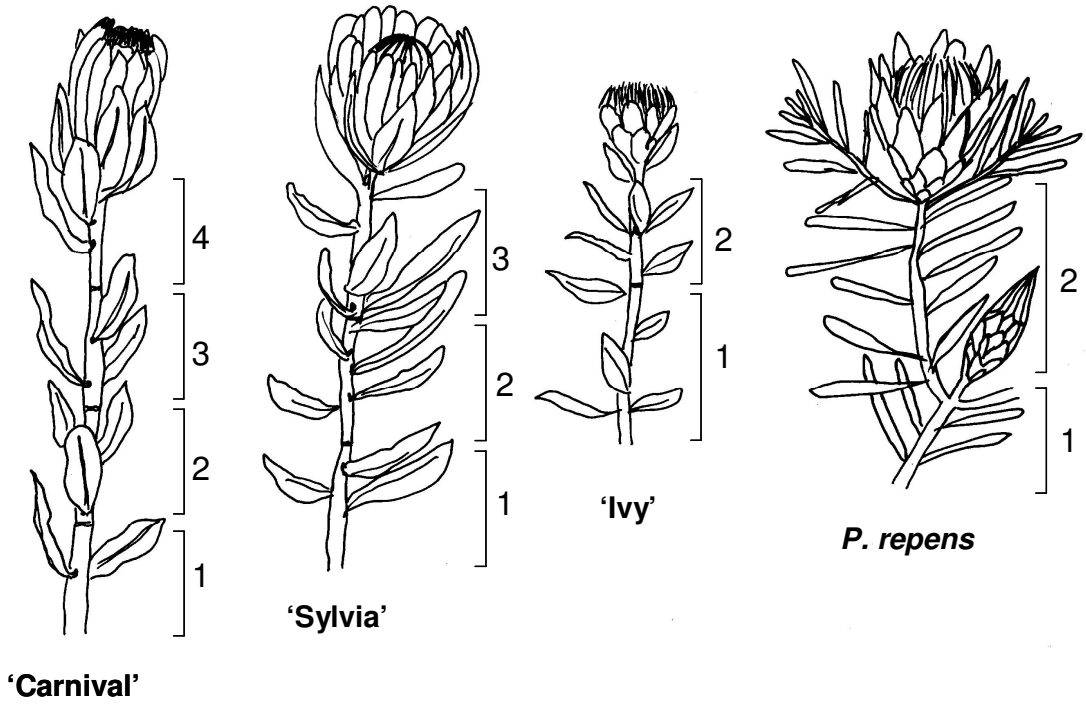


Figure 2.1. Schematic representation of shoots with mature inflorescences indicating the succession of seasonal growth flushes for the *Protea* hybrids and species used in this study. Numbers represent the growth flushes starting from the oldest flush.

2.3.2. Inflorescence and leaf size measurements

Allometric analyses were performed on data for *Protea* species collated from Rebelo (2001) that included leaf width (w) and length (l), and inflorescence w at widest point. Averages were calculated based on the minimum and maximum values recorded. Similar measurements for hybrid *Proteas* were performed at the study site, and average values (n=10) were included in analyses. Leaf size was calculated as w x l and correlated to inflorescence w across the *Protea* genus.

2.3.3. Respiration measurements

Shoots carrying developing inflorescences were excised from plants and kept in the dark in a temperature controlled room for 1 h prior to analyses. Respiration rates were measured by excising developing floral buds or inflorescences from the shoots and immediately placing them in either 125 or 250 ml plastic cuvettes, depending on their size. Cuvette lids had one inlet and one outlet tube attached to allow for sampling of CO_2. Air with a constant CO_2 concentration was pumped into the cuvette and the flow rate (*ca.* 350 ml min^{-1}) into each cuvette was recorded. After allowing *ca*. 30 min for equilibration, air leaving the cuvette was measured with an ADC LCA-2 infrared gas analyzer (IRGA; The Analytical Development Co. Ltd., Hoddesdon, England). The delta-CO_2 concentration was recorded on a data logger (Vernier, Oregon, USA) every 0.1s for 30s and the average calculated over 10s to account for minor fluctuation in the output values. Floral tissues were subsequently dried at 70°C for 72 h, allowed to cool to room temperature and weighed. Respiration rates were expressed both as nmol CO_2 s^{-1} (i.e. per inflorescence) and as nmol CO_2 g^{-1} DW s^{-1} (i.e. specific respiration rates). Leaf areas of the flushes on the subtending shoot were measured with an LI–3100 area meter (LI-COR Biosciences Inc.).

Data from respiration curves were used to calculate the total carbon expenditure of inflorescences lost to respiration during development. Expended carbon was calculated by integrating the area under the respiration curves (CurveExpert 1.40) generated by plotting respiration rates (nmol CO_2 s^{-1}) against inflorescence DW (g). For these calculations, it was assumed that all *Protea* inflorescences took 100 d to form mature inflorescences (Gerber, 2001a), and developed at a constant temperature. Carbon input into structural development was calculated from mature inflorescences assuming that 51 ± 0.9% (w/w) of the DW of the structure comprised carbon (based on previous mass

spectrophotometry measurements on *Protea* inflorescences; Smart, 2005; MSc dissertation, University of Stellenbosch). The total carbon cost of *Protea* inflorescence development was determined by adding the respiratory and structural carbon costs.

2.3.4. Defoliation and inflorescence removal studies

Defoliation was performed on 'Carnival' and 'Sylvia' shoots carrying small, immature inflorescences subtended by four seasonal flushes. Un-manipulated shoots, identified at the time of defoliation, were used as control shoots. Leaves were removed from single growth flushes, 1 to 4 (Fig. 2.1) of 'Carnival' or half of the leaves from flush 3 or 4. Defoliation of 'Carnival' was performed in December (summer), when 'Carnival' inflorescences normally develop. For 'Sylvia', defoliation was performed in May (autumn) and treatments were: removal of single flushes (except flush 1 and 2 that were removed together), removing all leaves except those of a chosen flush and complete removal of all the leaves from a shoot. Photosynthetic rates of the remaining leaves were measured (see below) for 'Carnival' after 49 d and again after 57 d, and after 46 d for 'Sylvia'. The dimensions of the developing inflorescences were also measured at these time points using a calliper. Relative change in inflorescence volume was calculated using $\frac{\ln vol_2 - \ln vol_1}{t_2 - t_1}$, where t = time in days (d) and vol = volume of the inflorescence calculated for an oblate spheroid using $vol = \frac{1}{3}(\pi r^2) \times h$, where r = width/2 of inflorescence and h = overall height of inflorescence.

To assess the effect of sink strength reduction on source assimilation, shoots bearing inflorescences of similar sizes were identified and the inflorescences excised 14, 10, 6 and 0 d prior to measurement of photosynthesis (see below). Both developing (12.67 ± 0.50 g DW) and mature (22.39 ± 4.01 g DW) 'Carnival' inflorescences were removed from flowering shoots, to determine which developmental stage might elicit a photosynthetic response. Only developing inflorescences were removed from the other *Proteas*.

2.3.5. Leaf gas exchange analyses

Photosynthetic rates of leaves from each seasonal flush (Fig. 2.1) subtending the developing inflorescence were measured using a Licor 6400 portable photosynthesis system (LI-COR Biosciences Inc., Nebraska, USA). Leaves used for repeat measurements were marked with permanent marker away from site of measurement to allow identification in subsequence measurements. The conditions in the 'leaf' cuvette (standard 2x3 chamber with 6400-02 LED light source) were: light intensity 1500 µmol m^{-2} s^{-1}; flow rate 500 µmol s^{-1}; cuvette temperature set to within 0.5°C of the ambient air temperature at the start of the measurements. Photosynthesis was measured between 09:30 and 16:00 on sunny days with minimal cloud cover.

2.3.6. Statistical analyses

Linear correlations between inflorescence size and leaf size, and specific respiration rate and inflorescence DW were calculated by performing Pearson's correlation calculations. Non-parametric correlations between respiration rate and inflorescence DW was calculated by performing Spearman's rank order correlation calculations. All correlations were performed using Statistica (Version 10, StatSoft Inc., Tulsa, USA). Where appropriate, results were subjected to analysis of variance (ANOVA) to determine significant differences using Statistica (Version 10). *Post hoc* Fisher LSD (95%) multiple range tests were conducted to determine the statistical differences between treatments.

2.4. Results

2.4.1. Shoot characteristics and allometry

Across the *Protea* genus there was a weak positive correlation (r=0.34; P=0.0032; n=72) between leaf size (l x w) and average inflorescence w (Fig. 2.2). The *Protea* cultivars used in this study had inflorescence and leaf sizes comparable to the other members of the genus as well as their parental species, clustering around the fitted regression line. *P. cynaroides* with a inflorescence size of 210 mm and *P. punctata* with a inflorescence size of 22.5 mm fall above and below, respectively, the confidence range (95%) for this trend. Removing these from the correlation analysis only slightly improved the strength of the regression (r=0.36).

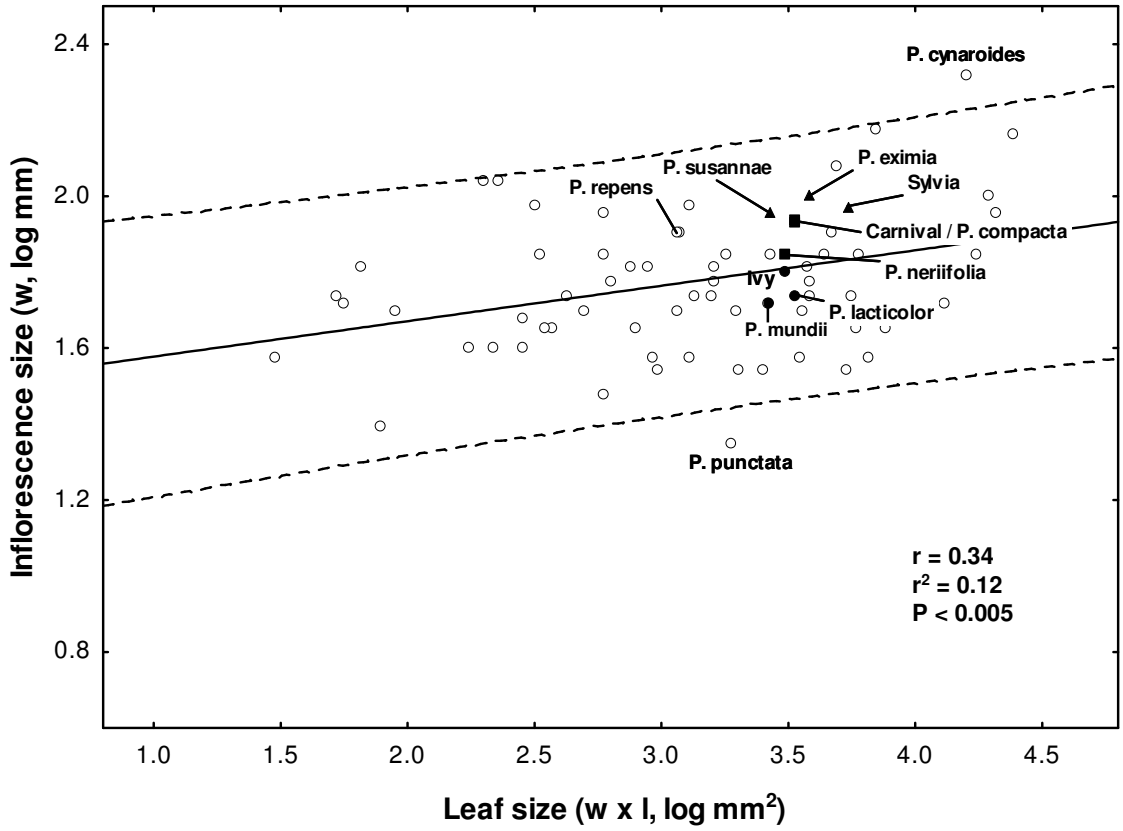


Figure 2.2. Correlation between leaf size (w x h) and flower size (w) for 69 species and three cultivars of the genus *Protea*. The Pearson's correlation coefficient (r), coefficient of determination (r^2) and significance (P) thereof are shown. The fitted regression is given by a solid line and its 95% confidence limits by dashed lines. Symbols indicating hybrid cultivars and their parental species are as follows: Ivy (●) hybrid of *P. lacticolor* (●) and *P. mundii* (●); Carnival (■) hybrid of *P. neriifolia* (■) and *P. compacta* (■); 'Sylvia' (▲) hybrid of *P. eximia* (▲) and *P. sussanae* (▲); and other species (○). Markers for 'Carnival' and *P. compacta* fall on the same position.

2.4.2. Inflorescence carbon costs and the effect of sink removal on photosynthetic rates

Carbon required for *Protea* inflorescence maintenance and development was determined by measuring the respiration rates of floral tissue at various stages of development (Fig. 2.3). For 'Sylvia', 'Carnival' and *P. repens* the respiration rates (nmol CO_2 s^{-1}) increased until the inflorescence was mature, after which it sustained a more stable rate. Respiration rates of 'Ivy', with the smallest inflorescence, did not reach a similar plateau during maturation of the inflorescence (Fig. 2.3). Specific respiration rates (nmol CO_2 g^{-1} s^{-1}) of *Protea* inflorescences decreased as their weight increased

(Fig. 2.4). 'Sylvia' inflorescences incurred the greatest respiratory rates (Table 2.1; Fig. 2.3 A), although their specific respiratory costs (nmol CO_2 g^{-1} s^{-1}) were not significantly different from those of 'Carnival' (Fig. 2.4 A and B). For 'Sylvia', 'Carnival', *P. repens* and 'Ivy' the carbon input into respiration and structural development of a mature inflorescence calculated from respiratory curves and dry weights were approx. 315, 221, 15 and 8 g, respectively. Respiratory costs contributed to 95% of the total carbon costs calculated for 'Sylvia' and 'Carnival' and 65 and 77% for *P. repens* and 'Ivy', respectively.

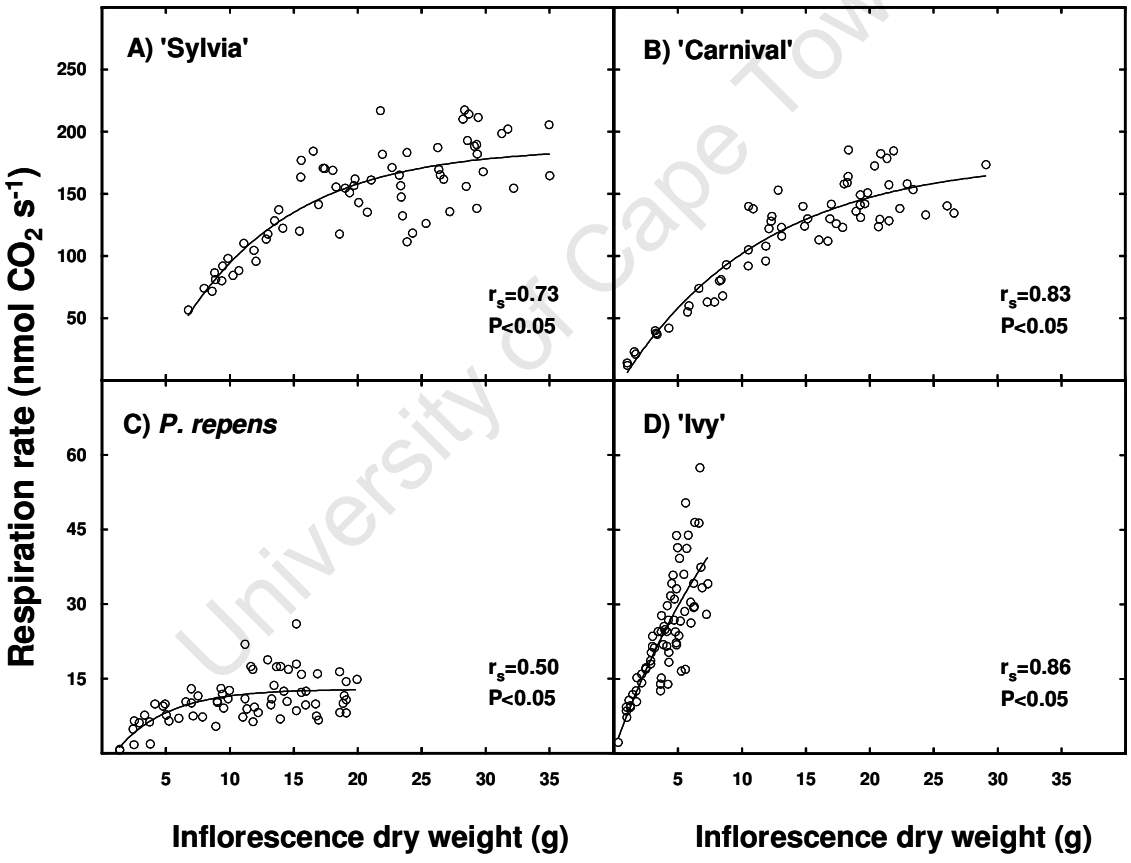


Figure 2.3. Respiratory CO_2 flux from *Protea* inflorescences at various stages of development. Note variation in scales. Spearman's rank order correlations were performed for non-parametric relationships using Statistica 10 (StatSoft Inc., Tulsa, USA) and Spearman's r values (r_s) are shown. 'Sylvia', n=67; 'Carnival', n=64; *P. repens*, n=67 and 'Ivy', n=70

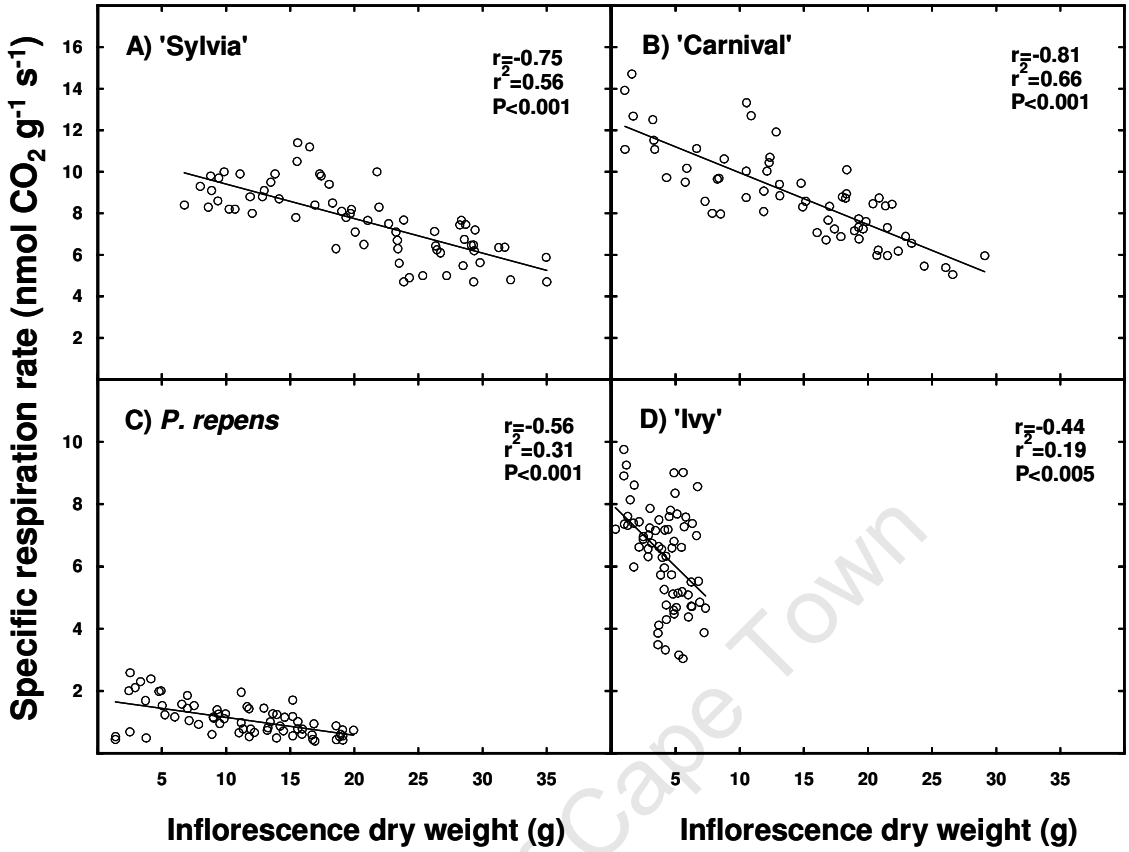


Figure 2.4. Specific respiration rates for developing *Protea* inflorescences expressed per dry weight of structures. Note variation in scales. Pearson's correlation coefficients (r), coefficients of determination (r^2) and significance (P) of regressions are indicated. 'Sylvia', n=67; 'Carnival', n=64; *P. repens*, n=67 and 'Ivy', n=70.

The seasonal flush subtending mature inflorescences of 'Carnival' had the greatest leaf area when compared to the other *Proteas* studied (Table 2.1). 'Sylvia', which developed the heaviest flower (*ca*. 28 g DW) had a lower leaf area, but similar leaf photosynthetic rates (µmol CO_2 m^{-2} s^{-1}) and total flush photosynthesis (µmol CO_2 s^{-1}) as 'Carnival'. In order to evaluate the capacity of the shoot to supply the photosynthate necessary for inflorescence development, the total flush photosynthesis was expressed versus the respiratory rates of the mature inflorescences (Table 2.1). 'Ivy' had the greatest carbon assimilation versus inflorescence respiration rates, whereas *P. repens*, 'Carnival' and 'Sylvia' had 58, 32 and 26% less than Ivy, respectively.

Table 2.1. Leaf area, inflorescence weights, leaf area per g inflorescence DW, photosynthetic and respiratory rates, total flush photosynthesis and photosynthesis versus specific respiratory rates of the inflorescences and subtending seasonal flush of flowering shoots carrying mature inflorescences for *P. repens* and the *Protea* cultivars 'Sylvia', 'Carnival' and 'Ivy'.

	'Sylvia'	'Carnival'	*P. repens*	'Ivy'
Leaf area flush^{-1} (cm^2)	481 ± 20 **a**	626 ± 15 **b**	209 ± 20 **c**	336 ± 16 **d**
Inflorescence DW (g)	28.0 ± 0.9 **a**	21.9 ± 0.5 **b**	15.6 ± 0.6 **c**	4.9 ± 0.2 **d**
Leaf area/Inflorescence DW (cm^2 g^{-1})	17 ± 0.9 **a**	29 ± 1.4 **b**	14 ± 1.6 **a**	71 ± 3.9 **c**
*Photosynthetic rate (µmol CO_2 m^{-2} s^{-1})	16.8 ± 0.9 **a**	15.0 ± 1.1 **a**	9.4 ± 1.3 **b**	15.5 ± 0.3 **a**
*Flush photosynthesis (µmol CO_2 s^{-1})	0.78 ± 0.08 **a**	0.95 ± 0.09 **a**	0.24 ± 0.05 **b**	0.54 ± 0.04 **c**
Inflorescence respiration (µmol CO_2 s^{-1})	0.183 ± 0.005 **a**	0.151 ± 0.009 **b**	0.017 ± 0.002 **c**	0.034 ± 0.008 **bc**
Photosynthesis/Respiration	5 ± 0.2 **a**	6 ± 0.3 **a**	11 ± 2 **abc**	19 ± 5 **c**

Data are means ± SE (n=20). Dissimilar letters indicate statistical significance determined by ANOVA followed by *post hoc* Fisher LSD (95%) multiple range tests. *Photosynthetic rates and flush photosynthesis is shown for the growth flush immediately subtending the inflorescence. Measurements for all the species were taken in March (summer).

There was no significant reduction in the photosynthetic rates of the leaves after the removal of a mature 'Carnival' inflorescence (Fig. 2.5 A). However, removal of a developing inflorescence resulted in a 14% reduction in the photosynthetic activity of the leaves on Flush 4 ($P<0.05$), 6 d after inflorescence removal compared to the control (0 d) (Fig. 2.5 A). Inflorescence removal for the other cultivars and *P. repens* was therefore performed on developing structures. Leaves of 'Ivy' on Flush 2, immediately subtending the inflorescence, also responded to inflorescence removal with significantly reduced (22%) photosynthetic rates ($P=0.004$) 6 d after inflorescence removal (Fig. 2.5 B). None of the other, older, flushes showed a detectable response to inflorescence removal (Table 2.2). There were no significant change in the photosynthetic rates of leaves in response to inflorescence removal in either 'Sylvia' or *P. repens* (Fig. 2.5 C and D; Table 2.2).

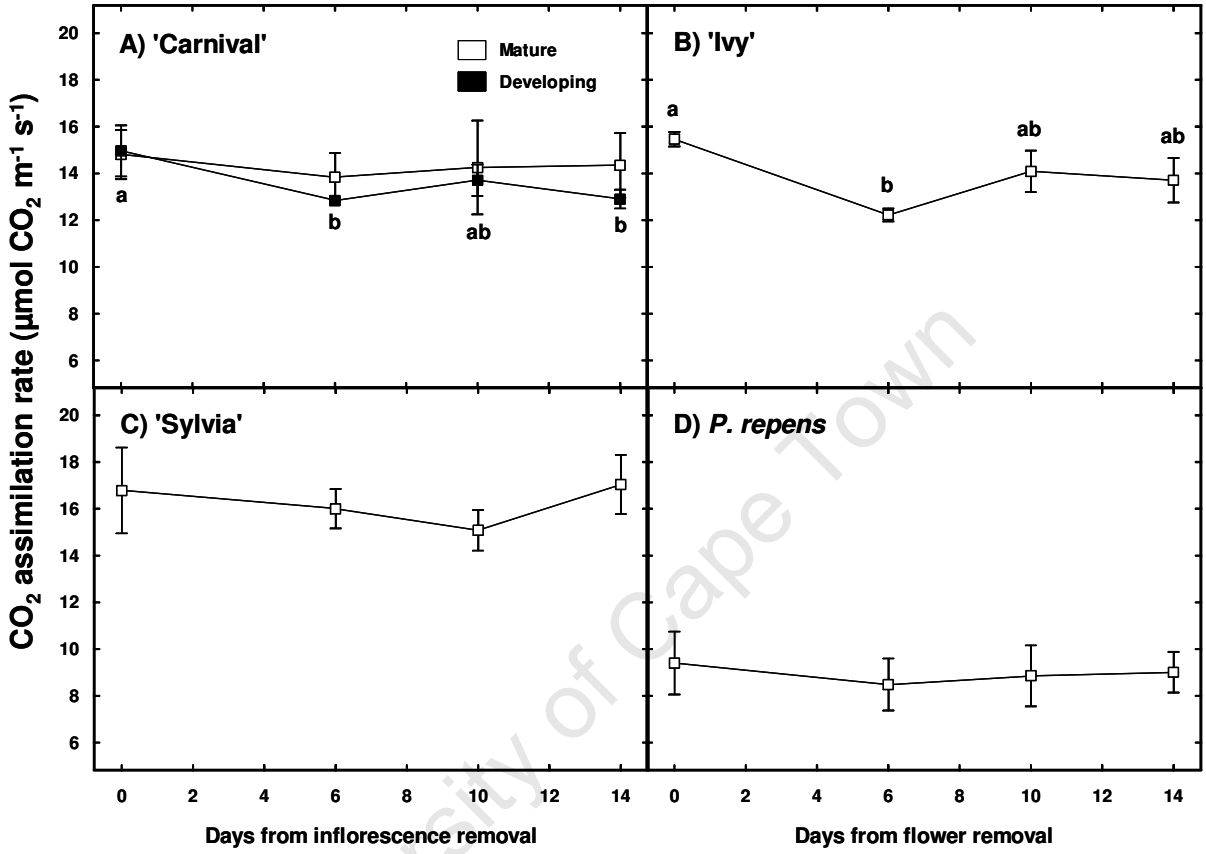


Figure 2.5. Photosynthetic rates of leaves on the growth flush immediately subtending the inflorescence 0, 6, 10 and 14 d after inflorescence removal. The effect of inflorescence removal on leaf photosynthesis for both developing (12.67 ± 0.50 g DW) and mature (22.39 ± 4.01 g DW) inflorescences was investigated only for 'Carnival'. Data are the means ± SE (n=5). Dissimilar letters below or above data points indicate statistically significant differences (P<0.05) determined by ANOVA *post hoc* Fisher LSD (95%) multiple range tests. Where letters have been omitted no significant differences were found.

Table 2.2. Photosynthetic rates of leaves from subtending flushes 14, 10, 6 and 0 days after inflorescence removal from 'Carnival' (both developing and mature), 'Sylvia', 'Ivy' and *P. repens* shoots.

		Days after inflorescence removal			
Hybrid / species	Flush measured *	0	6	10	14
'Carnival' (developing)	3	11.4 ± 1.8	8.9 ± 1.2	12.0 ± 1.4	10.4 ± 0.8
	2	8.0 ± 1.6	9.0 ± 0.8	8.6 ± 0.4	10.0 ± 1.4
'Carnival' (mature)	3	12.3 ± 1.3	11.7 ± 0.9	13.0 ± 1.0	11.6 ± 0.5
	2	11.7 ± 0.8	10.6 ± 0.9	12.9 ± 0.6	11.6 ± 0.7
	1	9.1 ± 0.5	8.9 ± 0.8	9.7 ± 1.4	10.0 ± 1.2
'Sylvia'	2	14.0 ± 1.1	14.0 ± 0.8	15.1 ± 1.1	16.3 ± 1.1
	1	11.8 ± 1.4	13.7 ± 1.4	11.7 ± 0.5	11.8 ± 1.6
'Ivy'	1	10.5 ± 0.6	10.1 ± 0.7	11.0 ± 1.5	11.3 ± 0.5
P. repens	1	5.3 ± 1.2	6.2 ± 1.1	7.3 ± 2.2	7.7 ± 0.8

Data are mean ± SE (n=5). *Flushes are numbered from oldest to youngest as per diagrammatic representation in Fig. 2.1. Data for the uppermost flush subtending the inflorescence is shown in Fig. 2.5. There were no significant differences (P>0.05) between photosynthetic rates determined by ANOVA followed by *post hoc* Fischer LSD multiple range tests. Data for each *Protea* and seasonal flush were analysed separately.

2.4.3. Effect of removal of foliar capacity on photosynthesis and inflorescence development

Defoliation of 'Carnival' shoots was performed when inflorescences weighed 0.52 ± 0.07 g DW with respiration rates of 4.74 ± 0.59 nmol CO_2 s^{-1} (n=5). Although the carbon demand at this stage was small, this increased significantly with increasing size (Fig. 2.3). Additional carbon would also be required for structural growth. The photosynthetic rates of the remaining leaves were measured after 49 and 67 d. There were no significant changes in the photosynthetic rates of remaining leaves at either of these time points (Fig. 2.6). Only shoots of which the 3rd and 4th growth flushes closest to the inflorescences were removed, and the control shoots, were measured at 67 d post defoliation. No significant differences in photosynthetic rates between 49 d and 67 d could be observed, even though the inflorescences increased in volume by 16.45 ± 1.06 cm^3 (n=33) in the 18 d between measurements.

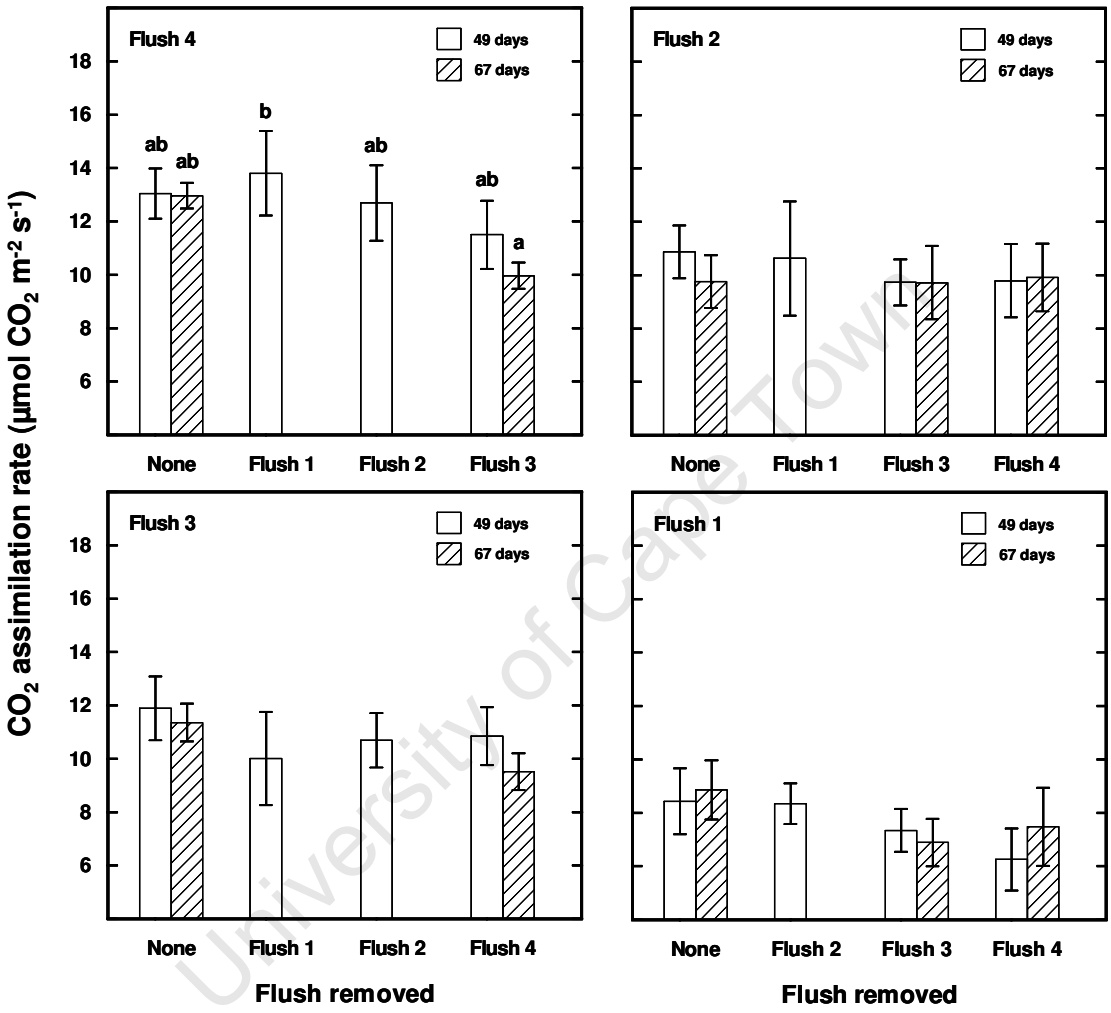


Figure 2.6. Photosynthetic CO_2 assimilation rates of remaining leaves 49 and 67 d after defoliation of inflorescence-bearing 'Carnival' shoots. Growth flushes removed during defoliation are indicated on the x axis. The flush measured is indicated in the top left corner of the graph. Flushes are numbered as indicated in Fig. 2.1. Bars represent means ± SE (n=5) and dissimilar letters above bars indicate statistically significant differences (P<0.05) determined by ANOVA *post hoc* Fisher LSD (95%) multiple range tests. Where letters are omitted no significant differences were found.

'Sylvia' shoots were defoliated when small, florally-determined meristems were visible. Photosynthetic rates of leaves on flush 3 of un-manipulated inflorescence-bearing 'Sylvia' shoots, were significantly higher ($P<0.05$) than those on flush 4, but similar to those on flush 2 (Fig. 2.7).

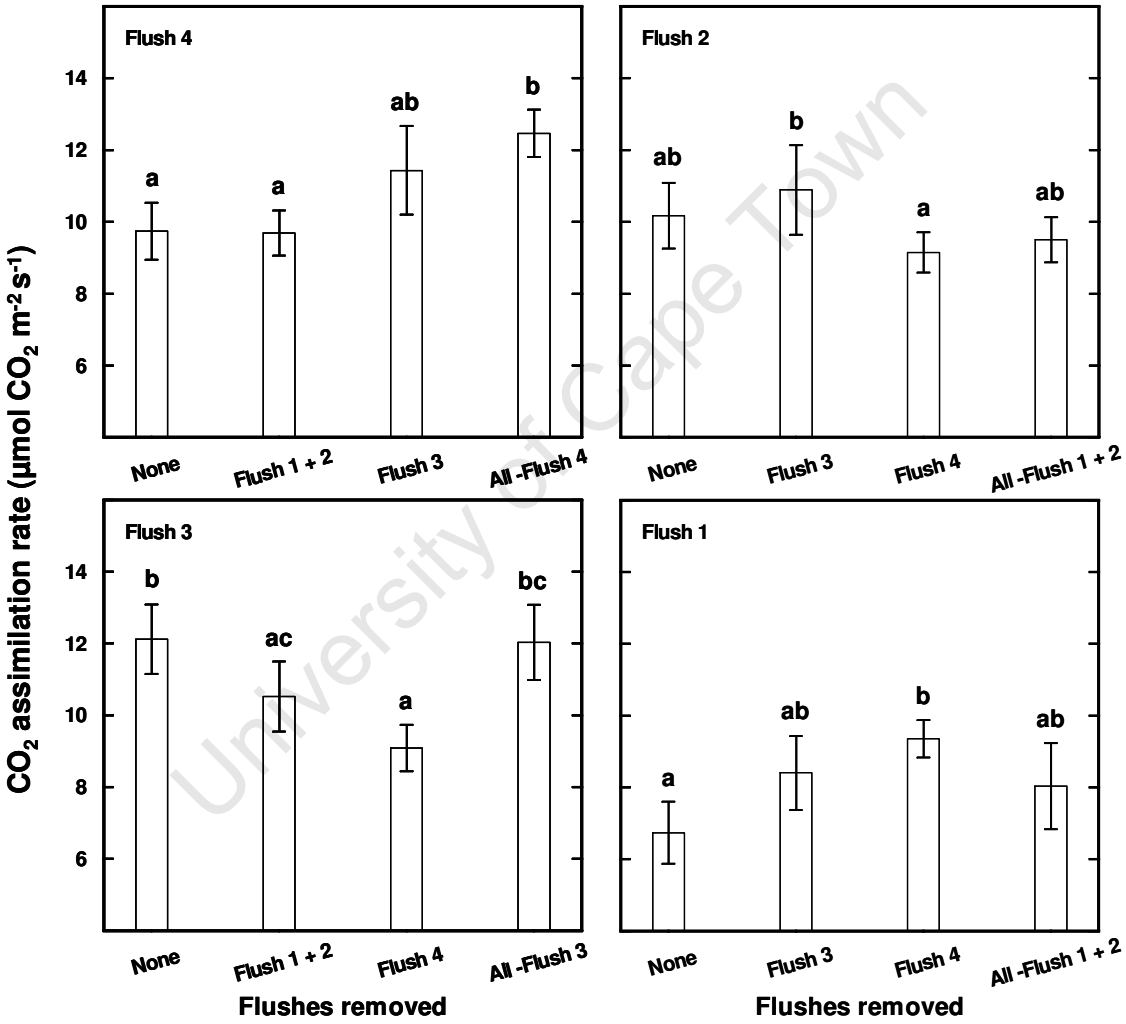


Figure 2.7. Photosynthetic CO_2 assimilation rates of remaining leaves on 'Sylvia' inflorescence bearing shoots 46 d after defoliation. The flush/es removed are indicated on the x axis. 'All –Flush' refers to the removal of all the leaves from the shoot except the flush measured. The flush measured is indicated in the top left corner of the graph. Flushes are numbered as indicated in Fig. 2.1. Bars represent means ± SE (n=5) and dissimilar letters above bars indicate statistically significant differences ($P<0.05$) determined by ANOVA *post hoc* Fisher LSD (95%) multiple range tests.

Leaves from the oldest flush, i.e. flush 1, had the lowest CO_2 assimilation rates. The flush thought to contribute most carbon to the developing inflorescence (flush 4), based on proximity to the sink tissue, showed increased photosynthetic rates with increasing severity of the defoliation treatment (Fig. 2.7). When only flush 4 was left on the shoot, the photosynthetic rate increased from 9.74 ± 0.79 µmol CO_2 m^{-2} s^{-1} (n=5) to 12.47 ± 0.65 µmol CO_2 m^{-2} s^{-1} (n=3; P=0.009). The photosynthetic rate of flush 3 decreased significantly (P<0.05) with the removal of the 4th flush and with removal of both the 1st and 2nd flushes. There was no increase in the photosynthetic rates of leaves on flush 3 when they were the only leaves remaining on the shoot. CO_2 assimilation rates of leaves on the 1st flush increased significantly (P<0.05) after removal of the 4th flush. Defoliation of inflorescence bearing 'Carnival' shoots did not result in a significant change in the inflorescence volume (Fig. 2.8 A) compared to the un-manipulated control. However, the relative change in inflorescence volume of 'Sylvia' was affected by leaf removal. Inflorescences carried on shoots with no leaves or with only leaves on flush 3 showed a significant reduction (P<0.05) in relative growth rate (Fig. 2.8 B).

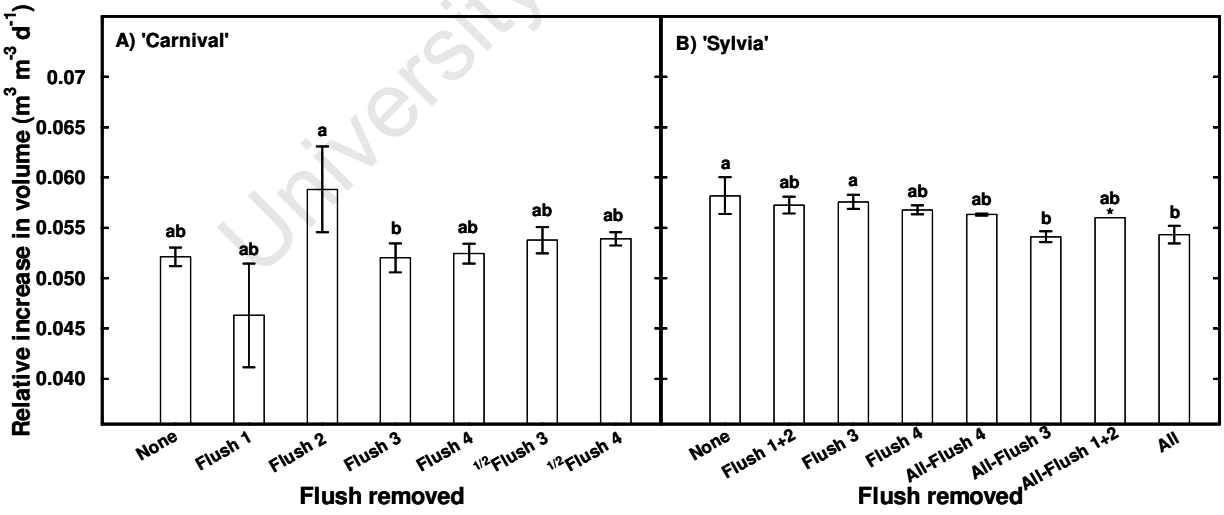


Figure 2.8. Relative increase of inflorescence volume following defoliation of the indicated flushes (x axis). Data for 'Carnival' and 'Sylvia' are shown and indicated in top left corner of graph. Inflorescence volumes were calculated for oblate spheroids from width (w) and height (h) measurements taken at the time of defoliation and after 49 d, for 'Carnival', and 46 d, for 'Sylvia'. Bars represent means ± SE (n=5) and dissimilar letters above bars indicate statistically significant differences (P<0.05) determined by ANOVA *post hoc* Fisher LSD (95%) multiple range tests.

2.5. Discussion

Proteas develop especially large reproductive structures with some cultivars such as 'Sylvia' carrying inflorescences weighing close to 30 g in dry weight (Table 2.1). These structures are both structurally and metabolically costly to develop. Results from the current study emphasises the high carbon costs of developing and maintaining these reproductive structures. For all *Proteas* studied, the specific respiration rates are highest during early development, as indicated by the fastest growth rates and highest metabolic activity (Fig. 2.4). Total respiration rates (nmol CO_2 s^{-1}) increased until maturation, after which changes were smaller (Fig. 2.3). At this stage all the structural development had been completed and carbon is only required for maintenance and nectar production. Similar results were found for developing pistachio (*Pistacia vera* L.) reproductive and vegetative organs (Marra *et al.*, 2009). Interestingly the same pattern was not evident for 'Ivy' inflorescences, the smallest used in this study (Table 2.1). This may indicate that 'Ivy' contains more metabolically active tissue at maturity compared to the inflorescences of other *Proteas*.

The highest specific respiration rates measured for *Protea* inflorescences were those carried by 'Carnival' (*ca.* 14 nmol CO_2 g^{-1} $s^{-}1$), while the highest total respiration rates were measured from fully developed 'Sylvia' inflorescences (ca. 200 nmol CO_2 s^{-1}). These specific rates were not higher than flowers from other species. For example, specific respiration rates of *Diplacus aurantiacus* were *ca*. 60 nmol CO_2 g^{-1} s^{-1} (Williams *et al.,* 1985). The total respiration rates of Orchid flowers (*Spiranthes cernua*), 85 nmol CO_2 s^{-1} (Antlfinger and Wendel, 1997), were similar to respiration rates from the small flowered hybrid 'Ivy' (*ca*. 60 nmol CO_2 s^{-1}), but higher than those of *P. repens* inflorescences (*ca*. 20 nmol CO_2 s^{-1}). However, the longevity of inflorescences determines the period for which carbon input is required, and needs to be considered when estimating carbon costs of flowering (Ashman and Schoen, 1994 and 1997). *Protea* inflorescences have a lengthy developmental period that may take up to 100 d (Gerber, 2001a) and fruits are often serotinous (Rebelo, 2001). Although the carbon costs of *Leucadendron* (Proteaceae) cones were not especially high (Cramer and Midgley, 2009), carbon and water are continuously lost by these serotinous structures. *Protea* inflorescences also produce copious amounts of sugar-rich nectar to attract birds for pollination which further increase their carbon costs (Nicolson and Van Wyk, 1998; Rebelo, 2001; Nicolson, 2002;). At maturity *P. neriifolia* inflorescences produce up to

10 ml nectar (Dai and Paull, 1995) which may contain up to 25% (w/w) sugar (Nicolson, 2002). Nectar production results in a dramatic sugar demand, at the expense of carbon available for the development of other sink tissues, and has been reported to consume up to 36.6% of carbon fixed by photosynthesis of milkweed (*Asclepias syriaca*) leaves daily (Southwich, 1984). *Protea* inflorescences therefore require a large and continuous photosynthate supply to successfully complete reproductive development.

Leaf and flower sizes across the *Protea* genus were correlated in an attempt to determine if a larger photosynthetic capacity could account for a larger reproductive effort, i.e. larger inflorescence. A weak positive correlation between leaf and flower size was evident across the *Protea* genus (Fig. 2.2), and these results indicated that variation in leaf size accounted for only 12% of the variation in inflorescence size. The weak correlation observed in this study may indicate that other factors, other than leaf size, influence inflorescence size (Langlade *et al*., 2005). One of the main determinants of inflorescence size and shape is pollination type (Schoen and Dubuc, 1990; Delph *et al.,* 1996; Jennions, 1996; Glaettli and Barrett, 2008) and this is also evident across the *Protea* genus. Bird and rodent pollinated inflorescences are mostly large to support the weight of the animal during feeding, whereas the smaller *Proteas* are mostly insect pollinated (Rebelo, 2001). Leaf size may also be environmentally determined as smaller leaved plants, for example, have greater fitness in dry environments (Ninnemets, 2001; Ackerly, 2004; Yates *et al*., 2010). This environmental adaptation is especially evident for *P. aristata* and *P. canaliculata*, which inhabit areas with high temperatures (>35°C) and low rainfall (*ca*. 500 mm year^{-1}; Rourke, 1980). These species have similar leaf areas (2.5 cm^2), however *P. aristata* develop inflorescences that are 16 times bigger than those of *P. canaliculata* (Le Maitre and Midgley, 1991). Allometric relationships between inflorescence and leaf mass for *Protea* have however, been reported by other authors (Midgley and Bond, 1989; Le Maitre and Midgley, 1991). For another Proteaceae genus, *Leucadendron*, allometric relationships between 'cone' and leaf mass and also 'cone' number and leaf size (w x l) have also been shown (Bond and Midgley, 1988; Midgley and Bond, 1989; Midgley, 2010). Based on the allometric relationships described in these studies and the high carbon costs associated with reproductive development, the functional relationship between leaves and inflorescences of *Proteas* was further investigated.

Why would the size of vegetative structures be related to those of reproductive ones? In general, allometric relationships indicate a physiological link between form and function (Niklas, 1994). As the main function of leaves is carbon assimilation, and inflorescences are mostly heterotrophic, leaf size may be related to inflorescence size, to ensure sufficient leaf area for carbon assimilation (Primack, 1987). Do *Protea* leaves assimilate enough carbon to support their large, 'carbon hungry' inflorescence throughout development as well as fulfil the carbon needs of other developing sinks to support polycarpic growth? Changes in the rate of photosynthesis in response to source-sink alterations are often used to assess the carbon status of plants (Hall and Milthorpe, 1978; Mondal *et al.*, 1978; Krapp and Stitt, 1995; Layne and Flore, 1995; Iglesias *et al.*, 2002; McCormick *et al.*, 2006; Franck *et al.*, 2006), but responses to source/sink manipulation are not observed in all cases (Herold, 1980; Dorchin *et al.*, 2006; Matloobi *et al.*, 2008; Gauci *et al.*, 2009).

After inflorescence removal, a reduction in photosynthesis was only observed for 'Ivy' and 'Carnival' subtending leaves and not for 'Sylvia' and *P. repens*. The age of the inflorescence removed also affected the magnitude of the photosynthetic response. After removal of a mature 'Carnival' inflorescence no reduction of photosynthesis was observed, however, photosynthetic rates were reduced when a developing inflorescence (ca. 12 g DW) was removed. This is because the mature inflorescences no longer have developmental costs, and photosynthate would only be required for further maintenance and nectar production. Similarly, no change in the photosynthetic rates of subtending leaves after 4 and 8 d were observed after removal of rose floral buds (Matloobi *et al.*, 2008). These authors suggest that the carbon made available from the reduction in sink strength would be translocated to the next strongest sink. Data from this study suggest a similar mode of action for wild growing *P. repens*. Commercial cultivars are pruned to produce lengthened shoots resulting in a greater leaf area subtending the developing inflorescence and an increased carbon assimilatory capacity. Thus the source-sink dynamic in a commercial system is different from that of wild-growing species. Together with having a much lower leaf capacity than the commercial cultivars (Table 2.1), *P. repens* also has small, narrow leaves with low photosynthetic rates. The wild growth habit also requires the simultaneous support of developing, mature and serotinous inflorescences as well as developing flushes (Fig. 2.1). The lack of reduction in photosynthesis after inflorescence removal of *P. repens*

may indicate that it is source-limited and any available carbon will simply be redirected to other sinks.

'Sylvia' and 'Carnival' had comparable inflorescence carbon costs and CO_2 assimilatory capabilities (Table 2.1). However, inflorescence removal in 'Carnival' led to a reduction in photosynthetic rates of the subtending leaves suggesting a sink-regulated system, whereas 'Sylvia' shoots did not respond to sink reduction (Fig. 2.6 A and C). The most prominent difference between these two cultivars is their flowering times. 'Sylvia' plants develop inflorescences throughout the year with a peak in production during the summer months of January and February, whereas flowering in 'Carnival' is limited to the summer period between January and March. All year-round flowering would require a continuous photosynthate supply, even during unfavourable photosynthetic conditions associated with winter (short day length, low light availability and low temperatures). It is probably due to reduced photosynthate availability under these conditions that inflorescence development in autumn/winter takes 5 months in comparison to 3 months under more favourable spring/summer conditions (Gerber *et al.*, 2001c). Defoliated 'Sylvia' shoots burdened with inflorescence development during autumn/winter, developed inflorescences with reduced growth rates compared to intact shoots (Fig. 2.8 B). These results suggested that the transport of assimilates from subtending leaves is crucial for inflorescence development during this period. 'Sylvia' shoots also suffer from a post-harvest problem called 'leaf blackening' which is prevalent in *Protea* species and hybrids carrying large flowerheads (Stephens *et al.*, 2001) such as *P. nerifolia* (McConchie *et al.*, 1991) and *P. eximia* (Bieleski *et al.*, 1992) and is caused by carbohydrate deficiencies (Jones *et al.*, 1995). During the winter months of this study, leaf blackening was observed on attached 'Sylvia' shoots carrying large inflorescences, and it is postulated that this may be due to a reduction in the photosynthetic activity of source leaves at this time. Year-round flowering combined with the development of a large, carbon 'expensive' inflorescence may deplete carbohydrates creating a carbon limited system resulting in both pre- and postharvest 'leaf blackening' in 'Sylvia'.

Plants consist of many physiologically integrated shoot modules. Commercial *Protea* plants are comprised of several stems, termed bearers, branching from a main stem that may each carry up to three shoots (flowering and/or vegetative). Source leaves therefore need to supply carbon to many developing sinks; including roots, vegetative

and floral meristems. The simultaneous development of various sink tissues often leads to the accumulation of less biomass by growing structures because of resource investment trade-offs (Ashman, 1994; Lopez *et al.*, 2001; Weiner *et al.*, 2009; Fu *et al.*, 2010). Because of the multi-stemmed growth habit of *Protea* plants, carbon may be available from other sources when the availability from the closest source leaves is limited. For example, translocation of carbon from neighbouring vegetative stems to defoliated reproductive stems has been shown for the *Camellia sasanqua* (Oitate, 2011) and *Olea europaea* (Proietti *et al.*, 2006). Inflorescence volume of 'Sylvia' was affected by complete defoliation (Fig. 2.8 B), but inflorescences were not aborted, as carbon would have been imported from other shoots or from remobilization of stored reserves. Thus multi-stemmed *Protea* plants may only respond to proximal sink strength reduction by decreasing their photosynthetic rates if other sink-demands are met.

'Carnival' and 'Sylvia' developed the largest inflorescences with the highest respiratory costs measured in this study (Table 2.1; Fig. 2.3 and 2.4). 'Ivy' had the smallest inflorescence, but interestingly *P. repens* inflorescences had the lowest respiratory rates. *P. repens* inflorescences may contain less metabolically active tissue, but more structural tissue, accounting for the lower respiratory rates even though inflorescence dry mass is higher than that of 'Ivy'. Our results support the hypothesis that *Protea* inflorescences are large carbon sinks. For at least two of the cultivars studied, 'Ivy' and 'Carnival', carbon appears to be readily available. Although photosynthate is always in high demand for plant tissue development, other resources required for inflorescence development may also be limited in certain environments. In general, Proteaceae grow in nutrient poor environments (Lamont, 1982; Stock and Lewis, 1984; Rebelo, 2001) and develop a high number of nutrient rich seeds (Kuo *et al.*, 1982; Stock *et al.*, 1990; Witkowsky and Lamont, 1996; Henery and Westoby, 2001; Groom and Lamont, 2010). Nutrient availability may therefore be a limiting factor in both inflorescence and seed biomass development especially in un-cultivated, wild grown *Proteas* that would not be fertilised.

2.5.1. Conclusion

Protea inflorescences are carbon expensive structures. However, for two of the hybrids studied, 'Carnival' and 'Ivy', carbon was readily available for inflorescence development. These hybrids have ample leaf area available for carbon assimilation, and develop

inflorescences during the spring/summer months when conditions are optimal for photosynthesis. *P. repens*, displaying the wild growth habit of un-pruned *Proteas*, also develops inflorescences during 'optimal' conditions for carbon assimilation. However, because it carries less leaf area and supports multiple sink tissues simultaneously, carbon is limited in this system. The *Protea* hybrid 'Sylvia', develops the most carbon-expensive inflorescences measured in this study, yet produces inflorescences throughout the year. Hybridisation may have resulted in genetic alterations in key floral induction genes in 'Sylvia', resulting in the precocious flowering phenotype observed. Whereas, the other hybrids and *P. repens* appear to induce flowering in response to clearly defined environmental signals, presumably through floral inductive pathways such as the photoperiodic pathway in response to increased day lengths. Although the results from this study did not suggest that inflorescence induction in *Protea* is controlled by carbon availability, understanding the limitations of source tissues to support inflorescence development may be important in a horticultural setting. As the carbon status of *Proteas* would affect productivity of these plants, findings of this study may guide growers to apply pruning practices and/or hormone- and nutrient-applications when plants would be most responsive to these treatments.

CHAPTER 3: Expression of a *FLOWERING LOCUS T* orthologue, *ProFT*, in leaves and meristems of *Protea* cultivar 'Carnival'

3.1. Abstract

Seasonally controlled flowering and growth of perennial plants is achieved through the accurate perception of day-length and, to some extent, temperature changes throughout the year. The *Protea* hybrid 'Carnival' (*P. compacta* x *P. neriifolia*), which is cultivated commercially for its flowers, displays photoperiod-responsive flowering. It was hypothesised, that this may be mediated through the measurement of seasonal changes by a functional circadian clock and its interaction with homologues of the photoperiodic flowering pathway, which has previously been identified in model plants. Rhythmic delayed fluorescence (DF), an output of the circadian clock, was successfully measured in 'Carnival' and in both its parental species, indicating the presence of functional circadian clocks in these plants. To investigate which conditions may be inductive for flowering of 'Carnival', an orthologous *FT* gene, *ProFT*, was isolated. *ProFT* showed increased expression in 'Carnival' leaves during October (13 h light; 11 h dark) when floral organs were being pre-formed in the meristem. The diurnal expression profile of *ProFT* during October also showed a significant peak in expression at 22:00 (3 h after dusk; Zeitgeber time 16), but was absent from tissues sampled during other months. *ProFT* expression was five-fold higher in florally determined buds compared to leaves, and low levels were present in vegetative meristems analysed. These results suggest that *Pro*FT may act as a floral inducer in 'Carnival', but based on spatial expression data is also likely to also play a role in inflorescence development and growth architecture.

3.2. Introduction

The Proteaceae genera *Protea*, *Leucadendron* and *Leucospermum* that are native to South Africa produce large colourful inflorescences that are cultivated and marketed as cut-flowers both locally and abroad (Criley, 2007). Most wild-grown *Proteas* flower in spring and summer, with a few species flowering in autumn (Rebelo, 2001). Flowering times of commercially developed hybrids, however, differ broadly with some even flowering outside of their parental species' flowering times (Gerber *et al.*, 2001c). Similar variation in flowering times has also been reported across species and hybrids from other Proteaceae genera, such as *Grevillea* (Joyce and Beal, 1999) and *Banksia* (Fuss

and Sedgley, 1991). Little is known about the physiological and molecular factors that influence the transition to flowering in *Protea*. However, other Proteaceae such as cultivars from *Leucospermum* (Malan and Jacobs; 1990) and *Leucadendron* (Hettash and Jacobs, 2006) have been reported to initiate flowering in response to short days. Because of the differing flowering times across the *Protea* genus, it is unlikely that a single signal is responsible for floral initiation across the genus. Although, evidence suggests that at least for *Protea* spp., which are less widely distributed than other spp. and display a smaller range of flowering periods, floral initiation may be environmentally cued. This is based on the fact that *Protea* spp. with broad geographic distributions display much broader flowering periods than narrowly distributed spp. (Rebelo, 2001). The cueing of flowering in *Protea* is likely to depend on genetic controls that are similar to those described for other species.

In *A. thaliana* the *FLOWERING LOCUS T* (*FT*) gene acts as a floral integrator of both environmental and endogenous signals that induce flowering. *FT* encodes a small 19.8 kDa protein in *A. thaliana* (Giakountis and Coupland, 2008) with high homology to the phosphatidylethanolamine-binding proteins (PEBP), also known as Raf1 kinase inhibitor proteins that are present in many organisms. These proteins have been implicated in various signalling cascades that influence a broad range of biological processes (Banfield and Brady, 2000; Vallée *et al.*, 2002). Translocation of the FT peptide from the site of induction in the leaves to the shoot apical meristem has been demonstrated for *A. thaliana* (Corbesier *et al.*, 2007; Mathieu *et al.*, 2007), cucurbits or squash (*Cucurbit moschata*; Lin *et al.*, 2007), tomato (*Solanum lycopersicon*; Shalit *et al.*, 2009) and rice (*Oryza sativa*; Tamaki *et al.*, 2007; Aki *et al.*, 2008). In the shoot apical meristem it interacts with the FLOWERING LOCUS D (FD) protein to up-regulate the meristem identity genes *APETALA1* (*AP1*) and *FRUITFULL* (*FUL*; Abe *et al.*, 2005; Wigge *et al.*, 2005), resulting in floral organ formation. The function of orthologous *FT* genes to induce flowering appears to be conserved between annuals and perennial trees. However, *FT* orthologues in woody perennial trees also fulfil roles in the control of growth and dormancy of meristems (Böhlenius *et al.,* 2006; Gyllenstrand *et al.,* 2007; Hsu *et al.,* 2011). Unlike annual plants, woody perennials also express multiple paralogous *FT* genes with differing spatial and temporal expression patterns (Böhlenius *et al.,* 2006; Gyllenstrand *et al.,* 2007; Hsu *et al.,* 2011; Igasaki *et al.,* 2008; Kotoda *et al.,* 2010; Nishikawa *et al.,* 2007; Shen *et al.,* 2011; Zhang *et al.,* 2010 & 2011). It is

likely that FT functions similarly in Proteaceae species displaying seasonally defined flowering and sympodial growth.

Flowering times of plants are often confined to specific seasons, as is also the case in many *Proteas*. This is achieved through accurate day-length measurement by a light-sensitive circadian clock, and interaction with components of the photoperiod flowering pathway (Fowler *et al.*, 1999; Park *et al.*, 1999; Suárez-López *et al.*, 2001). Circadian clocks are physiological 'time-keepers' that generate persistent rhythms with a *ca.* 24 h period and are used by many organisms (e.g. animals, plants, fungi and bacteria) to anticipate predictable environmental changes. This anticipatory ability in plants is key to their survival, because of their sedentary nature. The plant circadian clock is composed of a number of negative and positive regulatory feedback loops between interacting partners of the classical core oscillator; *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), the two morning expressed genes, and *TIMING OF CAB1* (*TOC1*) the evening element (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Green and Tobin, 1999; Mizoguchi *et al.*, 2002). Expression of the *CONSTANS* (*CO*) gene is circadianly regulated by GIGANTEA (GI), a direct output from the clock (Fowler *et al.*, 1999; Park *et al.*, 1999; Sawa *et al.*, 2007), and therefore has a rhythmic expression profile peaking in the late afternoon (Suárez-López *et al.*, 2001; Sawa *et al.*, 2007). Stable CO protein is present during long-day conditions in *A. thaliana* (Yanovsky and Kay, 2003; Valverde *et al.*, 2004) and subsequently up-regulates *FT* expression in the leaf vasculature (Samach *et al.*, 2000; Mathieu *et al.*, 2007). The day-length sensitivity of Proteaceae (Malan and Jacobs, 1990; Hettash and Jacobs, 2006) probably operates through similar clock-dependent mechanisms.

Flowering-time in some *Protea* cultivars, such as 'Carnival' (*P. compacta* x *P. neriifolia*) that was used in this study occurs at defined times of the year. 'Carnival' has a narrow flowering period, January to March (summer in the Southern Hemisphere) after initiating flowering during the elongation of the spring flush between September and October (Gerber *et al.*, 2001a). Its parental species, however, have much broader and different flowering times and distribution patterns across the Cape Floristic Region. *P. compacta* inhabits a small area on and around the mountains of the South Western Cape, South Africa, and flowers develop between April and September with a peak between May and June (Rebelo, 2001). *P. neriifolia* has a much broader distribution than *P. compacta*,

encompassing the South-Western to Eastern Cape, and flowers between February and November. This is a very lengthy flowering period, however, there is a variation in the peak flowering time of *P. neriifolia* plants across its distribution region. The westerly occurring plants will flower in autumn, while the more easterly distributed plants flower in spring. This suggests that environmental differences between these regions may result in varying flowering responses, or that the *P. neriifolia* populations inhabiting the western regions are genetically distinct from those occurring in the Eastern Cape. A high degree of genetic variation in *P. neriifolia* has previously been reported in segregating seedlings from self-pollinated plants (Coetzee and Littlejohn, 2001). These seedlings showed a high level of phenotypic variability, which included factors such as growth habit, flower colour and size, and leaf morphology. 'Carnival' is clonally propagated, and therefore no genetic variation is to be expected between individual plants. Together with displaying a narrow and defined flowering period, 'Carnival' also depends on seasonal change to enter a dormant state during unfavourable winter conditions.

Because 'Carnival' has a narrow and defined flowering time it is plausible that floral induction may be regulated by an environmental signal such as seasonal change. It was hypothesised that this transition may be dependent on the circadian clock, similarly to the *CO*/*FT* module in *A. thaliana*. An *FT* orthologue, *ProFT* (*ProteaFT*), was isolated from 'Carnival' and its diurnal and seasonal expression profiles measured. Delayed fluorescence was used to investigate the presence of a functional circadian clock in 'Carnival' and its parental species. *ProFT* expression in meristematic tissues at various stages of development was also measured.

3.3. Materials and methods

3.3.1. Plant material, sampling and growth conditions

Protea cultivar 'Carnival' (*P. compacta x P. neriifolia*), grown as described in Section 2.3.1, was used for gene isolation and expression analyses. Leaf tissue used for diurnal and seasonal gene expression profiling was collected from three independent plants over a 24 h period. Leaves were collected every two hours between 08:00 to 18:00 and every four hours between 18:00 to 06:00, with a final collection at 08:00. Dates of sampling were: 10 to 11 April, 6 to 7 June, 12 to 13 August, 8 to 9 September and 12 to 13 October 2006. *Protea* meristematic tissues used for expression analyses were

collected between 14:30 and 15:00 on the following dates: 'wavy leaf' (WL), 9th May; 'onset dormancy' (OD), 9th May; 'dormant' (D), 3rd August; 'onset of growth' (OG), 6th September; 'active growing' (AG), 9th May; 'floral meristem' (FB), 3rd August in 2007. The meristems represented various developmental stages, depicted in Fig. 3.6 and defined within text, and were named based on their developmental and morphological characteristics. Tissue was excised from the shoot, frozen in liquid nitrogen and stored at -80°C until analysis. For meristematic tissue the outer layers of leaves or bud-scales were removed to expose the meristematic region before freezing. Weather data for 2006 and 2007 was provided by the ARC Institute for Soil, Climate and Water from a weather station situated at Bonfoi, Stellenbosch, South Africa (33°93'S; 18°78'E).

3.3.2. Delayed fluorescence (DF) measurements of circadian rhythms in Protea *leaf segments*

Leaves from *P. compacta*, *P. neriifolia* and 'Carnival' were used for DF measurements. For *P. compacta* and *P. neriifolia*, potted plants were acquired and entrained to 12 h light and 12 h dark at *ca.* 22°C for at least two weeks preceding DF. 'Carnival' shoots were excised from plants grown in field conditions and placed into modified Hoagland's solution (Hoagland and Arnon, 1950) under entraining conditions for a week preceding DF measurement. Modified Hoagland's solution contained: 7 mM $Ca(NO_3)_2$; 5 mM KNO_3; 2 mM KH_2PO_4; 2 mM $MgSO_4$; 45 µM H_3BO_3; 9 µM $MnCl_2$; 0.7 µM $ZnSO_4$; 0.4 µM $CuSO_4$; 0.14 µM $NaMoO_4$; 27.9 µM EDTA; 28 µM $FeSO_4$ and 1 mM KOH (pH 5.5). Leaves were rinsed under sterile dH_2O, cut into *ca*. 1 cm^2 pieces and floated on sterile dH_2O. DF was imaged using a Lumina *in vivo* imaging system (IVIS®) (Caliper Life Sciences Inc., Hopkinton, MA, USA). Leaf segments were placed in the instrument before dawn and DF measurement performed under constant dark (DD) conditions at 22°C.

Chlorophyll excitation was achieved by illuminating leaf segments with red light (excitation filter, 640 nm) for 60s with the lamp level set on high. A 1s delay was allowed before DF measurement to ensure the removal of all actinic light. DF was captured in the dark over 60s with the Cy5.5 (695-770 nm) emission filter in place. Measurements were repeated after a 60 min delay for a total of 96 cycles. The camera settings were: binning, 4; f-stop, 2 and field of view 12.5 cm. Spectral emission and kinetics of DF were determined on *P. compacta* leaf segments. For spectral emission measurements the

following emission filters were used: GFP (515-575 nm); DsRED (575-650 nm); Cy5.5 (695-770 nm) and ICG (810-875). Decay kinetics of DF was measured by exposing leaf segments to light for 10 min and measuring DF at 10s intervals for 80s.

The DF images were analysed using Living Image 3.2 (Caliper Life Sciences) software to identify and measure the intensities of the regions of interest. Total counts from fluorescent images were corrected for decreasing fluorescence by fitting a linear regression and subtracting the Y value of the equation from the raw data (Gould *et al.*, 2009). Data were corrected for variation in leaf segment sizes by dividing total count data by the area (in cm^2) of the region of interest. Corrected data was subjected to fast Fourier transformed non-linear least square analysis (Plautz *et al.*, 1997) using BRASS (Biological Rhythm Analysis Software, www.amillar.org) to generate period and relative amplitude error (RAE) estimates from each time course series.

3.3.3. Extraction of nucleic acids and reverse transcription of RNA

DNA used for PCR amplification was extracted following a modified CTAB extraction protocol (Gawel and Jarret, 1991). Modifications included a second chloroform:isoamylacohol (24:1) extraction before precipitation of gDNA from the supernatant. Precipitation was achieved by addition of an equal volume of isopropanol including 10 µg ml^{-1} RNase A and gDNA was allowed to precipitate at -20℃ overnight. Pelleted DNA was washed twice with 75% ethanol preceding drying in a 37℃ heating block and re-suspension in 50 µl sterile dH_20. RNA samples for expression analyses were extracted following Smart and Roden (2010). Quantity and quality of nucleic acids were determined by Nanodrop ND-100 spectrophotometry and gel electrophoresis. Seasonal, diurnal leaf RNA samples were converted to cDNA using the Enhanced Avian HS RT-PCR kit from Sigma-Aldrich (Missouri, USA). The manufacturer's protocol was followed; except 250 ng of RNA was reverse transcribed using half reaction volumes. Reverse transcription of the meristematic RNA samples was performed using the Improm-II™ Reverse Transcription System (Promega, Madison WI, USA) as per manufacturer's protocol using random primers (Promega).

3.3.4. Isolation of the partial ProFT *gene and 5'UTR region*

A combination of degenerate and thermal asymmetric interlaced (TAIL) PCR (Liu and Whittier, 1995; Liu *et al.*, 1995) methods was used to identify and isolate the partial

ProFT gene and 5'UTR sequences from 'Carnival' gDNA and cDNA. Degenerate primers to homologous regions of various *FT* orthologous genes were designed and kindly provided by Eric Banda (Molecular and Cell Biology, University of Cape Town). Primer sequences were:
dgEBft24F, 5' ggTTgTTggCCgTgTggTAggAgA(C/T)gT(A/C/g/T)(C/T)T(A/C/g/T)gA 3' and dgEBft447R-2, 5' AAgTTCAgCAAAATCTCTAgTgTTAAAgTT(C/T)Tg 3'. The PCR was performed twice; firstly with 0.5 µl cDNA or 100 ng gDNA as template, and secondly with 1 µl of a 10x dilution of the primary PCR as template. The primary and secondary PCR's were performed in 30 µl volumes, with the following components: 1x Phusion GC buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 µM forward primer, 0.75 µM reverse primer and 0.6 units Phusion Hot Start high fidelity polymerase (Finnzymes, Thermo Fisher Scientific, MA, USA). The following reaction conditions were followed: initial denaturation at 98°C for 30s; cycling (35x) 98°C for 10s, 60°C for 20s and 72°C for 20s (cDNA) or 75s (gDNA); final elongation at 72°C for 10 min.

The 5' end of *ProFT* including *ca.* 790 bp of sequence upstream of the translation initiation codon (ATG) was amplified from 'Carnival' gDNA by TAIL-PCR. Reaction conditions and arbitrary primers used were as described by Liu and Whittier (1995) using 50 ng 'Carnival' gDNA as template. Reactions were performed using GoTaq polymerase (Promega, Madison WI, USA) with the following gene specific primers: 5ProFT_GS3, 5' TATCCATTCgCTACCTCCCTATTg 3'; 5ProFT_GS2, 5' gATCTCAAC CCTAggTTggTTAAC 3' and 5ProFT_GS1, 5' gTgTAAAAggTCCgAAggTCATTg 3' for primary, secondary and tertiary PCR reactions. PCR products of interest were excised and extracted from agarose gels, cloned into pJET1.2 (Fermentas, Burlington, Canada) and sequenced (Macrogen Inc., Seoul, Korea). *In silico* 5'UTR analyses were performed using the RegSite Plant database (Softberry Inc., NY, USA), to predict the transcription start site (TSS) and promoter position, and the PlantCARE database (Lescot *et al.*, 2002) to identify *cis*-acting regulatory elements.

3.3.5. Phylogenetic analyses

The coding sequence of *ProFT* was translated *in silico* using DNAMAN (version 4.13, Lynnon Biosoft, Quebec, Canada) and the derived amino acid sequence used for phylogenetic analysis. Sequences used in phylogenetic analyses were chosen based on lowest *E*-values obtained after a protein-protein blast (BLASTp) of the deduced

amino acid sequence of *Pro*FT. FT orthologues from *A. thaliana* and rice (*Oryza sativa*) were included while MOTHER OF FT (MFT) and TERMINAL FLOWER1 (TFL1) from *A. thaliana* were used as outliers. A multiple sequence alignment was performed using ClustalW (Thompson *et al*., 1994). The evolutionary relationships of 23 amino acid sequences of FT orthologues were determined using the Neighbour-Joining method (MEGA5, Centre of Evolutionary Functional Genomics Biodesign Institute, Arizona State University, Arizona, US). Bootstrap values were derived from 1000 replicate runs and the percentage of replicate trees in which the associated proteins would cluster together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and the units are the number of amino acid substitutions per site. The tree was drawn to scale, see included scale bar, with the branch lengths in the same units as the evolutionary distances.

3.3.5. ProFT *expression in meristematic and leaf tissues*

A combination of semi-quantitative PCR (semi-qPCR) and quantitative real-time PCR (qRT-PCR) was used to measure *ProFT* expression levels in leaf and meristem tissue. All primers used for gene expression studies were designed to span exon-intron-exon boundaries and were thus unable to amplify gDNA, which was confirmed by the absence of amplification when gDNA was used as template. *ProFT* primers were designed to bind regions where the various genes in the *FT/TFL*-like family from *A. thaliana* had low homology to each other, thus reducing the possibility of amplifying any of the other *FT/TFL*-like genes. Semi-qPCR was performed on diurnal and seasonal leaf samples with GoTaq polymerase (Promega) using the following gene specific primers:

ProFTsqPCR_F (5' CTTCggACCTTTTACACACTgCT 3') and ProFTsqPCR_R (5' TTTgCCgCCATCCTggAACAT 3') to amplify a 241 bp *ProFT* product. Similarly designed primers that amplified a 301 bp product from the reference gene, *eIF4A* (*eukaryotic initiation factor 4A*), were used to normalise expression levels. These primers were: EIF4A_F1 (5' CTCATCAATgCATgg(A/T)gACATgCCTCA 3') and EIF4A_R (5' TggCATTTCATCAATCTgggTACTgTA 3'). Reactions were performed in a 25 µl volume with the following components: 1x GoTaq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM forward primer, 0.3 µM reverse primer, 0.75 units GoTaq polymerase and 0.5 µl cDNA as template. The following amplification conditions were followed: initial denaturing at 95°C for 3 min; cycling (35x for *ProFT* and 33x for *eIF4A*) 95°C for

30s, 59°C for 30s and 72°C for 30s; final elongation at 72°C for 7 min. For cycle optimisation see Appendix B (Fig. B1 and B2). The PCR products were separated on a 1.5% (w/v) agarose gel and visualised by ethidium bromide (0.5 µg ml^{-1}) staining in 1x TAE. Intensities of the bands were determined by Quantity One (version 4.6.3, Bio-Rad) 1-D imaging and analysis software.

qRT-PCR was performed on cDNA samples from meristems, October leaf samples and the 22:00 leaf samples from the various months. The Dynamo flash SYBR green qPCR kit (Finnzymes) was used for qRT-PCR. qRT-PCR was performed in a 10 µl reaction volume containing: 1x Dynamo master mix, 0.5 µl forward primer, 0.5 µl reverse primer and 1 µl cDNA as template. Primers used for the amplification of *ProFT* were: ProFTsqPCR_F2 (5' gAAggTACCACAACAAATCTAgg 3') and ProFTsqPCR_R (see above) amplifying a 139 bp product. *eIF4A* was used as reference gene and amplified with EIF4A_F2 (5' gTCgTTTTggACg(C/T)AAgggTgTTgC 3') and EIF4A_R (see above) amplifying a 107 bp fragment. Reactions were performed in a Rotor-Gene™ 6000 (Corbett Life Science Pty. Ltd., Sydney, Australia) instrument using 60°C as annealing temperature and following cycling conditions as per manufacturer's instructions (Finnzymes). Real-time PCR efficiencies (*E*) and cycle numbers required to reach fluorescence threshold (Cq, also known as Ct or Cp) were determined using LinRegPCR (Ramakers *et al.*, 2003) software. Relative expression levels were calculated as described by Pfaffl (2001) using the following equation:

$$ratio = (E_{\mathrm{Pr}oFT})^{\Delta Cq_{\mathrm{Pr}oFT}(control-sample)} / (E_{eIF4A})^{\Delta Cq_{eIF4A}(control-sample)}$$.

The sample with the highest *ProFT* Cq value, indicating lowest level, was used as control. These control samples were 'dormant bud' samples for meristematic expression, 18:00 samples for the diurnal October profile and June samples for the seasonal change in *ProFT* expressed in leaves at 22:00. For melt curves and optimisation see Appendix B and Fig. B3.

3.3.6. Statistical analyses

Where appropriate, results were subjected to analysis of variance (ANOVA) to determine significant differences using Statistica (Version 8, StatSoft Inc.) followed by *post hoc* Fisher LSD (95%) multiple range tests.

3.4. Results

3.4.1. DF exhibited circadian rhythmicity in 'Carnival' and its parental species

Experimental conditions for DF measurements of leaf segments using the Lumina *in vivo* imaging system were optimised before using it to determine circadian rhythmicity. The emission spectra of DF showed peak emission between 695 and 770 nm (Fig. 3.1 A), consistent with emission from chlorophyll *a* (Gould *et al.*, 2009). While, decay kinetics confirmed that 60s was sufficient to capture post illumination fluorescence (Fig. 3.1 B).

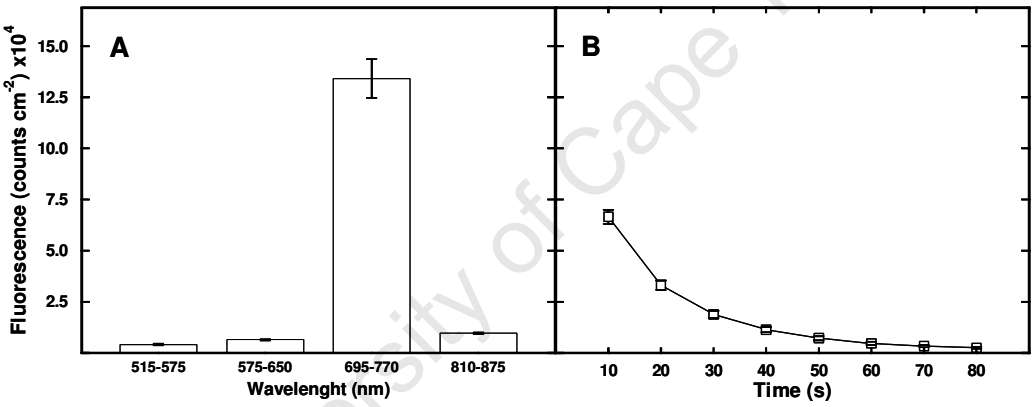


Figure 3.1. Spectral emission and kinetics of delayed fluorescence (DF) in *P. compacta* leaf segments measured in a Xenogen IVIS® Lumina (Caliper Life Sciences). Data represent average counts cm^{-2} leaf segment ± SE (n=4). **A**. Spectral emission of DF measured by exposing leaf segments to red light (excitation filter, 640 nm) for 60 s followed by DF measurement under dark conditions with a 60 s exposure using different emission filters alternatively in repeated DF measurements. **B**. DF decay kinetics determined by exposing leaf segments to light for 10 min and measuring DF at 10 s intervals for a total of 80 s.

For *P. compacta* and 'Carnival' *ca.* 60% of the leaf segments analysed exhibited robust circadian rhythmicity, whereas rhythms could only be extracted from 35% of *P. neriifolia* segments (Table 3.1). *P. compacta* and 'Carnival' DF traces, showing the average normalised DF plotted against time, also exhibited more robust rhythms compared to those of *P. neriifolia* when visually inspected (Fig. 3.2 A-C). *P. neriifolia* period estimates of individual leaf segments also had higher RAE values (Fig. 3.2 E) compared to those of *P. compacta* (Fig. 3.2 D). RAE values indicate rhythm robustness, with 0 representing a perfect cosine fit and 1 being not statistically significant (Gould *et al.*, 2006 and 2009). Only rhythmic traces with a RAE<0.6, considered significant in other studies (Hazen *et al.*, 2005), were therefore included in period estimate analyses. *P. compacta*, *P. neriifolia* and 'Carnival' displayed slightly different free-running periods (Table 3.1), although these differences were not statistically significant (P>0.05).

Table 3.1. Period length and relative amplitude error (RAE) estimates of delayed fluorescence (DF) measurements of *P. compacta*, *P. neriifolia* and 'Carnival' leaf segments determined by BRASS (biological rhythm analysis software).

	Protea species / hybrid cultivar		
	P. compacta	*P. neriifolia*	'Carnival'
Number of leaf segments analysed	20	20	22
Number of leaf segments with rhythmic DF	12	7	13
Period length (h)	26.7 ± 1.0	28.1 ± 1.8	27.2 ± 1.5
RAE	0.47 ± 0.09	0.45 ± 0.06	0.45 ± 0.03

Data represents means ± SE of DF from rhythmic leaf segments with RAEs<0.6.

3.4.2. Identification of ProFT *from 'Carnival'*

A partial sequence with homology to other *FT*-like sequences, identified from a nucleotide BLAST search, was successfully amplified from 'Carnival' leaf cDNA and gDNA. This partial sequence information was used to design primers for TAIL-PCR amplification of the remaining 5' coding sequence as well as 792 bp of the 5' sequence upstream of the predicted translation start sequence (ATG). Alignment of the deduced amino acid sequence of *Pro*FT to other FT orthologues from angiosperm species (Fig. 3.3 A), showed that at least a further 78 bp is required from the 3' end of the gene to obtain the complete gene sequence. This conclusion was based on the peptide lengths of the FT orthologues from other species. Proteins used in the sequence alignment and phylogenetic analysis and their GenBank accession numbers, given in brackets, are as follow: *Platanus* x *acerifolia Pa*FT1 (ACX34055) and *Pa*FT2 (ACX34072); *Cucurbita moschata* FTL1 (ABR20498) and FTL2 (ABR20499); *Cucurbita maxima* FT1 (ABI94605) and FT2 (ABI94606); *Cucumis sativus* FT-like (BAH28253); *Malus* x *domestica Md*FT1 (BAD08340) and *Md*FT2 (ADP69290); *Prunus mume* FT-like (CAQ16124); *Pyrus pyrifolia* FT (BAJ11577); *Photinia serratifolia* FT (AEO72028); *Oryza sativa* Hd3a (BAB61027); *Lichi chinensis* FT1 (AEU08960) and FT2 (AEU08961); *Populus nigra Pn*FT1 (BAG12899), *Pn*FT2 (BAG12900), *Pn*FT3 (BAG12904) and *Pn*FT4 (BAG12902); *A. thaliana* FT (NP_176726), TFL (P93003) and MFT (Q9XFK7); *Vitis labrusca* x *Vitis vinifera Vv*FT (ABN46891); *Ficus caricus* FT-like (BAI60052). Not all these species were included in the multiple sequence alignment. As the amino acid sequences for *Platanus* x *acerifolia Pa*FT1 and *Pa*FT2 were 100% identical, only *Pa*FT1, hereafter referred to as *Pa*FT, was included in the sequence alignment and phylogenetic analysis (Fig. 3.3 A).

*Pro*FT from 'Carnival' had the highest similarity, 77%, to *Pa*FT from the woody perennial hybrid London plane tree (*Platanus* x *acerifolia*) and 75, 74 and 71% similarity to FT orthologues from grapevine (*V. labrusca x V. vinefera*), fig (*F. caricus*) and apple (*Malus* x *domestica*), respectively. *Pro*FT was less similar (66% and 65%) to FT from the herbaceous annual *A. thaliana* and Hd3a from the monocot rice (*Oryza sativa*). The deduced *Pro*FT amino acid sequence contained the core amino acids, Tyr85 and Gln140 (indicated with red triangles; Fig. 3.3 A), identified as conferring specific activity to the protein (Ahn *et al.*, 2006; Carmona *et al.*, 2007) and discriminating it from proteins of the other FT/TFL-like family members.

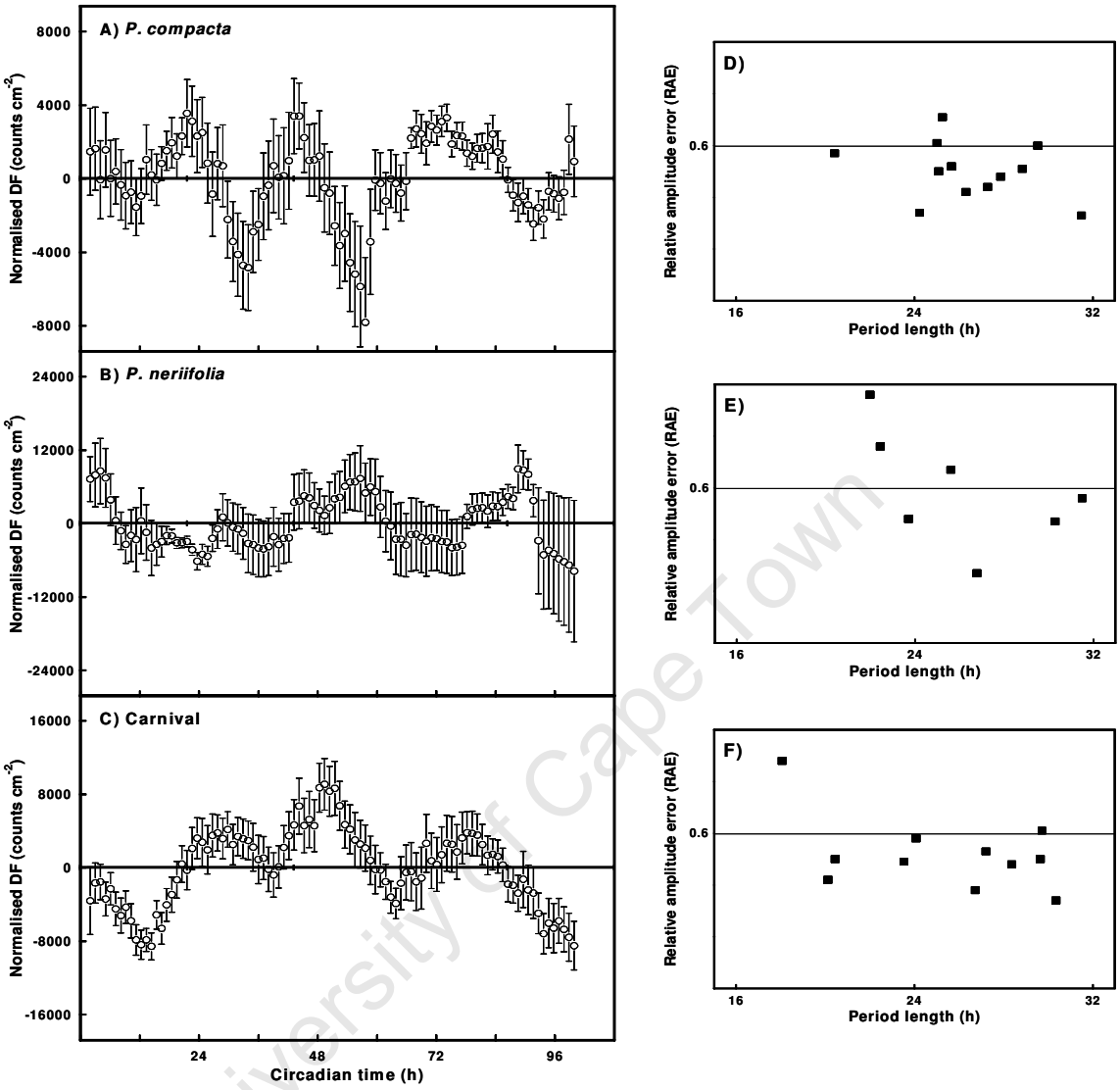


Figure 3.2. Rhythmic delayed fluorescence (DF) traces from *Protea* leaf segments measured hourly over *ca.* four days in constant dark (DD) conditions. Plants were entrained under 12 h light 12 h dark conditions at 22℃ for two weeks, *P. compacta* and *P. neriifolia*, or one week, 'Carnival', preceding DF measurementGraphs represent normalised averages ± SE for *P. compacta* (n=20; **A**), *P. neriifolia* (n=20; **B**) and 'Carnival' (n=22; **C**). **D-F**. Period estimates plotted against relative amplitude errors (RAEs) determined for rhythmic DF traces (Table 3.1) by Biological Rhythms Analysis Software (BRASS) for *P. compata* (n=12; **D**), *P. neriifolia* (n=7; **E**) and 'Carnival' (n=13; **F**).

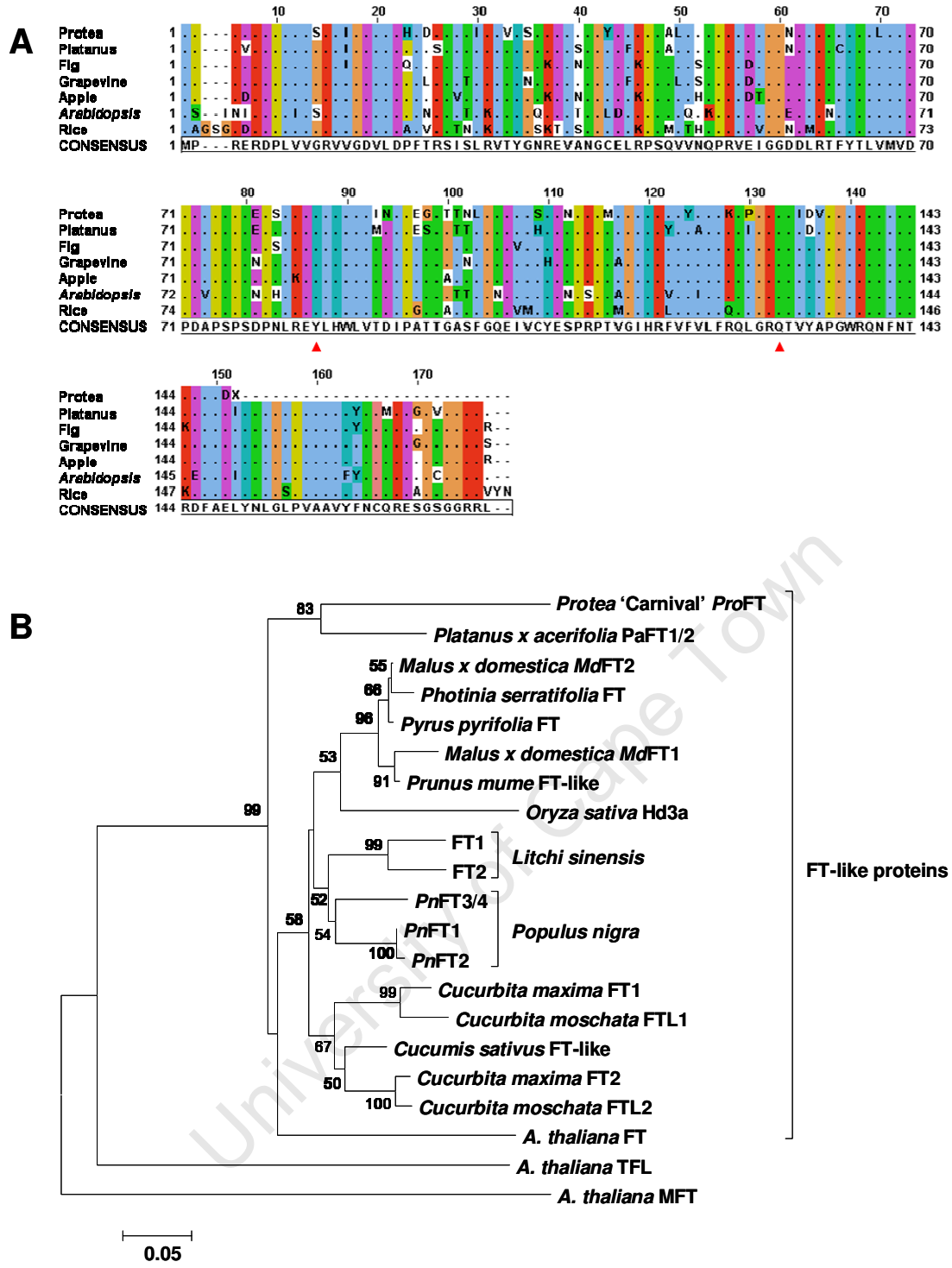


Figure 3.3. A. Alignment of *Pro*FT deduced amino acid sequence with that of FT orthologues from other woody perennial species, *A. thaliana* and *O. sativa* . Protein sequences used for alignments were taken from GenBank and accession numbers are given in text. The consensus sequence is shown and dissimilarities in the aligned sequences indicated. Red triangles indicate the amino acid residues conserved in the FT orthologues (Ahn *et al.*, 2006). Alignments were performed in CLUSTALW2 and graphically enhanced with Jalview. **B. Phylogenetic tree showing the evolutionary relationship of *Pro*FT to other orthologous FT proteins.** The FT-like subfamily is indicated in parenthesis on the right. *Pro*FT was placed at the top of the tree. GenBank accession numbers of proteins used in analysis are given in text. Branches are labelled with bootstrap values determined from 1000 re-samplings and only those over 40% are shown. Branch lengths are drawn to scale, see scale bar, and indicates evolutionary distances as the number of amino acid substitutions per site. These were computed using the Poisson correction method. Phylogenetic analyses were performed in MEGA5.

*Pro*FT grouped with the FT-like clade when the phylogenetic relationship of *Pro*FT to orthologous FT proteins from other angiosperms was determined (Fig. 3.3 B). *Pro*FT was aligned to 23 other amino acid sequences including FT orthologues from *A. thaliana* and rice. MOTHER OF FT (MFT) and TERMINAL FLOWERING LOCUS1 (TFL1) sequences were also included to show the phylogenetic separation of the three FT/TFL1 protein clades. The FT-like sequences formed a distinct group with a high bootstrap support value, thus separating them from MFT and TFL proteins (Fig. 3.3 B). The FT-like clade was further divided into four groups with FT from the annual model *A. thaliana* resolving on its own branch. Interestingly FT from the monocotyledonous rice plant, Hd3a, grouped with woody perennial species but occupied a separate branch in this group. *Pro*FT resolved in a strongly supported (83% bootstrap value) sub-clade with *Pa*FT from *Platanus* x *acerifolia*, a hybrid of *Platanus orientalis* and *Platanus occidentalis* (common name: London plane).

In silico promoter analysis using the PlantCARE database (Lescot *et al.*, 2002) predicted various *cis*-regulatory elements in the 792 bp sequence isolated from the 5' upstream region of *ProFT* (Table 3.2). The RegSite Plant database (Softberry Inc., www.softberry.com) predicted a 317 bp 5'UTR region and a TATA-box at position -24 based on the predicted transcription start site (TSS). Seven putative light responsive elements were present between -459 and +99 relative to the predicted TSS (Table 3.2). These included: a Box IV (*Petroselinum crispum*) at position -340; two Box I's (*Pisum sativa*) at positions -228 and +159; a CATT-motif (*Zea mays*) at position -280; a G-box (*Antirrhinum majus* and *Daucus carota*) at position -459; an I-box (*Larix laricina*) at position -255; an Sp1 element (*Oryza sativa*) at position +99 and various As-2-boxes (*Nicotiana tabacum*) at positions +22, +25, +28 and +31.

Table 3.2. Possible *cis* acting regulatory elements identified by PlantCARE (Lescot *et al.*, 2002) in the 5' *ProFT* sequence upstream of the transcription start site (TSS) predicted by the RegSite Plant database.

CIS regulatory element	Organism/s	Position from TSS	Strand	Sequence	Function	Reference
ABRE	*A. thaliana*	-459	-	TACGTG	*cis*-acting element involved in the abs*cis*ic acid responsiveness	Yamaguchi-Shinozaki and Shinozaki (1993); Baker *et al.* (1994)
ARE	*Z. mays*	-413	-	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction	unpublished
Box 4	*Petroselinum crispum*	-380	+	ATTAAT	part of a conserved DNA module involved in light responsiveness	Lois *et al.* (1989)
Box I	*Pisum sativum*	-228 and +159	+ and -	TTTCAAA	light responsive element	Kuhlemeier *et al.* (1987)
Box-WI	*Petroselinum crispum*	+57	-	TTGACC	fungal elicitor responsive element	Rushton *et al.* (1996)
CATT-motif	*Z. mays*	-280	-	GCATTC	part of a light responsive element	Sheen (1991)
CGTCA-motif	*H. vulgare*	-185	-	CGTCA	*cis*-acting regulatory element involved in the MeJA-responsiveness	Rouster *et al.* (1997)
G-box	*Antirrhinum majus; Daucus carota*	Both -459	+ and -	CACGTA; TACGTG	*cis*-acting regulatory element involved in light responsiveness	Argüello-Astorga and Herrera-Estrella (1998)
GCN4_motif	*Oryza sativa*	-110	-	TGAGTCA	*cis*-regulatory element involved in endosperm expression	Kim and Wu (1990)
I-box	*Larix laricina*	-255	-	GTATAAGGCC	part of a light responsive element	Terzaghi and Cashmore (1995)
MBS	*A. thaliana*	-427	-	TAACTG	MYB binding site involved in drought-inducibility	Yamaguchi-Shinozaki and Shinozaki (1993)
Skn-1 motif	*O. sativa*	-186	+ and -	GTCAT	*cis*-acting regulatory element required for endosperm expression	Takaiwa *et al.* (1991)
Sp1	*O. sativa*	+99	+	GGGCGG	light responsive element	Litts *et al.* (1992)
TATCCAT/C-motif	*O. sativa*	-28	+	TATCCAT	GA regulated response	Morita *et al.* (1998)
TC-rich repeats	*Nicotiana tabacum*	+13	-	ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness	Diaz-De-Leon *et al.* (1993)
TCCACCT-motif	*N. tabacum*	+252	-	ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness	unpublished
TGA-box	*G. max*	-185	+	TGACGTAA	part of an auxin-responsive element	Hagen *et al.* (1991)
TGACG-motif	*H. vulgare*	-185	+	TGACG	*cis*-acting regulatory element involved in the MeJA-responsiveness	Rouster *et al.* (1997)
As-2-box	*N. tabacum*	+22; +25; +28 and +31	-	GATAatGATG	involved in shoot-specific expression and light responsiveness	Diaz-De-Leon *et al.* (1993)

Only *cis*-regulatory elements with a matrix score of >=5 are shown Lower case letters in sequences indicate non-conserved nucleotides in the recognition site. Numbering is from the putative TSS.

3.4.3. Seasonal and diurnal changes of ProFT *expression levels in 'Carnival' leaves*

ProFT expression levels were low throughout the autumn (April), winter (June) and spring (August/September) and no clear diurnal pattern of expression could be discerned for *ProFT* during these months (Fig. 3.4 A-D). However, during early summer (October), *ProFT* transcript levels started to increase and a clear peak in expression could be discerned at 22:00 (ZT 16). This peak in *ProFT* expression was significantly higher ($P<0.05$) than that of any other time points measured (Fig. 3.4 E). These results were confirmed using freshly extracted RNA and more sensitive qRT-PCR analyses, which yielded similar results (Appendix B; Fig. B4). As the expression of *ProFT* peaked at 22:00 in October, expression levels for this time point were analysed by qRT-PCR across all the months sampled (Fig. 3.5). October had significantly higher overall *ProFT* expression levels ($P<0.05$) compared to the other months analysed.

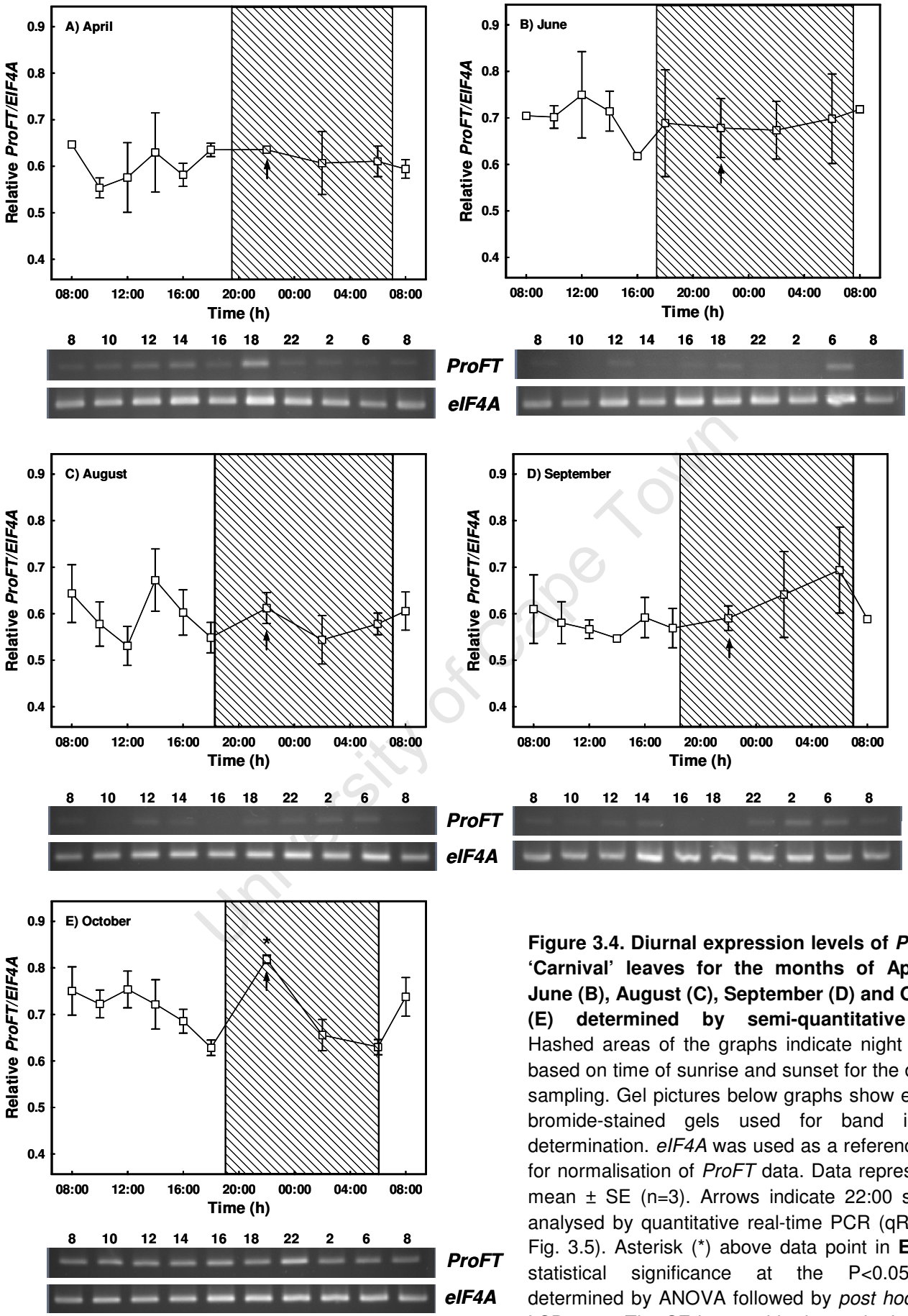


Figure 3.4. Diurnal expression levels of *ProFT* in 'Carnival' leaves for the months of April (A), June (B), August (C), September (D) and October (E) determined by semi-quantitative PCR. Hashed areas of the graphs indicate night periods based on time of sunrise and sunset for the dates of sampling. Gel pictures below graphs show ethidium bromide-stained gels used for band intensity determination. *eIF4A* was used as a reference gene for normalisation of *ProFT* data. Data represent the mean ± SE (n=3). Arrows indicate 22:00 samples analysed by quantitative real-time PCR (qRT-PCR; Fig. 3.5). Asterisk (*) above data point in **E** shows statistical significance at the P<0.05 level determined by ANOVA followed by *post hoc* Fisher LSD tests. The SE bar on this data point is smaller than the marker.

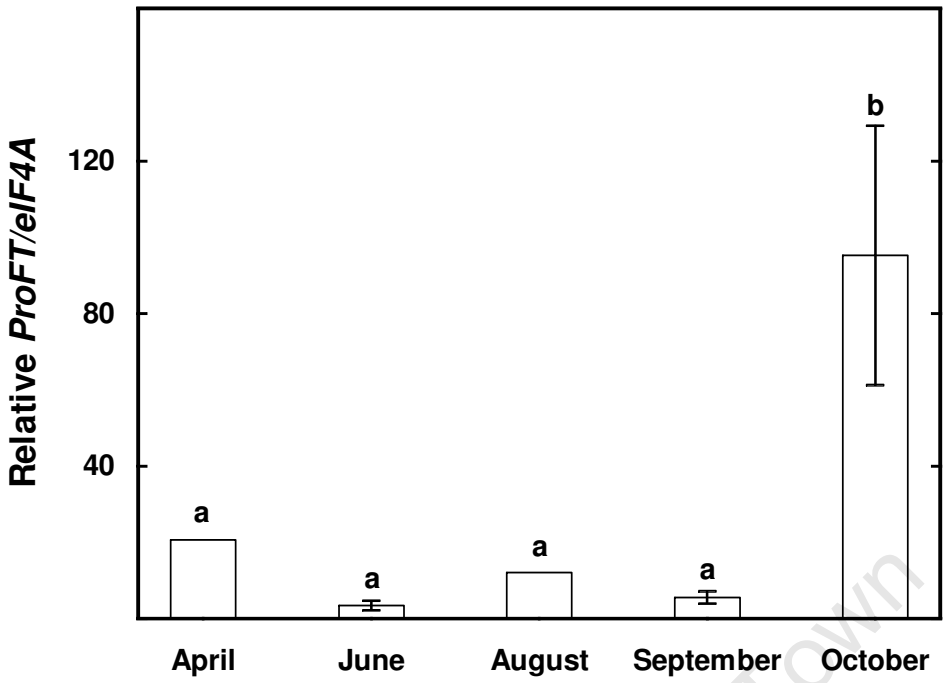


Figure 3.5. *ProFT* expression levels at 22:00 in leaves of field grown 'Carnival' for the months indicated. Gene expression was determined using quantitative real-time PCR and *ProFT* expression levels were normalised to those of the reference gene *eIF4A*. June was found to have the lowest *ProFT* expression levels and was used as 'control' to calculate relative expression of the other samples. PCR efficiencies were calculated using the LinRegPCR software (Ramakers *et al*., 2003). Bars represent means ± SE (n=3 and n=2 where error bars are absent). Dissimilar letters above bars indicate statistically significant differences (P<0.05) between samples determined by ANOVA followed by *post hoc* Fisher LSD tests.

3.4.4. Meristematic expression of ProFT

'Carnival' shoots are comprised of many seasonal growth flushes (Fig. 2.1). These flushes are formed during favourable growing conditions (OG; Fig. 3.6 A), such as increased day-length and temperature. During these periods, growth flushes pre-formed in the apical meristem begin to elongate. After the extension of these flushes during the active growth phase (AG; Fig. 3.6 B) the leaves are young, soft and have a 'wavy' appearance (WL; Fig. 3.6 C). At the 'WL' stage the meristem is visible and after 'hardening-off' of the leaves the meristem may follow three possible developmental actions:

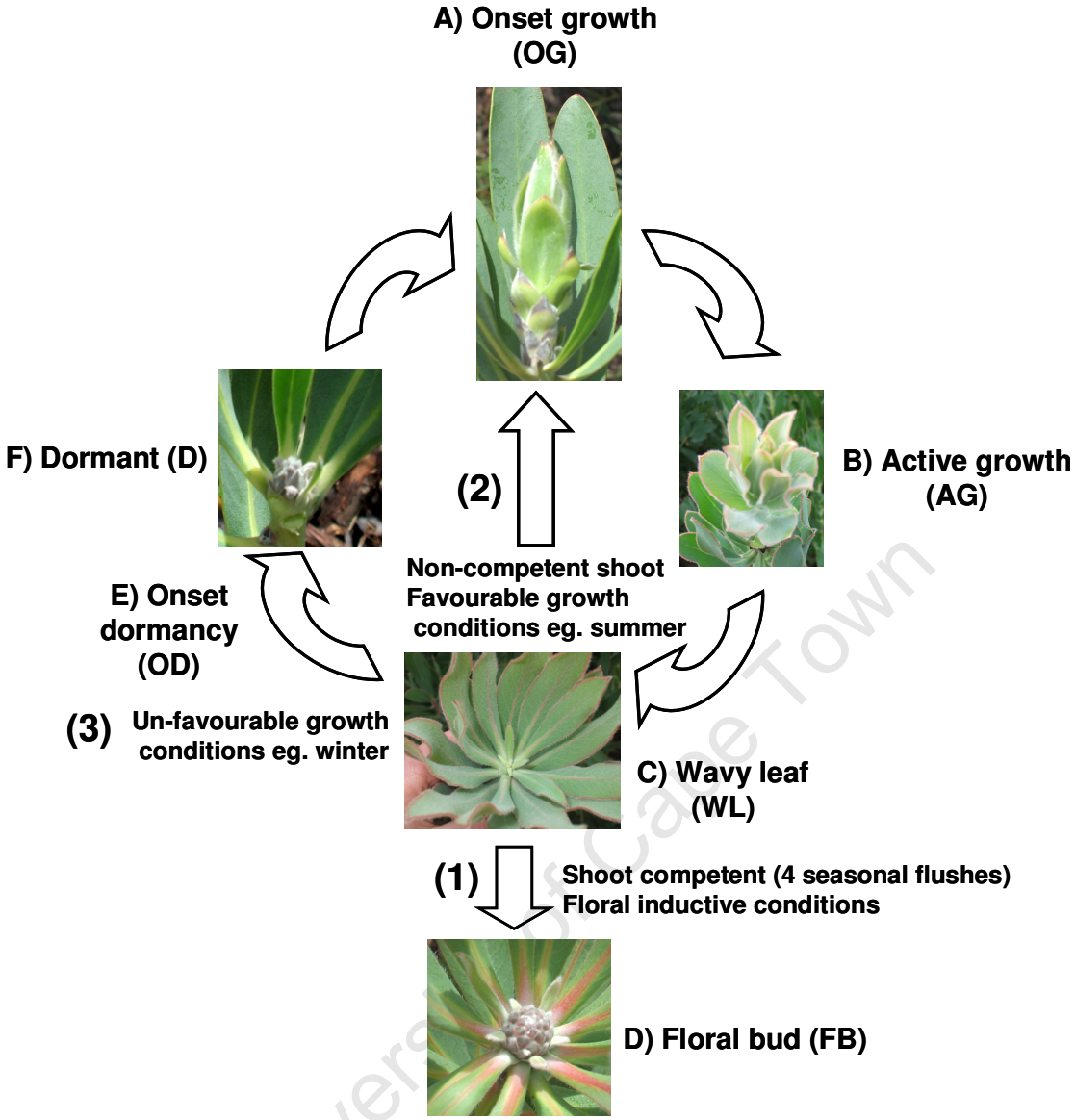


Figure 3.6. Diagram representing the seasonal growth of 'Carnival' shoots. During favourable growth conditions, such as summer, autumn and spring, seasonal growth flushes are formed on shoots. The vegetative meristem elongates to form a 'torpedo'-shaped elongating flush here termed the 'onset growth' (OG) bud (**A**). This flush soon elongates further and 'active growth' (AG; **B**) can be observed. After extension of the flush the immature leaves have a 'wavy leaf' (WL; **C**) appearance. Depending on the age of the shoot (1, 2, 3 or 4 flush) and the environmental conditions preceding flush elongation, one of three developmental paths could be followed at this stage. **1**) 'Floral buds' (FB; **D**) may be developed if the flush is subtended by 3 previously formed growth flushes. **2**) If conditions are favourable for growth, another seasonal flush may be developed (**A-C**). **3**) During unfavourable conditions such as short days, reduced irradiance and low temperatures associated with the onset of winter, shoots may enter a period of dormancy. Growth is suspended and bud set achieved during 'onset dormancy' (OD; **E**). A number of grey-coloured bract-like scales are formed around the bud to protect it during this 'dormant' (D; **F**) period. After the winter period, 'onset of the growth' (OG; **A**) of a new flush is started early in spring.

1) If the shoot is competent to flower, i.e. is preceded by 3 - 4 growth flushes and florally inductive conditions persist, floral buds may develop (FB; Fig. 3.6 F); 2) During favourable growth conditions the meristem may develop another growth flush; or 3) under unfavourable conditions, such as winter, the meristem enters a dormant period (onset dormancy (OD), Fig. 3.6 E; dormant (D), Fig. 3.6 F).

ProFT expression in meristematic tissue was measured at the various developmental stages and compared to the expression in leaves. *ProFT* expression was 10 fold higher ($P<0.05$) in florally determined meristems compared to the other vegetative developmental stages (Fig. 3.7). *ProFT* expression in leaves measured at 22:00 in October was twice as high as in vegetative meristem tissues however; it was still 5 fold lower than in floral buds (Fig. 3.7). *ProFT* expression in 'WL' meristems was not measured.

Weather data for 2007, during which the meristematic tissues were collected, indicated that the average temperatures in March, April and May of 2007 (indicated with arrows in Fig. 3.8) were *ca*. 1°C warmer than the corresponding months in 2006. During leaf sampling in 2006 no 'out-of-season' flush development or flowering was observed, but in 2007 actively growing and florally determined meristems were collected in May and August respectively. Some dormant meristems were observed in August 2007, indicating that some shoots still entered a dormant state whereas others developed another seasonal flush in late autumn and subsequently flowered in August.

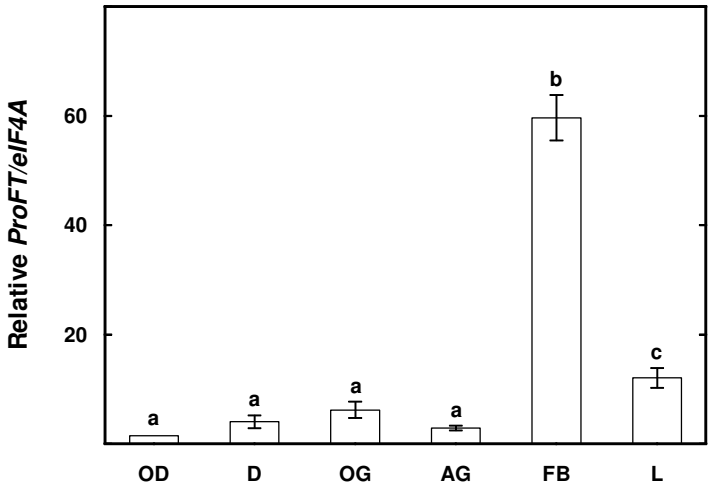


Figure 3.7. Quantitative real-time PCR analysis of *ProFT* expression in 'Carnival' meristematic tissues at various stages of development as depicted in Fig. 3.6. Expression of *eIF4A* was used for normalisation. Letters below bars indicate the developmental stage: OD, onset dormancy; D, dormant; OG, onset growth; AG, active growth; FB, floral bud and L, leaves. Leaf samples used for expression analyses were collected at 22:00 on the 12th October 2006. OD was found to have the lowest *ProFT* expression levels and was used as 'control' to calculate relative expression of the other samples. Expression levels were determined as described in Fig. 3.5. Bars represent means ± SE (n=3 and n=2 where error bars are absent). Dissimilar letters above bars indicate statistically significant differences (P<0.05) between samples determined by ANOVA followed by *post hoc* Fisher LSD test.

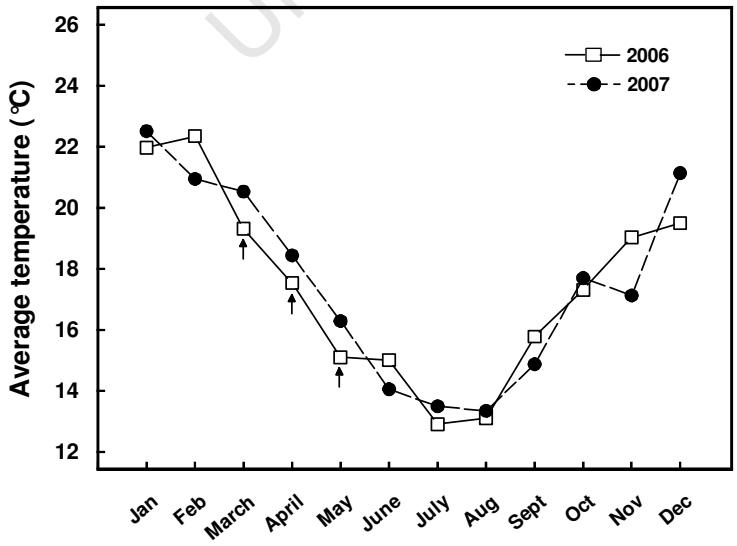


Figure 3.8. Average temperature in degrees Celsius (°C) calculated from hourly temperature for the two years of study, 2006 and 2007. Data was kindly provided by the ARC- Institute for Soil Climate and Water from a weather station situated at Bonfoi (33°93'S; 18°78'E) in close proximity to the experimental site at Protea Heights, Stellenbosch, South Africa (33°54'S; 18°48'E). Arrows indicate the three months in 2007, leading into the winter period, which had higher average temperatures than the corresponding months in 2006.

3.5. Discussion

As for other species investigated, FT orthologous proteins also occur in Proteaceae. *Pro*FT from 'Carnival' shared greater than 70% homology with other woody perennial FT orthologues on the deduced amino acid level. Conserved amino acid residues, which confirm it as an orthologue of FT, and not of any of the other members of the FT/TFL-like family, were present in the *Pro*FT deduced amino acid sequence (Fig. 3.3). FT orthologous proteins showed a high level of evolutionary conservation, and *Pro*FT aligned strongly (high bootstrap support) with other FT proteins in phylogenetic analyses (Fig. 3.3 B). The *Pro*FT peptide was phylogenetically most closely related to *Pa*FT from London plane (*Platanus* x *acerifolia*) which is from the same plant order, Proteales, as *Protea* (Carpenter *et al*., 2005; von Balthazar and Schönenberger, 2009). The genome of London plane contains two paralogous *FT*-like genes, *PaFT1* and *PaFT2*, which encode the same peptide (Zhang *et al*., 2011). Alternative splicing of the *PaFT* pre-mRNAs resulted in expression of numerous *PaFT* variants which displayed distinct spatial and temporal expression patterns. Alternative splicing of *FT*-like pre-mRNAs has also been reported in sunflower (*Helianthus annuus*; Blackman *et al*., 2010) and maize (*Zea mays*; Danilevskaya *et al*., 2008) and may be involved in controlling the temporal and spatial expression of *FT*-like genes. However, no alternative splicing of *ProFT* was observed in 'Carnival' as only one product was seen on agarose gels after PCR amplification from cDNA. Despite this, like *ProFT* expression in 'Carnival' *PaFT* transcripts were present in London plane bud and inflorescence tissue.

ProFT showed *ca*. six-fold higher expression in young florally determined inflorescences, compared to the highest expression levels measured in leaves (Fig. 3.7), while expression in meristematic tissue at other stages of development (Fig. 3.6) remained low. The expression detected in the vegetative meristems may be a result of *ProFT* expression in developing embryonic leaves contained within meristems, as was also the case in tomato (Lifschitz *et al*., 2006) and has been suggested for poplar (Rinne *et al*., 2011). Although *FT* expression in *A. thaliana* is exclusively maintained in leaf vasculature and excluded from the SAM, later in development it can be detected in the vasculature of inflorescences, flowers and siliques (Kobayashi *et al*., 1999; Takada and Goto, 2003). Therefore, the detection of *ProFT* expression in developing 'Carnival' inflorescences is not that unusual.

'Carnival' plants undergo successive periods of active growth, dormancy and flowering. The meristem enters a dormant state during the winter (June-August in the Southern Hemisphere), which is characterised phenotypically by enclosure of the meristem in scale-like bracts (Fig. 3.6 F). Dormancy is released (also referred to as 'budbreak') during early spring (September) and a new seasonal flush is formed. In cultivated and pruned 'Carnival' plants, this spring flush carries the developing inflorescence (Gerber *et al*., 1995) if it is preceded by three previously formed flushes (Greenfield *et al*., 1994). 'Carnival' inflorescence development occurs between November and February and flowering is initiated during the elongation of the spring flush, September-October (Gerber *et al*., 2001a). High levels of *ProFT* were present in the florally determined meristems during inflorescence differentiation (Fig. 3.7). In tomato, the *FT* orthologue (*SINGLE FLOWER TRUSS; SFT*) and the floral inhibitor orthologue of *TERMINAL FLOWERING LOCUS1*, *SELF-PRUNING* (*SP*), have been implicated in the maintenance of a sympodial, perennial growth habit (Lifschitz *et al*., 2006; Shalit *et al*., 2009). Shalit *et al*. (2006) suggest that the balance between SFT and SP peptides in the apical meristem is required to keep it in a vegetative state. A similar role for *ProFT* in meristem maintenance may be responsible for sympodial growth in 'Carnival' as the high levels of *ProFT* present in young florally determined terminal meristems precedes loss of apical dominance and development of axilliary buds into side shoots. These are manually removed in cultivated *Proteas* to produce lengthy stems, but develop shoots which carry the next season's flowers in wild grown species. Similar loss of apical dominance has been observed in transgenic plants ectopically expressing *FT* orthologues (Hsu *et al*., 2011; Kotoda *et al*., 2010; Shen *et al*., 2011; Zhang *et al*., 2011). These findings lend support to the suggested involvement of *FT*-like genes in determining growth architecture and suggest a possible role for *ProFT* in conveying apical dominance to florally determined terminal meristems of *Protea*.

ProFT was expressed in 'Carnival' meristematic tissue during vegetative development and dormancy (Fig. 3.7). However, there were no significant differences in *ProFT* expression levels between these phases. *ProFT* levels were only measured at one time point for each developmental stage and therefore fluctuations in expression experienced for brief periods may not have been observed during this study. As *FT* is proposed to play a role in the onset and release of dormancy in woody perennial plants (Böhlenius *et al*., 2006; Gyllenstrand *et al*., 2007; Holliday *et al*., 2008; Horvath *et al*.,

2008), differences in *Pro*FT expression were expected between meristematic tissue at the different developmental stages surrounding dormancy (OD, D and OG). There was an increase in expression at the release of dormancy (OG), however, it was not significantly different from the levels at the other developmental stages. The involvement of *ProFT* in onset and release of dormancy in 'Carnival' may be better assessed by measuring *ProFT* expression levels continuously during this period, as Rinne *et al*. (2011) found that *FT* levels dropped rapidly in poplar after bud burst, associated with the release from dormancy.

During 2007, when meristematic tissue was collected for expression analyses, there were higher than average monthly temperatures in March, April and May leading up to winter (Fig. 3.8). This resulted in the early flowering of some 'Carnival' shoots, and allowed for collection of floral buds during August, the winter period when shoots are usually dormant. Early flowering was the result of shoots displaying out-of-season development of new growth flushes during autumn when shoots are usually preparing for dormancy by ceasing growth and initiating bud set. During diurnal leaf sampling in 2006, no out-of-season flush development was observed. It is possible that early flowering in 'Carnival' was induced by the more favourable environmental conditions (i.e. higher temperature and presumed higher light intensities), during this time. Timing of flowering in *A. thaliana* is also temperature sensitive and this response appears to be mediated in a *FT* dependent manner (Franklin, 2009). *FT* expression is up-regulated in response to the down-regulation of two floral repressors namely *FLOWERING LOCUS M* (*FLM*; Balasubramanian *et al*., 2006) which responds to extreme temperature fluctuations, and *SHORT VEGETATIVE PHASE* (*SVP*; Hartmann *et al*., 2000) which responds to less extreme temperature fluctuations, as also found in this study, through the thermosensory pathway. Roles for *SVP* orthologues in determining temperature-sensitive meristem identity in other plant species have also been suggested (Brill and Watson, 2004; Lee *et al*., 2007; Travaskis *et al*., 2007). *SVP* is under clock-dependent regulation through GI (Yoshida *et al*., 2009) and represses *FT* transcription by binding to CArG motifs in the *FT* promoter region (Lee *et al*., 2007). These authors found that at higher ambient temperatures the expression levels of *SVP* were reduced leading to the up-regulation of *FT*. SVP-mediated control of *ProFT* expression levels, by an orthologous gene in 'Carnival', may play a role in the 'out-of-

season' flowering observed during 2007, although, this would require further investigation.

Diurnal *ProFT* expression in 'Carnival' during October, the month with the highest expression levels, showed a significant ($P<0.05$) peak in expression at 22:00 (ZT16) when this time point was compared across the other months analysed (Fig. 3.5). Flowering in 'Carnival' is induced during the elongation of the spring flush, which coincided with the increased *ProFT* expression levels during October. This increase in *ProFT* expression may be as a result of floral inductive photoperiodic conditions, but the up-regulation of *ProFT* through other pathways cannot be excluded. The expression pattern of *ProFT* corresponded to the peak of *FT* expression in *A. thaliana* under LDs which is mediated by CO stabilisation observed between 10 and 16 h after dawn (Jackson, 2009). The presence of active and stable CO transcriptionally activates *FT* through binding to a CO consensus responsive motif ('TGTG(N2-3)ATG') in the *FT* promoter (Adrian *et al.*, 2010; Tiwari *et al.*, 2010). Orthologous *CO* genes are proposed to play similar roles in woody perennial species such as grapevine (*V. vinifera*; Almada *et al.*, 2009). Although no CO responsive motive was identified in the 792 bp identified upstream of the *ProFT* translation start codon, it may be present further upstream. Also, it is possible that in 'Carnival' a CO orthologue may bind through a different motif than those of the herbaceous plants studied. The longest day-length for which *ProFT* expression was measured was for the month of October and this may suggest that a *CO* orthologue in 'Carnival' could be responsible for up-regulation of *ProFT* expression at this time. Isolation and expression profiling of a *CO* orthologue from 'Carnival' could provide more evidence for the regulation of *ProFT* by the photoperiodic pathway. Unfortunately a *CO* orthologue from 'Carnival' was not found during this study. In *A. thaliana*, *CO* expression is controlled by the circadian clock that is critical in ensuring accurate photoperiod responsive flowering.

Circadian period and RAE estimates were successfully extracted from DF data of *Protea* leaves, indicating the presence of a functional circadian clock. Photoperiodic flowering is generally controlled by the circadian clock, and defects in the clock often affect flowering time in *A. thaliana* (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Green *et al.*, 2002). DF of barley (*Hordeum vulgare*), maize (*Zea mays*), lettuce (*Lactuca sativa*), *Capsella bursa-pastoris* and *Kalanchoë fedtschenkoi* leaf segments were

previously found to display robust, high-amplitude rhythms with tight periods and low RAE in constant light free-running conditions (Gould *et al.*, 2009). However, DF in this study decreased to low levels during the period of analysis and had low amplitudes and large errors. This is largely attributable to the fact that DF was measured under continuous dark conditions. Unlike the experimental set-up of Gould *et al*. (2009) the Xenogen lumina instrument, used in this study, does not allow the measurement of fluorescence under constant light conditions. Continuous darkness often causes the damping of plant circadian output rhythms after one or two cycles (Wang and Tobin, 1998) and DF is an output that rapidly damps in the dark (Gould *et al.*, 2009). Circadianly regulated genes such as the phytochromes (*PHYA* to *PHYE*; *CRY1* and *CRY2*; Tóth *et al.*, 2001) have also been found to lose rhythmicity faster under constant darkness compared to constant light. However, rhythmic DF was observed in *Protea* leaf cuttings suggesting that this is a suitable method for investigating the circadian clocks of these plants, especially in the absence of a transformation system to introduce reporter genes, and genome sequence information to follow gene expression.

DF rhythms of *P. neriifolia* were less robust than those of *P. compacta* and 'Carnival'. A number of factors may have contributed to the variation in rhythmicity between different leaf segments. Chlorophyll is not evenly distributed across leaf surfaces and this result in heterogeneity and patchiness of chlorophyll fluorescence. Across the leaf surface of *Kalanchoë daigremontiana* chlorophyll fluorescence showed spatial and temporal variation (Rascher *et al*., 2001), and some species used in the DF study by Gould *et al.* (2009) also showed variable DF between leaf segments. The position on the leaf from which the segment is taken for DF analysis could therefore also influence results. More robust rhythms, with smaller variation between leaf segments and lower RAEs, may be achieved if leaf segments are excised from leaf areas that show robust and consistent rhythmic DF. The age of the leaves from which segments are excised may also influence the number of hours for which robust DF measurements could be collected under DD free-running conditions. For these measurements we used both young and old leaves and found that in young leaves chlorophyll fluorescence quickly dampened due to the presence of high levels of phenolics in *Protea* (Jones *et al*., 1995; Coetzee and Littlejohn, 2001) resulting in browning of the cut tissues.

'Carnival' DF rhythms displayed a broad spread of periods with low RAEs that may be a result of hybridisation. A very broad period range of leaf movement periods and flowering times was also reported for *A. thaliana* recombinant inbred lines (RILs) generated by crosses of Cape Verde Island (Cvi), Columbia (Col-0) and Landsberg *erecta* accessions (Alonso-Blanco *et al.*, 1998; Swarup *et al.*, 1999). This suggested that, although the parental species had very similar circadian periods and flowering times to each other, the segregation of alleles involved in period-length determination and photoperiodic flowering could result in variation within the progeny. Although there is a large difference in flowering time between the parental *Protea* species and the commercial hybrid, flowering times of each is still largely confined to certain periods. The 'tight' flowering time of 'Carnival' is probably due to the fact that once a hybrid has been selected for cultivation it is clonally propagated, therefore ensuring genetic identity. This narrow flowering time of 'Carnival' together with the fact that it responds to seasonal changes by entering a dormant state during unfavourable conditions and by initiating growth during favourable conditions, indicates that 'Carnival' is able to anticipate seasonal change, suggested the presence of a functional circadian clock confirmed by DF results. A functional circadian oscillator has been found essential for the correct timing of seasonal traits such as entry and release from dormancy as well as cold hardiness in a deciduous poplar hybrid, *P. tremula* x *P. tremuloides*, (Ibáñez *et al.*, 2010). Thus the circadian oscillator in 'Carnival' may play a role in regulating the transitions between the different growth states observed in 'Carnival'.

In an effort to identity potential *cis*-regulatory elements (CREs) in the 5' DNA regulatory region of *ProFT* that may regulate expression, homology to known CREs were identified using the PlantCARE database. These included seven light response elements including an I- and G-box, common in promoters of light-regulated genes (Argüello-Astorga and Herrera-Estrella, 1998; Giuliano *et al.*, 1988) and postulated to be involved in hormone signalling (Menkens *et al.*, 1995). An I-box was also identified in the *A. thaliana FT* promoter region (Adrian *et al.*, 2010). Hormone responsive elements were also identified and included: abscisic acid (ABRE), methyl jasmonate (CGTCA and TGACG), gibberelic acid (TATCCAT/C) and auxin (TGA) sensitive motifs. Hormones have also been implicated in the control of flowering in many perennial trees (reviewed by Bangerth, 2009). An ABRE element has also been identified in the promoter region of *MFT*, which regulates seed germination through ABA and GA signalling (Xi *et al.*,

2010). ABA is also involved in the regulation of dormancy in meristems (Rohde *et al.*, 2002; Ruonala *et al.*, 2006) and *ProFT* expression in the meristematic tissue of 'Carnival' at various stages of development (Fig. 3.7) suggests that it may play a role in the regulation of dormancy as proposed for *FT* orthologues from other woody perennial plants. No cytokinin-response element was identified in the *ProFT* regulatory region analysed, however, application of the synthetic cytokinin benzyladenine (BA) has been suggested for the manipulation of flowering time in 'Carnival' (Hoffman *et al.*, 2009). As no other studies have reported the presence of a cytokinin-response element associated with *FT*, the floral response observed after BA treatment of *Protea* may be independent of *FT* or rely on the regulation of floral inductive targets either upstream or downstream of *FT* in the flowering pathway. Recent work by D'Aloia *et al.* (2011) showed that the treatment of *A. thaliana* plants with BA resulted in the induction of flowering through expression of the *FT*-like family member *TWIN SISTER OF FT* (*TSF*) and not *FT*. However, the authors did not assess whether BA application directly up-regulates *TSF*, or if *TSF* is up-regulated by a gene downstream in the floral inductive pathway in which case a BA responsive element is not required. As only 792 bp sequence upstream of the translation initiation codon (ATG) of *ProFT* was isolated and analysed in this study other regulatory motifs may have been missed. Further characterisation of the *ProFT* promoter may lead to the identification of other relevant *cis*-regulatory elements required for the temporal and spatial expression of *ProFT. In silico* identified response elements also require further experimental work to confirm functionality. Experiments such as Pro_{ProFT}*::reporter-gene* fusions expressed ectopically in a model system such as *A. thaliana* may be used to investigate the functionality of these response elements, but did not form part of the current study.

3.5.1. Conclusion

An *FT* orthologue, *ProFT*, was isolated from a floriculturally important *Protea* hybrid 'Carnival'. The spatial and temporal expression of *ProFT* suggested that it may play diverse roles in 'Carnival' growth and development. DF measurements confirmed the presence of a functional circadian clock, which may regulate the seasonal periods of growth, dormancy and flowering observed in 'Carnival'. *ProFT* expression patterns in leaf tissue at the time of floral transition suggested that *ProFT* may be involved in inducing this phase change in 'Carnival'. Flowering times of *Protea* species and commercial hybrids differ significantly and thus a single environmental stimulus

responsible for floral initiation across *Proteas* is highly unlikely. *ProFT* expression may therefore be regulated through multiple pathways including: circadian, light- and temperature-dependent pathways, as suggested for orthologous *FT* floral integrators in other plant species.

CHAPTER 4: Cloning and expression analysis of *ProLFY*, a meristem identity gene from the *Protea* hybrid 'Carnival'

4.1. Abstract

Four paralogous genes with sequence similarity to the meristem identity gene *LEAFY* (*LFY*) of *Arabidopsis thaliana* were identified in the *Protea* cultivar 'Carnival' (*P.compacta x P.neriifolia*). The deduced amino acid sequences of *Protea* LFY (*Pro*LFY) paralogues displayed highly conserved N- and C-terminal regions comparable to that of other LFY proteins. Paralogous sequences showed 97-99% similarity at nucleotide and deduced amino acid level and these differences may be important in defining functionality of the different *Pro*LFY proteins during growth and development. To determine if these *ProLFY* paralogues are involved in floral transition and development, three paralogues were heterologously expressed in *A. thaliana* Col-0 wild-type and *lfy-2* mutant plants. These three paralogues had the greatest sequence dissimilarities which may suggest different functionality. Expression of paralogue, *ProLFY-WL4*, rescued the low fertility of the *lfy-2* mutant by restoring seed yield to wild-type levels and resulted in the conversion of 'leaf-like' inflorescences to flowers with a wild-type appearance. *ProLFY-WL4* expression levels during 'Carnival' meristem development confirmed a role for this paralogue in inflorescence development. The other two paralogues used in heterologous *A. thaliana* expression studies, *ProLFY-L5* and *ProLFY-D1*, did not rescue fertility or the abnormal floral phenotype of *lfy-2*. However, transgenic plants developed either smaller leaves when expressing *35S::ProLFY-L5*, or larger leaves when expressing *35S::ProLFY-D1*, compared to the non-transgenic *lfy-2* plants. Together these results suggest that *ProLFY* paralogues, as in other species, may be involved in both floral and vegetative development of *Protea*. A role for *ProLFY* paralogues in inferring floral complexity to *Protea* inflorescences could not be established in this study and would require further investigation into their spatial and temporal expression patterns.

4.2. Introduction

Across the angiosperms, there is a diverse array of floral shapes and sizes resulting from co-evolution with pollinators and their specific preferences and morphology (Bowman *et al.*, 2007; Koes, 2007). This is especially evident across the Proteaceae family, named after the mythological Greek god Proteus who could change shape at will

(Rebelo, 2001). Cultivated *Proteas* have large colourful inflorescences and are predominantly bird-pollinated (Wright *et al.*, 1991; Rebelo, 2001; Hargreaves *et al.*, 2004). Flowerheads, or inflorescences, are comprised of many (200 - 240) individual florets (Gerber *et al.*, 2001a) which are spirally arranged on a flat or pointed receptacle and surrounded by involucral bracts that create the colourful display. Limited work has been done on the genetic control of floral transition and inflorescence development in *Proteas*, however key meristem identity genes have been studied in other plant species.

In the model annual *Arabidopsis thaliana*, floral inductive conditions result in the up-regulation of the meristem identity gene *LEAFY* (*LFY*), which together with *APELATA1* (*AP1*) is responsible for the switch from vegetative to floral development (Mandel and Yanofsky, 1995; Weigel et al., 1992; Weigel and Nilsson, 1995). This transition is achieved through the up-regulation of the homeotic (floral organ identity) genes by LFY and AP1 (Hong *et al.*, 2003; Lohmann *et al.*, 2001; William *et al.*, 2004). Expression and transgenic studies involving meristem identity gene orthologues from woody perennial plants suggest they also fulfil a role in the transition from vegetative to reproductive growth. As most woody perennials display lengthy juvenile phases before reaching adulthood and competence to flower, many studies attempting to reduce the length of the juvenile phases of horticulturally important plants have been undertaken (Wilkie *et al.*, 2008; Bangerth, 2009). Studies aimed at creating 'fast flowering' plants have led to the identification of numerous *LFY* orthologues from woody perennial species (Meeks-Wagner, 1996; Smyth, 1996; reviewed in Martín-Trillo and Martínez-Zapater, 2002).

In the woody perennial *Sophora tetraptera*, the up-regulation of the *LFY* and *AP1* orthologues, *StLFY* and *StAP1*, coincided with early flower development suggesting roles in floral transition (Song et al., 2008). Similar expression patterns were also reported for *LFY* orthologues from *Eucalyptus globulus* (Southerton et al., 1998) and *Eucalyptus grandis* (Dornelas et al., 2004). Increased expression levels of *LFY* orthologues *ALF* and *MEL* from kiwifruit (*Actinidia deliciosa*; Walton et al., 2001) and *Metrosideros excelsa* (Sreekantan et al., 2004) corresponded with the timing of floral initiation and development in the meristem, suggesting these genes play a role in flowering. The heterologous expression of *A. thaliana LFY* in poplar (*P. trichocarpa*; Weigel and Nilsson, 1995), citrus (*Citrus sinensis*; Peña *et al.*, 2001) and rice (*Oryza sativa*; He et al., 2000) resulted in precocious flowering in these species, suggesting

that *LFY* orthologues are functionally conserved and little diversification of the protein has occurred between species (Maizel et al., 2005). However, the interaction of LFY proteins with downstream target genes seem to differ between species, as many plants overexpressing *LFY* genes display abnormal vegetative and floral development (He *et al.*, 2000; Rottmann *et al.*, 2000; Peña *et al.*, 2001; Zhang *et al.*, 2008; Flachowsky *et al.*, 2010). These dissimilarities in downstream interactions may be important in generating the diversity of floral architectures seen across the angiosperms.

LFY is present as a single copy in most plant genomes (Melzer *et al.*, 2010; Moyroud et al., 2009), however, in some polyploid species multiple copies do occur. For example the genome of the allotetraploid *Nicotiana tabacum* harbours two *LFY* copies, *NFL1* and *NFL2*, but based on spatio-temporal expression patterns of these genes it appear that little diversification of function has occurred between these (Kelly *et al.*, 1995). Apple (*Malus x domestica*), which is a polyploid as the Maloid family from which it evolved between 10 and 7 million years ago is polyploid (Harris *et al.*, 2002), has three copies of *LFY* (*AFL1*, *ALF2* and *AFL1a*; Wada et al., 2007). However, unlike *LFY* orthologues in *N. tabacum* diversification of *ALF* function between these copies has been suggested based on differences in their temporal and spatial expression patterns. These functions could, however, not clearly be defined in the study by Wada et al. (2007). Two *LFY* copies are also present in maize (*Zea mays*; Bomblies et al., 2003), *Jonopsidium aceaule* (Shu et al., 2000) and *Idahoa scapigera* (Sliwinski et al., 2007) and these are proposed to play a role in determining floral architecture. In other plant species such as *E. globulus* and *Sophora tetraptera* two *LFY* copies are present in their genomes however one of each of these copies had been reduced to a pseudogene (Southerton et al., 1998; Song et al., 2008).

The complex floral structures carried by *Protea* plants, presumably require the expression of numerous homeotic genes under the control of meristem identity genes orthologous to *LFY* and *AP1* responsible for floral transition. A *LFY* orthologue may fulfil similar roles in *Protea* to *LFY* orthologues in other species which include: floral transition, patterning and development. In a previous study a partial sequence of *Protea LEAFY* (*ProLFY*), previously named *PROFL*, was identified (Smart, 2005; MSc dissertation, University of Stellenbosch). This sequence information was used to design primers to isolate the complete coding sequence of *ProLFY* from the commercially

grown *Protea* hybrid 'Carnival' (*P.compacta x P.neriifolia*). Multiple paralogous *ProLFY* genes were identified and subsequently heterologously expressed in *A. thaliana* to determine functional homology to *LFY* and study the effect of *ProLFY* expression on flowering time and floral phenotype. Results from this study were used to elucidate possible roles for these paralogues in *Protea*.

4.3. Materials and methods

4.3.1. Plant material, sampling and growth conditions

Protea cultivar 'Carnival' (*P.compacta x P.neriifolia*) grown as described in section 2.3.1 was used for gene isolation and expression analyses. *Protea* meristematic tissues were collected as described in section 3.3.1 and the meristems represented various developmental stages depicted in Fig. 3.6. Leaf tissue was collected on 3rd August 2007. Tissue was excised from the shoot, frozen in liquid nitrogen and stored at -80°C until analysis.

A. thaliana wild-type Col-0 and *lfy-2* mutant seeds, obtained from the European Arabidopsis Stock Centre (NASC; stock numbers N1092 and N6229), were surface-sterilised with 2% (v/v) sodium hypochlorite (Bleach) and 0.025% (v/v) Tween-20 for 5 min with gentle agitation and then rinsed thrice with sterile distilled H_2O. Sterilised seeds were plated onto Murashige and Skoog (MS; Murashige and Skoog, 1962) basal media with vitamins (pH 5.8) (Highveld Biological Pty. Ltd., Lyndhurst, South Africa) and 0.8% (w/v) agar under sterile conditions. Plated seeds were stratified for 72 h in the dark at 4°C and subsequently incubated at 21°C under cool white fluorescent light (*ca*. 80 µmol photons m^{-2} s^{-1}) under 16 h light: 8 h dark regimes. Once seedlings had reached a suitable size (*ca*. 4 rosette leaves, 2 weeks) they were transplanted into soil either individually when used for transformations or phenotype analyses (T_1 and T_2 transgenic seeds) or, when multiple possible 'transgenic' (T_0) seedlings were to be screened, by lifting whole agar slabs containing seedlings out of the plates and placing them on the soil. Plants were grown in a peat:vermiculite (1:1) mixture supplemented with 0.165% (volume fertiliser/volume soil) of Wonder 2:3:2 (N:P:K) fertiliser (Efekto, South Africa) .

4.3.2. Extraction of nucleic acids

'Carnival' genomic DNA (gDNA) was extracted from leaf tissue using a modified CTAB method. For Southern analysis, 3 g of tissue ground to a fine powder in liquid nitrogen using a sterile mortar and pestle was added to 15 ml pre-warmed (60°C) extraction buffer in 50 ml Beckman centrifuge tubes (Beckman Coulter Inc., California, USA). Extraction buffer components were as follows: 2% (w/v) CTAB, 2% (w/v) PVP-40 (polyvinylpyrrolidone), 100 mM Tris (pH 8.0), 25 mM EDTA, 2 M NaCl, 3.5 mM spermidine and 2% (v/v) β-mercaptoethanol. Tubes were incubated at 60°C for 30 min and intermittently vortexed to facilitate extraction. All further steps were performed at room temperature (*ca*. 22°C). Following incubation, tubes were centrifuged at 10 700 xg for 10 min. The supernatant was removed to a new tube and extracted twice with an equal volume of phenol:chloroform (1:1) centrifuging as before. DNA was precipitated by the addition of two volumes of isopropanol. Tubes were mixed gently and centrifuged at 5 000 xg for 10 min. The resulting DNA pellet was washed with 70% (v/v) ethanol, air dried and re-suspended in 500 µl TE buffer. Precipitated RNA was degraded by the addition of 10 µg ml^{-1} RNase A followed by incubation at 37°C for 30 min. RNase A was removed by extracting with an equal volume of phenol:chloroform (1:1), mixed well and centrifuged at 10 700 xg for 10 min. DNA was precipitated with 2.5 M ammonium acetate (pH 7.7) and 2.5 times the final volume of absolute ethanol. After centrifugation at 10 000 xg for 10 min the DNA pellet was washed with 70% ethanol, air dried and re-suspended in 100 µl sterile dH_2O.

DNA used for PCR amplification was extracted on a smaller scale following a modified CTAB extraction protocol (Gawel and Jarret, 1991) as described in 3.3.3. *A. thaliana* gDNA extractions for transgene screening and derived cleaved amplified polymorphic sequence (dCAPS) analyses from transgenic lines were performed as in Edwards *et al.* (1991). RNA used for cDNA library construction was extracted using a large-scale isolation method used for pine (*Pinus radiata*; Azevedo *et al.*, 2003). RNA samples for expression analyses were extracted following Smart and Roden (2010). Quantity and quality of nucleic acids were determined by Nanodrop ND-100 spectrophotometry and gel electrophoresis.

4.3.3. Southern analysis

'Carnival' DNA (10 µg) was restricted with *Sac*I, *Bam*HI, *Hin*dIII, *Xba*I, *Xho*I or *Eco*RI using 50 units of enzyme and allowing complete restriction overnight. The completion of the restriction was confirmed by gel electrophoresis. Restricted DNA was electrophoresed at 3 V cm^{-1} for 1 h followed by 2 V cm^{-1} for 6 h in a 0.8% (w/v) agarose gel with TAE buffer. The gel was stained in a 0.5 µg ml^{-1} ethidium bromide solution to allow visualisation. Following visualisation the gel was submerged, firstly in denaturing buffer (0.5 M NaOH, 1 M NaCl) and then in neutralising buffer (0.5 M Tris (pH 7.4), 3 M NaCl) for 15 min each with shaking. DNA was immobilised onto a N^+ hybond membrane (Amersham Biosciences (*now GE Healthcare*), Uppsala, Sweden) by downward capillary blotting with 10x SSC. DNA was crosslinked to the membrane at 1200 mJ s^{-1} using an Amersham UV-Crosslinker (RPN 2500/2501) and rinsed in 2x SSC. Pre-hybridisation and hybridisation were performed in Church and Gilbert (1984) hybridisation buffer. Pre-hybridisation was performed in 20 ml buffer for 3 h at 60°C. A previously identified 450 bp cDNA fragment of *ProLFY* (Fig. 4.1) was labelled with [α-^{32}P]dCTP (Amersham), with a specific activity of 3000 mCi $mmol^{-1}$, using the Megaprime™ DNA labelling system (Amersham) as per manufacturer's description. Un-incorporated radioactive residues were removed by passing the labelled probe through a PCR purification column (QIAquick, Qiagen, Hilden, Germany). The probe was denatured at 95°C for 5 min and immediately added to the pre-hybridisation solution. Hybridisation was allowed to continue for *ca*. 15 h at 60°C. Following hybridisation the membrane was washed firstly with a 2x SSC, 0.1% (w/v) SDS solution for 15 min at 55°C, followed by a 20 min wash at 50°C with 2x SSC, 0.1% (w/v) SDS and finally 20 min at 50°C with 0.5x SSC, 0.1% (w/v) SDS. The membrane was rinsed in 2x SSC and sealed in a plastic bag prior to exposing it to universal (RX-U/HR-HA) Fuji X-ray film (Fujifilm, Tokyo, Japan). Exposure was allowed to continue for 7 d at -80°C before developing the film.

4.3.4. Reverse transcription of RNA and cDNA library construction

A cDNA library of 'Carnival' floral meristematic tissue was constructed with the Novagen OrientExpress cDNA library construction system (Merck, Darmstadt, Germany). An Oligo(dT) primer was used for cDNA synthesis and the λSCREEN expression system was used for subsequent cloning. The library was constructed as per manufacturer's manual with the following modification: 100 µg total RNA, instead of 4 µg poly(A)$^+$ RNA,

was used for cDNA synthesis, assuming that poly(A)$^+$ mRNA comprises 4% of total RNA. cDNA used for the amplification of *ProLFY* paralogues and DGGE analyses was reverse transcribed from 250 ng RNA with the Sigma Enhanced Avian HS RT-PCR kit (Sigma-Aldrich, Missouri, USA) as per manufacturer's instructions except that all reaction volumes were halved. For semi-quantitative expression analyses of *ProLFY-WL4*, 1 µg of RNA was reverse transcribed with random primers using the Improm-II™ Reverse Transcription System (Promega, Madison WI, USA) to generate cDNA.

4.3.5. Isolation of ProLFY *from 'Carnival'*

A previously identified partial 450 bp sequence of *PROFL,* re-named *ProLFY*, amplified from 'Carnival' floral meristematic cDNA with the LFY3F[1] and LFY3R[2] primers (Smart, 2005; MSc dissertation, University of Stellenbosch) was used to design homologous primer sequences to obtain the *ProLFY* full length sequence. Numbers shown in bold superscript (in the text) after primer names indicate their binding sites on the schematic representation of the *ProLFY* gene (Fig. 4.1), corresponding to sequences in Table 4.1.

Figure 4.1. Schematic representation of the *ProLFY* gene from *Protea* cultivar 'Carnival'. Primer binding sites, directionality and identity are shown. Numbers assigned to primers correspond to those in Table 4.1 and are indicated in bold superscript when mentioned in text. Exons are shown as rectangles and introns as lines. Hatched portions of exon 2 and 3 indicate the previously identified *ca*. 450 bp partial sequence used as probe for Southern hybridisation (Fig. 4.2) and to design primers for thermal asymmetric interlaced–PCR (TAIL-PCR) and cDNA library amplification.

Table 4.1. Primer sequences used for isolation of the full length *ProLFY* sequence from *Protea* cultivar 'Carnival' gDNA by TAIL-PCR (5' region) and by amplification from a meristematic cDNA library (3' region).

Number	Primer name	Sequence (5'→3')	Forward/Reverse
1	LFY3F	CAgAgggAgCACCCgTTCATTgTgAC	Forward
2	LFY3R	gACg(A/C)AgCTT(g/T)gT(g/T)gg(A/g)ACATACCA	Reverse
3	LFY3(2)R	CgTCACCTTggTggggCATTTTTC	Reverse
4	LFYTAIL2R	TCTgAACATgAATCAAgAAATCACggCACTgCT	Reverse
5	MSPROFL R1	ggCTCTgTCACgATgAACgggTgCT	Reverse
6	PROFL_GS1R	TCCCCATCgCCTCCTTCTCTTgTT	Reverse
7	PROFL_GS2R	CCTgggAgAgTgCATCgAgATCgTT	Reverse
8	PROFL_GS3R	CgTCgggACTCTTCCTCTTCgAgAA	Reverse
9	LFYTAIL1F	gggAgCTTggAggCAggCCTgCTACCA	Forward
10	Screen-1-rgt-R*	gTTTAgAggCCCCAAggggTTAT	Reverse
11	LFY3(2)F	CCCCTTgTTgCCATggCAgCC	Forward
12	ProLFY_F	ATggATCCCgACACgTTCTC	Forward
13	ProLFY_R	TTACAggCggTggTAgCC	Reverse
14	ProLFY_DGGE_R	***CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCCT*** TCCTgggAgAgTgCATCgA	Reverse
15	WL_ProLFY_F	TTCAAATgggATCCAAgAggAg	Forward
16	WL_ProLFY_R	CACCgTgAACCCTAgCTCC	Reverse

*Sequence not shown on schematic representation as it is homologous to part of the right λSCREEN-1 vector arm used for construction of the cDNA library (Novagen, Merck). Bold italics in the ProLFY_DGGE_R primer indicate the GC-clamp sequence added to the 5' end of the amplified fragment to facilitate denaturing gradient gel electrophoresis (DGGE; Sheffield *et al.*, 1989).

Two approaches were followed to isolate the complete *ProLFY* sequence. Firstly, thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier, 1995; Liu *et al.*, 1995) was used to obtain the 5' region of the sequence. Secondly, PCR amplification from a floral meristematic cDNA library was used to obtain the 3' region. Two rounds of TAIL-PCR were required to amplify the complete 5' region of *ProLFY*. GoTaq polymerase (Promega, Madison WI, USA) was used for all TAIL-PCR reactions. Reaction conditions and arbitrary primers used were as described by Liu and Whittier (1995) using 50 ng 'Carnival' gDNA as template. The first TAIL-PCR reaction was performed with gene specific primers: LFY3(2)R**[3]**, LFYTAIL2R**[4]** and MSPROFL R1**[5]** for primary, secondary and tertiary PCR respectively, while the second used PROFL_GS1R**[6]**, PROFL_GS2R**[7]** and PROFL_GS3R**[8]**. The 3' region was amplified from a floral meristematic cDNA library using a *ProLFY* sequence specific primer, LFYTAIL1F**[9]**, and a primer

homologous to the right phage arm (Screen-1-rgt_R[10]). Preceding amplification, the cDNA library was treated at 99°C for 5 min. Amplification was performed in a 30 µl reaction volume using the Phusion high fidelity polymerase Hot Start system from Finnzymes (Thermo Fisher Scientific, MA, USA) with the following components: 1x HF buffer, 0.2 mM dNTPs, 0.3 µM forward primer, 0.3 µM reverse primer, 1.2 units Phusion polymerase and 2 µl of the denatured cDNA library as template. Following an initial denaturing step at 98°C for 30s, the following cycling conditions were repeated 35 times: 98°C, 10s; 63°C, 20s and 72°C, 45s. Cycling was followed by a final elongation step at 72°C for 10 min. Sequence identity was confirmed by nested PCR using LFY3(2)F[11] and Screen-1-rgt-R[10]. Fragments of interest from various PCR reactions were cloned into pJET1.2 (Fermentas, Burlington, Canada) and sequenced (Macrogen Inc., Seoul, Korea).

4.3.6. Cloning of ProLFY *for heterologous expression in* A. thaliana

Forward and reverse primers (LFY_F[12] and LFY_R[13]) were designed to amplify the complete coding sequence (cds) from 'Carnival' meristematic cDNA. Amplifications were performed with Accutaq (Sigma-Aldrich) in 30 µl reaction volumes with the following components: 1x buffer (containing 2.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.4 µM forward primer, 0.4 µM reverse primer, 1.2 units Accutaq polymerase and 2 µl cDNA as template. PCR products were gel extracted and cloned into the pJET1.2 vector (Fermentas). Initially 34 clones were partially sequenced: WL, 5; OD, 5; D, 5; AG, 5; FB, 8 and L, 6. Eight clones (WL4, 6 and 8; D1, L1 and 5; FB3 and 7) were chosen to be sequenced fully using the LFY3F[1] primer. To produce *35S::ProLFY* sense constructs for heterologous expression in *A. thaliana*, four *ProLFY* clones (WL4, D1, L5 and FB7) and *A. thaliana LFY* (*AtLFY*), were cloned into the pEarlyGate201 plant expression vector (Early *et al.*, 2006). pEarlyGate201 was obtained from the Arabidopsis Biological Resource Center (ABRC, stock number: CB3-687). *AtLFY* was amplified from pIL8, a vector harbouring the *LFY* gene (kindly supplied by D. Weigel, Max Planck Institute, Tübingen, Germany), with the following primer pair:

aLFY_forward, 5' ATggATCCTgAAggTTTCACgAgT 3' and aLFY_reverse, 5' CTAgAAA CgCAAgTCgTCgCC 3'. PCR amplification was performed with the Phusion high fidelity polymerase Hot Start system from Finnzymes (Thermo Fisher Scientific) in a 50 µl reaction with the following components: 1x HF buffer, 0.2 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 2 units of Phusion polymerase and 30 ng of pIL8 vector

as template. The following reaction conditions were followed: initial denaturation, 98℃ for 30s; cycling (35x), 98℃ for 10s, 60℃ for 20s and 72℃ for 45s; final elongation, 72℃ for 10 min. Amplified *AtLFY* was subsequently gel extracted and cloned into the pJET1.2 vector. A pEarlyGate201 expression vector lacking the *ccdB* cytotoxic gene was generated by restricting the vector with *Sma*I and subsequently re-ligating the vector. The resulting vector (pEarlyGate201Δ*ccdB*, vector control) could be propagated in *E.coli* (DH5α) cells and was used to generate the transformation vectors expressing the *ProLFY* paralogues and *AtLFY* gene. *AtLFY* was excised from the pJET1.2 cloning vector with *Xho*I and *Nco*I to release the fragment of interest and subsequently directionally cloned into the pEarlyGate201*ΔccdB* vector using the same restriction sites. The *ProLFY* paralogues were directionally cloned into the *Sma*I (blunt) and *Xho*I sites of pEarlyGate201*ΔccdB* by restricting the *ProLFY* pJET1.2 clones with *Pvu*II (blunt) and *Xho*I. The presence of the inserts was confirmed by restriction digest. The *35S::ProLFY* paralogues, *35S::AtLFY* and pEarlyGate201*ΔccdB* (empty vector) were used to transform competent *Agrobacterium tumefaciens* strain GV3101 cells for *A. thaliana* floral-tissue transformation.

4.3.7. Phylogenetic analyses

The coding sequences of *ProLFY* paralogues were *in silico* translated using DNAMAN (version 4.13, Lynnon Biosoft, Quebec, Canada) and the derived amino acid sequences aligned to identify a consensus sequence to use for phylogenetic analysis. Sequences included in phylogenetic analyses were chosen based on lowest *E*-values obtained after a protein-protein blast (BLASTp) of the consensus, deduced amino acid sequence of *Pro*LFY. LFY orthologues from *A. thaliana* and rice (*Oryza sativa*) were included. Multiple sequence alignments and phylogenetic tree generation was performed as described in 3.3.5.

4.3.8. Denaturing gradient gel electrophoresis of ProLFY *paralogues*

Fragments subjected to DGGE were amplified from 'Carnival' gDNA and meristematic cDNA samples firstly with ProLFY_F[12] and PROFL_GS1R[16] primers using Phusion high fidelity polymerase in 30 µl reactions with the following components: 1x GC buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.7 units Phusion polymerase and 50 ng gDNA, *ca*. 30 ng plasmid DNA or 0.5 µl cDNA as template. The following cycling conditions were followed: initial denaturing, 98℃ for

30s; cycling (35x), 98°C for 30s, 63°C for 15s and 72°C for 15s; final elongation, 72°C for 10 min. A second PCR was performed using the same forward primer, ProLFY_F**12**, and ProLFY_DGGE_R**14** as reverse primer under the same PCR conditions as described above, except that 1 µl of a 10-fold dilution of the initial PCR was used as template. The GC-clamp added to the reverse primer (Sheffield *et al.*, 1989) is indicated in bold italics in Table 4.1. Fragments amplified from clones: FB3, FB7, L5, WL4 and D1 were mixed together and used as a marker. DGGE was performed with a Bio-Rad Dcode Universal Mutation Detection System (BioRad, Germany) as per manufacturer's description. Initially only the marker sample and individually amplified clones were subjected to DGGE. The samples were prepared by adding 10 µl of a 2x loading dye (70% (v/v) glycerol, 0.05% (w/v) bromophenol blue and 0.05 (w/v) xylene cyanol) to 10 µl of the PCR samples and applying them directly to a 20 - 60% denaturing gradient gel where 100% denaturant was 7 M urea and 40% (v/v) formamide. The gel was electrophoresed in 1x TAE at 60°C for 10 min at 180 V, followed by 2 h at 90 V and finally 150 V for 3 h. After electrophoresis the gel was stained in 1 µg ml^{-1} ethidium bromide 1x TAE, rinsed in 1x TAE and visualised. After the initial DGGE, 'Carnival' gDNA, cDNA and marker samples were subjected to DGGE with a 40 - 60% denaturing gradient as described above, but electrophoresed at 180 V for 10 min followed by 62 V for 18 h. The marker sample was allowed to form heteroduplexes by denaturing it at 95°C for 5 min, treating it at 65°C for 1 h and then allowing it to cool to room temperature (ca. 22°C) slowly. Post electrophoresis staining and visualising were performed as described above.

4.3.9. Genotyping of lfy-2 *mutants*

A derived cleaved amplified polymorphic sequence (dCAPS) marker was designed to distinguish between the Col-0 wild-type (wt) *LFY* and the ethylmethane sulfonate (EMS) generated *lfy-2* mutant. The point mutation in *lfy-2* is at 719 bp in the *LFY* sequence and caused by a C→T conversion (Fig. 4.6 A). Fragments were amplified from 1 µl of extracted DNA (Edwards *et al.*, 1991) with the dCAPSlfy-Forward-*BamH*I and dCAPSlfy-Reverse primer sequences shown in Fig. 4.6 A. Reactions were performed with GoTaq polymerase (Promega) in 30 µl reaction volumes with the following components: 1x GoTaq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM forward primer, 0.3 µM reverse primer and 1 unit GoTaq polymerase (added during the initial denaturing step). The following cycling conditions were followed: initial denaturing, 95°C for 5 min;

cycling (40x), 95°C for 30s, 58°C for 30s and 72°C for 30s; final elongation at 72°C for 7 min. PCR products, 10 µl, were restricted with 10 units of *Bam*HI (Fermentas) in a final reaction volume of 30 µl and incubated at 37°C overnight. Restricted fragments were electrophoresed in 2% (w/v) agarose gels containing 1 µg ml^{-1} ethidium bromide with 1x TAE at 5 V cm^{-1} for 2 h.

4.3.10. A. thaliana *transformation, flowering time and phenotype analyses*

A. thaliana Col-0 and *lfy-2* plants were transformed with *A. tumefaciens* using the floral dip method as described by Clough and Bent (1998). Potential transgenic T_0 plants were screened by spraying with 0.29 mg ml^{-1} BASTA (Bayer CropScience, Monheim, Germany; kind gift from Bayer CropScience Paarl) once at 1.5 weeks old and again at 2.5 weeks. The presence of the *BaR* (BASTA resistance) gene in surviving seedlings was confirmed by PCR using the following primer pair:
pat_f, 5' CCAgAAACCCACgTCATgCCAgTT 3' and pat_r, 5' CTACATCgAgACAAgCAC ggTCAACTT 3' in 20 µl reaction volumes with the following components: 1x GoTaq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM forward primer, 0.3 µM reverse primer, 0.5 units GoTaq polymerase and 1 µl gDNA (Edwards *et al.*, 1991). The following cycling conditions were followed: initial denaturation, 95°C for 5 min; cycling (35x), 95°C for 30s, 55°C for 30s, 72°C for 30s; final elongation at 72°C for 7 min. T_1 plants were screened for the *BaR* gene by PCR using the same PCR conditions as above. To determine if the *ProLFY* transgene segregated with the *BaR* gene, some T_1 plants were also screened with the ProLFY_F[12] and ProLFY_R[13] primers. Two transgenic lines from each of the *ProLFY* paralogues were selected from Col-0 and *lfy-2* T_1 plants and used to determine transgene segregation and phenotype of the T_2 population. For the empty vector control and *35S::AtLFY* Col-0 plants, segregation of only one transgenic line for each was analysed. Morphologic and phenotypic characteristics were reported for transgenic plants in which the presence of the transgene was confirmed. Days-to-flowering was determined at the appearance of the first visible floral buds in the meristem and the number of rosette leaves was counted at this time. Upon completion of flowering and complete drying of plants (*ca*. 4 weeks), seed weights were determined for *lfy-2* T_2 transgenics as well as untransformed Col-0 and *lfy-2* plants. Morphological floral characteristics were visualised using a Nikon stereoscopic zoom microscope SMZ1500 (Nikon Inc., New York, USA).

4.3.11. Semi-quantitative PCR expression profiling of ProLFY_WL4

Semi-quantitative PCR was performed on cDNA derived from 'Carnival' meristematic and leaf tissue. For all cycle optimisation see Appendix C (Fig. C1 and C2). The following primer pair was designed to specifically amplify the *WL4* paralogue: WL_ProLFY_F[15] and WL_ProLFY_R[16] amplifying a *ca*. 140 bp fragment. Specificity of the primers was determined by PCR using the WL4, L5, D1 and FB3 clones as template in 20 µl reactions with GoTaq polymerase. The following components were included in the reaction: 1x GoTaq buffer, 1.75 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM forward primer, 0.3 µM reverse primer, 10 pg plasmid DNA as template and 1 unit GoTaq polymerase (added at the initial denaturing step). The following cycling conditions were followed: initial denaturing, 95°C for 3 min; cycling (35 x), 95°C for 30s, 65°C for 30s, 72°C for 15s; final elongation at 72°C for 7 min. Semi-quantitative PCR of *ProLFY-WL4* was performed as described above with the following changes: 2 µl cDNA was used as template and only 34 cycles were performed. Transcript levels were normalised to *eIF4A* (*eukaryotic initiation factor 4A*). PCR reaction conditions for the amplification of *eIF4A* were as for *ProLFY-WL4* except that the annealing temperature was dropped to 59°C, only 31 cycles were performed and 1 µl cDNA was used as template. Amplification was performed with the following primer pair:

EIF4A F2, 5' gTCgTTTTggACC(C/T)AAgggTgTTgC 3' and EIF4A R, 5' TggCATTTCAT CAATCTgggTACTgTA 3', that amplified a *ca*. 100 bp fragment. The PCR products were separated on a 1.5% (w/v) agarose gel and visualised by ethidium bromide (0.5 µg ml^{-1}) staining in 1x TAE. Intensities of the bands were determined by Quantity One (version 4.6.3, Bio-Rad) 1-D imaging and analysis software.

4.3.12. Statistical analyses

Where appropriate, results were subjected to analysis of variance (ANOVA) to determine significant differences using Statistica (Version 8, StatSoft Inc.). *Post hoc* Fisher LSD (95%) multiple range tests were conducted to determine statistical differences between samples.

4.4. Results

4.4.1. Protea *hybrid 'Carnival' has many paralogous* ProLFY *genes*

Southern analysis of 'Carnival' gDNA, probed with a 450 bp fragment amplified from floral meristem cDNA (Fig. 4.1), suggests the presence of more than one copy of the *ProLFY* gene (Fig. 4.2). At least three bands could clearly be discerned in the *Sac*I, *Hin*dIII, *Xba*I and *Eco*RI restricted lanes, indicated by arrows, and the intensity of the top band in the *Xho*I digest may suggest the presence of two similar sized bands. The complete *ProLFY* sequence from 'Carnival' was successfully isolated using a combination of approaches. The 5' region of *ProLFY* was amplified using TAIL-PCR and the 3' region by PCR amplification from a floral meristem cDNA library using gene and vector specific primers. Upon identification of the complete sequence, full length *ProLFY* sequences were amplified from 'Carnival' cDNA generated by reverse transcription of RNA isolated from meristematic tissue at various stages of development (Fig 3.6). Initially, 34 clones were partially sequenced and thereafter 8 clones (D1, FB3, FB7, L1, L5, WL4, WL6 and WL8) were chosen to be fully sequenced based on dissimilarities in the partial sequences. A fragment corresponding to FB7 could not be identified from gDNA or cDNA during DGGE separation (Fig. 4.5) and was therefore omitted from alignments. Nucleotide acid and deduced amino acid sequence alignments of the paralogous *ProLFY* genes showed only minor differences with 97 to 99% identity (Fig. 4.3; Fig. 4.4; Table 4.2).

The *Pro*LFY consensus amino acid sequence showed 73, 68, 70, 68, 67 and 68% homology to LFY orthologues from the woody perennial species: platanus (*P. racemosa*; AAF77610.1), walnut (*Juglans regia*; ADL61865.1), quince (*Cydonia oblonga*; AB162037.1), cedar (*Cedrela fissilis*; AY633621.1), apple (AFL1 (AB056158.1) and AFL2 (AB056159.1))(Fig. 4.4). *Pro*LFY was also 69% homologous to the herbaceous monkey flower (*Mimulus guttatus*; AY524036.1). *Pro*LFY showed 64% homology to LFY from *A. thaliana* (AF466801.1) and 50% to RFL from rice (*O. sativa*; AB005620.1). Numbers in parentheses indicate the accession numbers for the proteins from GenBank. Regions with high homology were mostly limited to the conserved N- and C-terminal regions (Fig. 4.5). Blue and red lines above the sequence alignment indicate these regions. Amino acid residues key to LFY function, identified by Maizel *et al.* (2005) and Hamès *et al.* (2008) are indicated by characters below the residues. All

these key amino acid residues were conserved in *Pro*LFY, however a number of dissimilar residues were present within these conserved regions.

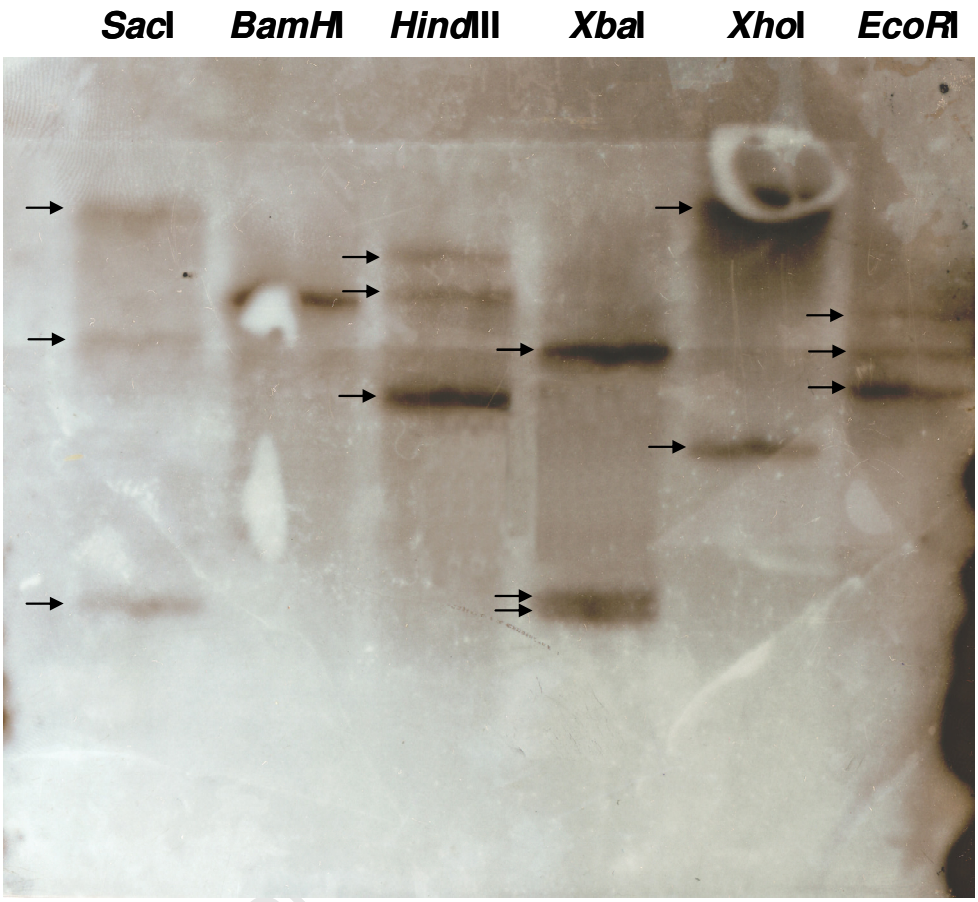


Figure 4.2. Southern hybridisation of gDNA from *Protea* cultivar 'Carnival' restricted with either *Sac*I, *Bam*HI, *Hin*dIII, *Xba*I, *Xho*I or *Eco*RI probed with a partial sequence of *ProLFY*. Restricted gDNA, immobilised on a N^+ hybond membrane (Amersham), was probed with the radioactively labelled (α-^{32}P[dCTP]) 450 bp sequence of *ProLFY* previously amplified from floral meristematic cDNA (Fig. 4.1). Arrows indicate bands of interest.

The evolutionary relationship of the conserved *Pro*LFY sequence to LFY orthologues from other species was determined by phylogenetic analyses. Sequences shown in the protein alignment (Fig. 4.4), together with those from: mango (*Magifera indica*; ADX97316); cucumber (*Cucumis melo* subsp. Melo; ADN33854); painted nettle (*Solenostemon scutellarioides*; ABP87785); longan (*Dimocarpus longan*; ABP02007);

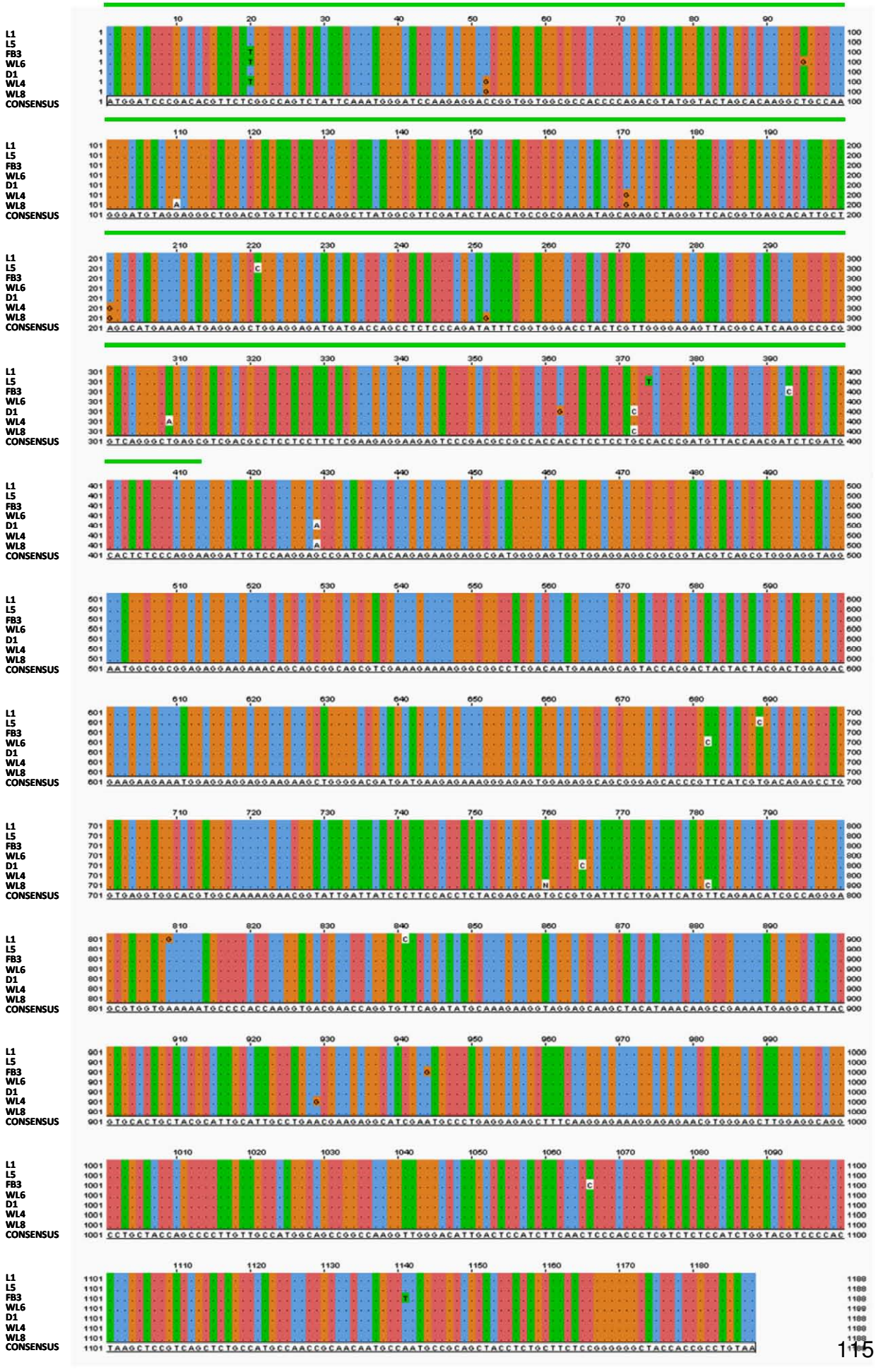
butterfly bush (*Buddleja davidii*; ABA43519 (LFY1a); ABA43620 (LFY1b); ABA43522 (LFY2); ABA43523 (LFY3)); monkey flower (*Mimulus ringens*; AA545975); mountain walnut (*Carya cathayensis*; ABI58284); Chinese chestnut (*Castanea mollissima*; ABB83126); orange (*Citrus sinensis*; AAR01229); poplar spp. (*Populus tricocarpa*; AAB51533 and *P. tremula*; AEK26561); pea (*Pisum sativum*; AAC49782); almond (*Prunus dulcis*; AAY30859); barrel clover (*Medicago truncatula*; AAX22220); pepper (*Capsicum annuum*; AB518396) and violet (*Viola pubescens*; ABU54075 (LFY); ABV59208 (LFY2)) were included in phylogenetic analyses. The proteins that shared the highest BLASTp scores (lowest E-values) with *Pro*LFY formed a well-supported clade (85%) in phylogenetic analyses, while *A. thaliana* LFY and rice RFL formed clear outlier groups. *Pro*LFY formed a strongly supported (84%) subclade with *Para*LFY from platanus (Fig. 4.6).

Table 4.2. Percentage sequence similarities representing the number of identical residues between *ProLFY* sequences on nucleotide and deduced amino acid level, determined after CLUSTALW alignment.

	L1	L5	FB3	WL6	D1	WL4	WL8
L1	-	99.5 (98.7)	99.4 (98.4)	99.5 (98.7)	99.4 (99.2)	99.3 (98.7)	99.0 (98.2)
L5	99.5 (98.7)	-	99.3 (98.2)	99.4 (98.4)	99.4 (98.9)	99.2 (98.4)	98.9 (97.9)
FB3	99.4 (98.4)	99.3 (98.2)	-	99.4 (98.7)	99.2 (98.7)	99.2 (98.7)	98.8 (97.7)
WL6	99.5 (98.7)	99.4 (98.4)	99.4 (98.7)	-	99.4 (98.9)	99.4 (98.9)	98.9 (97.9)
D1	99.4 (99.2)	99.4 (98.9)	99.2 (98.7)	99.4 (98.9)	-	99.1 (98.9)	99.2 (98.4)
WL4	99.3 (98.7)	99.2 (98.4)	99.2 (98.7)	99.4 (98.9)	99.1 (98.9)	-	99.2 (98.4)
WL8	99.0 (98.2)	98.9 (97.9)	98.8 (97.7)	98.9 (97.9)	99.2 (98.4)	99.2 (98.4)	-

Similarities on deduced amino acid level are shown in brackets.

Figure 4.3 (follows on next page). Nucleotide sequence alignments of cloned *ProLFY* paralogous cDNA sequences isolated from meristematic tissue at various stages of development (defined in Fig. 3.6): FB3 (floral meristem); WL4,6,8 (wavy leaf meristem); D1 (dormant meristem). L1 and 5 were amplified from leaf cDNA. The consensus sequence is shown and dissimilarities between paralogous genes indicated. The green line above the sequence shows the portion of the sequence amplified from gDNA and cDNA for DGGE analysis. Alignments were performed in CLUSTALW2 and graphically enhanced with Jalview 2.6.1 (Waterhouse *et al.* 2009).


L1
L5
FB3
WL6
D1
WL4
WL8
CONSENSUS

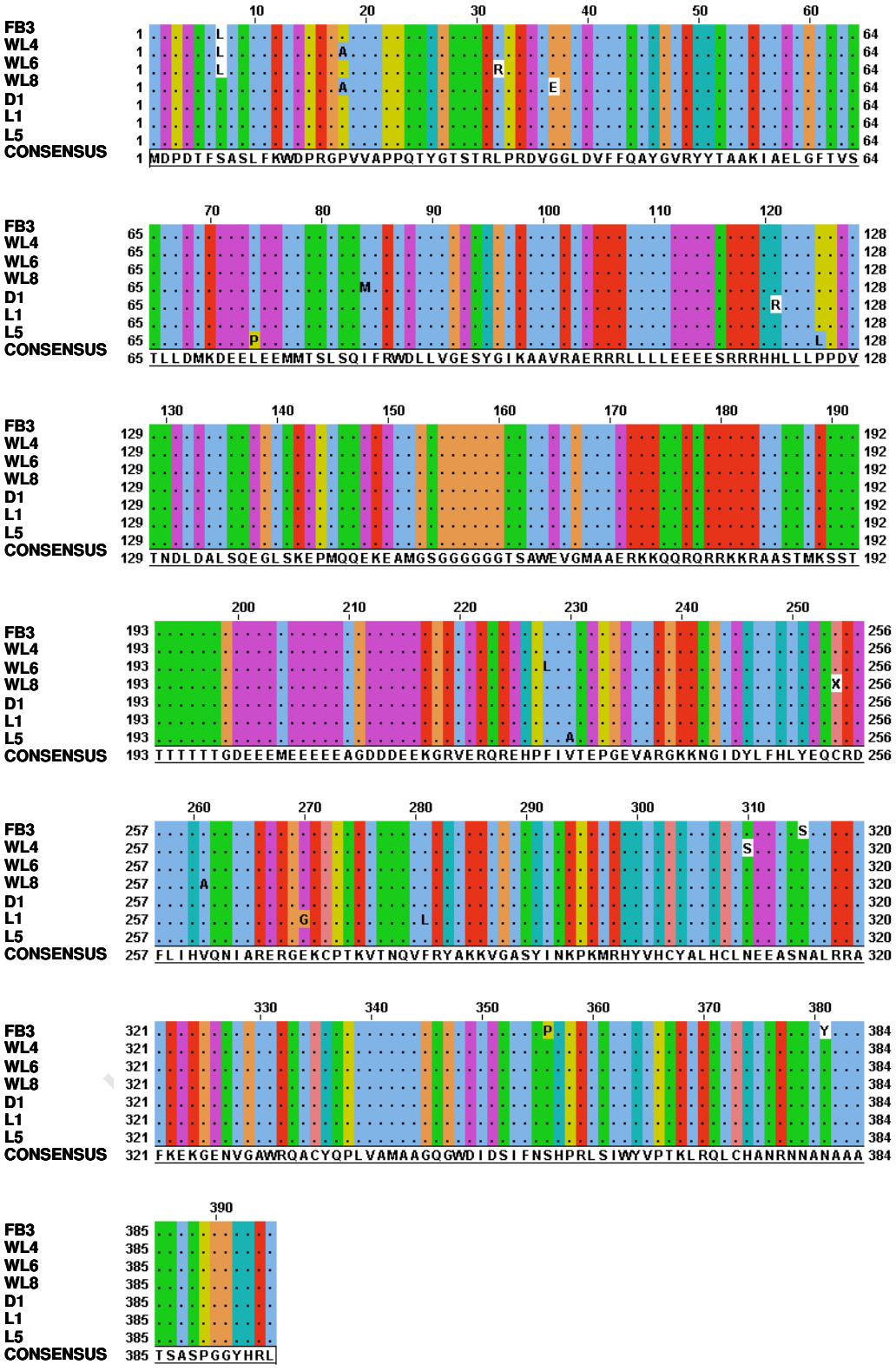


Figure 4.4. Deduced amino acid sequence alignments of cloned *ProLFY* paralogous cDNA sequences shown in Fig. 4.3. The consensus sequence is shown and dissimilarities between paralogous genes indicated. Alignments were performed in CLUSTALW2 and graphically enhanced with Jalview 2.6.1.

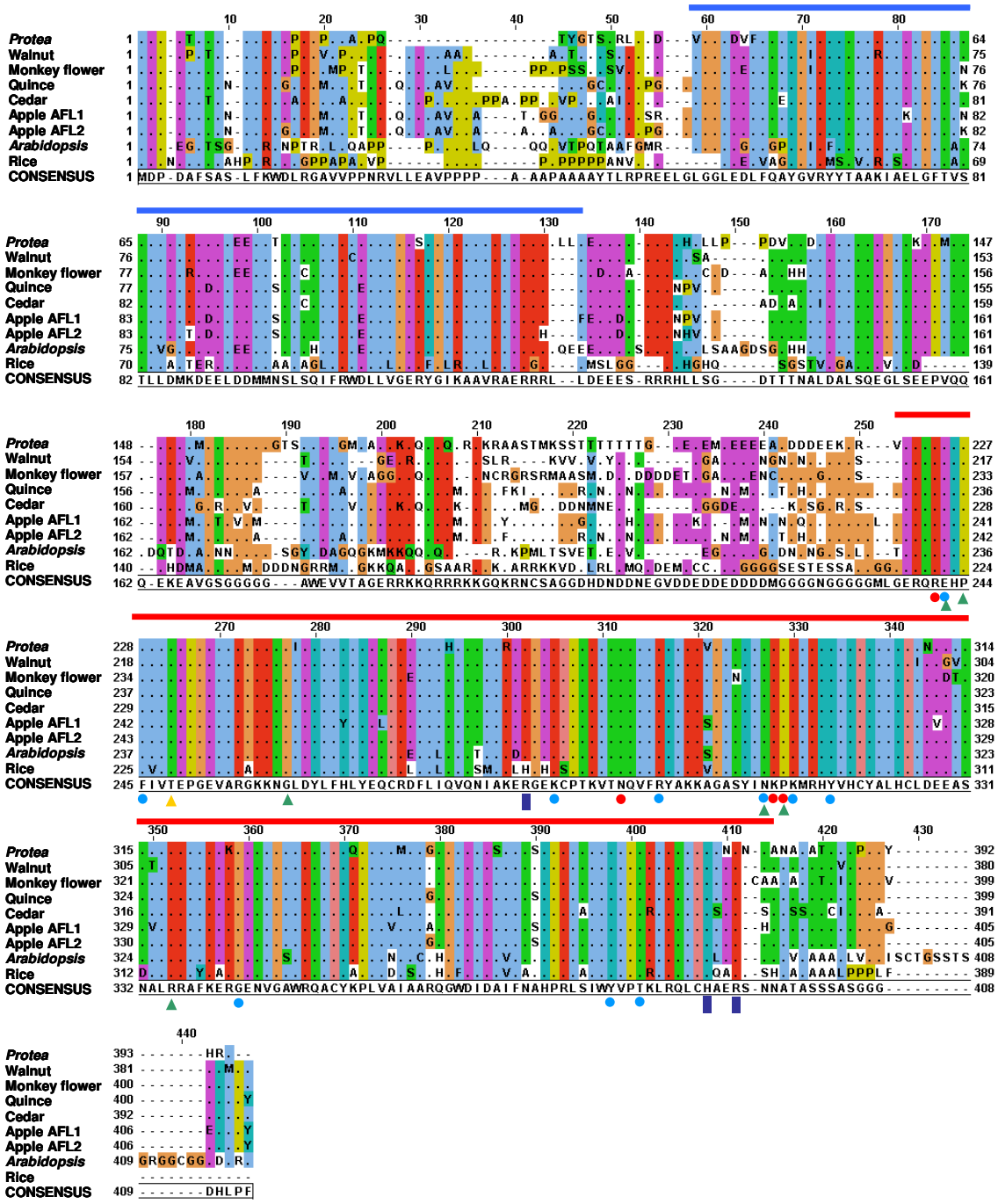


Figure 4.5. Alignment of the consensus deduced amino acid sequence from *Protea, ProLFY*, with orthologous *LFY* genes from other species. GenBank accession numbers and scientific names of species used in alignment are given in the text. The consensus sequence is shown and dissimilarities in the other sequences indicated. Alignments were performed in CLUSTALW2 and graphically enhanced with Jalview. Conserved domains and residues identified by Maizel *et al.* (2005) and Hamès *et al.* (2008) are shown. Blue and red lines above amino acids indicate the conserved N- and C-domains respectively. Red circles show amino acids involved in interaction with DNA bases and blue circles those involved in interaction with the DNA backbone. Blue rectangles indicate residues involved in protein dimerisation and green triangles show the position of *A. thaliana lfy* mutations and the yellow triangle the position of the amino acid substitution of *lfy-2* (Thr→Ile).

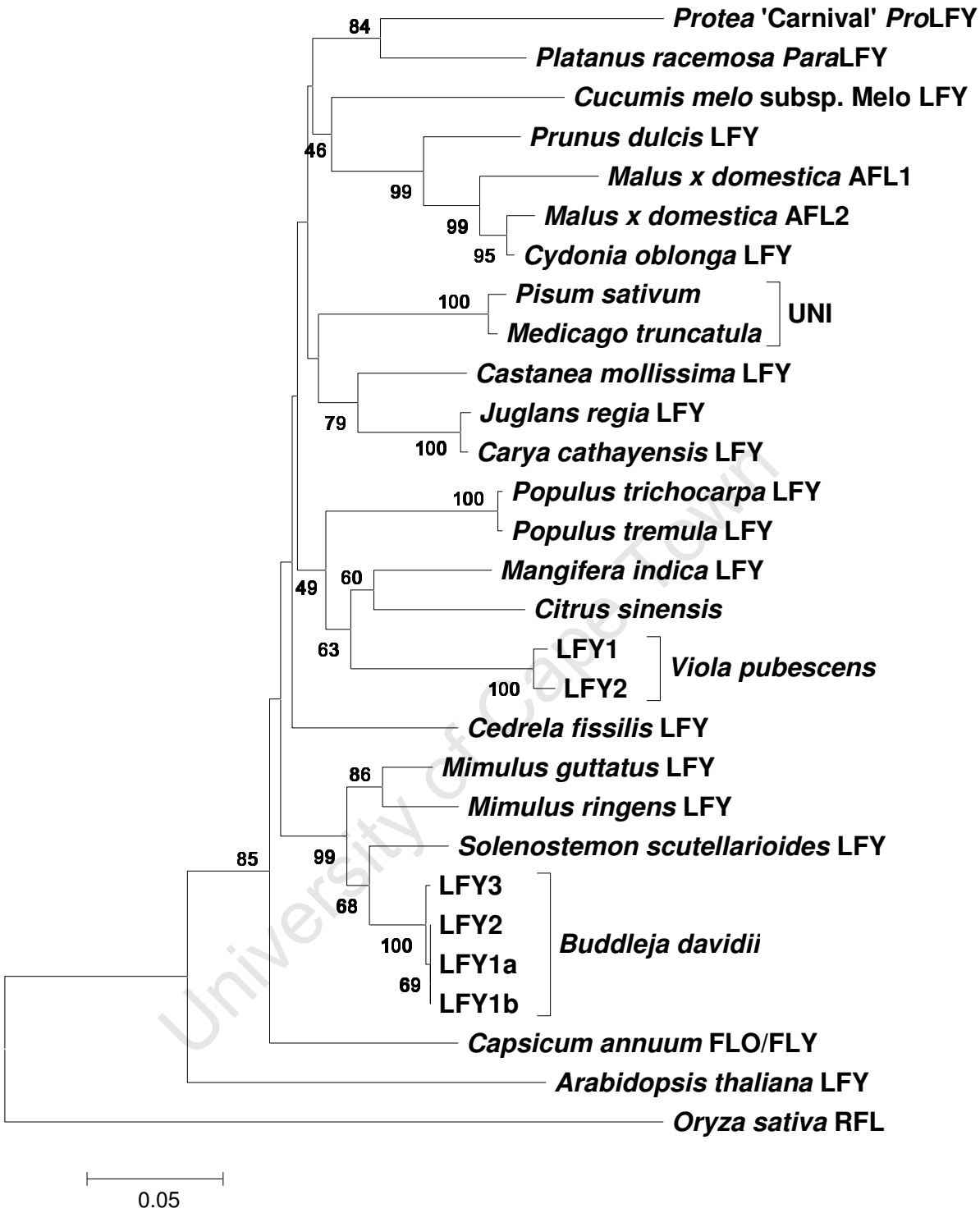


Fig. 4.6. Phylogenetic tree showing the evolutionary relationship of *Pro*LFY to other orthologous LFY proteins. *Pro*LFY was placed at the top of the tree. GenBank accession numbers of proteins used in analysis are given in text. Branches are labelled with bootstrap values determined from 1000 re-samplings (>40%). Branch lengths, computed using the Poisson correction method, are drawn to scale (see scale bar). These indicate evolutionary distances as the number of amino acid substitutions per site. Phylogenetic analyses were performed in MEGA5 and alignments preceding analyses were performed with ClustalW (Thompson et al., 1994).

A portion of the amino (N) terminal region of *ProLFY*, shown as a green line above the nucleotide sequence alignment (Fig. 4.3), was amplified from 'Carnival' gDNA and cDNA. These PCR products were subsequently subjected to DGGE analysis. The amplified region contained numerous SNPs which allowed the separation of similar sized fragments by DGGE. DGGE indicated the presence of at least 4 paralogous *ProLFY* genes within the 'Carnival' genome (Fig. 4.7 B). Three of these corresponded to known sequences: FB3/D1, L5 and WL4 used as markers for band identification (Fig. 4.7 A). Homology between the amplified sequences caused the formation of heteroduplexes during the PCR amplifications from gDNA and cDNA. Marker fragments could be forced to form heteroduplexes by denaturing them and allowing heteroduplex formation at 65°C, thus identifying the higher bands visible on the DGGE gel as heteroduplexes. There was no apparent tissue specific expression of particular *ProLFY* paralogues when cDNA samples from dormant meristems, wavy leaf meristems, floral meristems or leaf tissue was compared at the transcript level (Fig. 4.7 B).

4.4.2. Expression of ProLFY *paralogous genes in* A. thaliana *Col-0 and* lfy-2

A. thaliana lfy-2 plants flowered later and with more rosette leaves than Col-0 plants ($P<0.05$; Fig. 4.8), consistent with flowering times reported for *lfy-2* mutants by Page *et al.* (1999). Mutant, *lfy-2*, flowers had a 'leaf-like' appearance with only a few wild-type flowers developing late during flowering (Fig. 4.10 B and C; Fig. 4.11 A) similar to the phenotypic appearance of *lfy-2* reported by Schultz and Haughn (1991). However, the greatest dissimilarity between Col-0 plants and *lfy-2* mutants was a five-fold lower seed yield ($P<0.05$) from *lfy-2* mutants compared to Col-0 plants (Fig. 4.8).

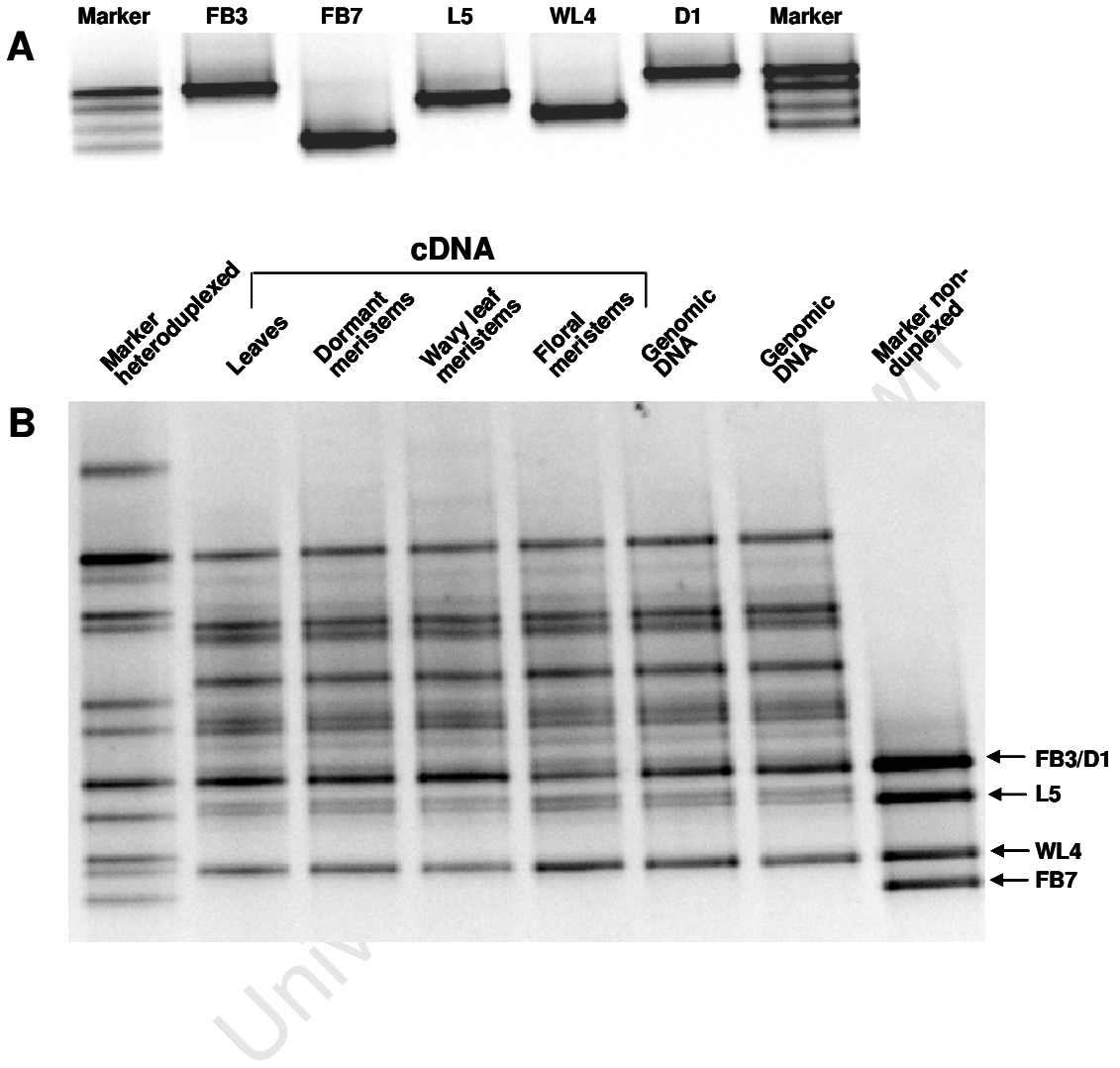


Figure 4.7. DGGE analyses of the 5' region of the *ProLFY* gene from *Protea* cultivar 'Carnival'. The fragment amplified is indicated by the green line above the nucleotide sequence alignment in Fig. 4.3. **A**. A marker was generated by mixing the amplified fragments from FB3, D1, L5, WL4 and FB7 clones and used to identify *ProLFY* paralogues amplified from gDNA and cDNA subsequent to electrophoreses in a 20-60% denaturing gel. **B**. Denaturing gel gradient (40-60%) electrophoresis of PCR products amplified from gDNA and cDNA samples. cDNA samples were generated from leaf and meristematic tissue at various stages of development i.e. dormant, wavy leaf and floral buds. The marker sample was heteroduplexed by denaturing it at 95°C for 5 min, allowing heteroduplex formation at 65°C for 1 h and cooling it to room temperature slowly.

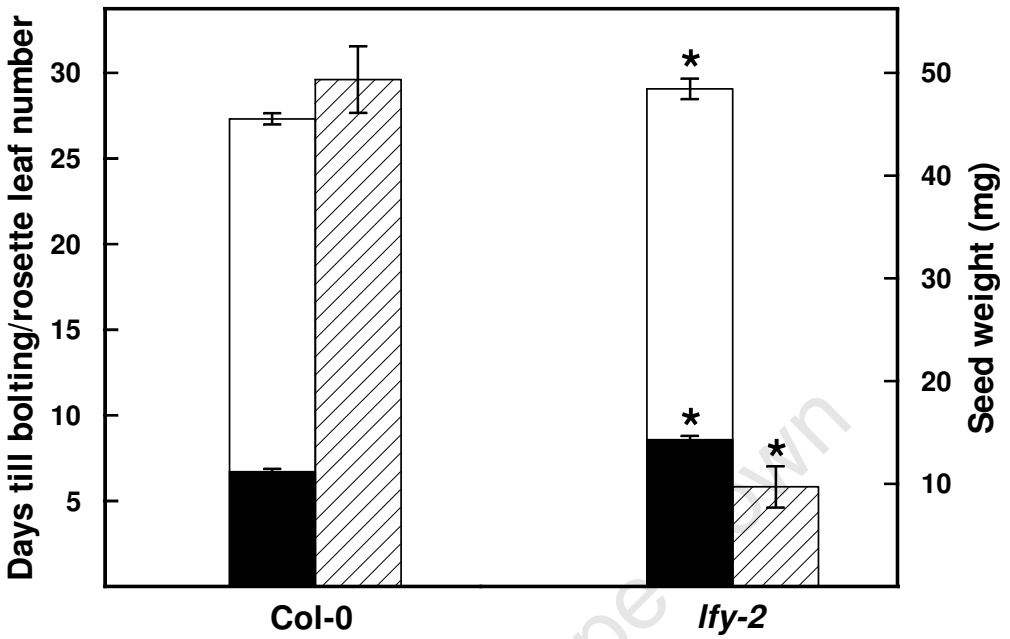


Figure 4.8. Phenotypic characteristics of the *A. thaliana lfy-2* mutant. Days to bolting (white bars), rosette leaf number at bolting (black bars) and seed weight (hatched bars; mg) of *A. thaliana* ecotype Col-0 and *lfy-2* mutant plants. Asterisks (*) indicate significant differences (P<0.05) determined by ANOVA followed by *post hoc* Fisher LSD tests. Bars represent means and error bars indicate SDev (n=20).

Paralogous *ProLFY* genes: *WL4*, *L5* and *D1* were heterologously expressed in Col-0 and *lfy-2* plants under control of the cauliflower mosaic virus 35S promoter (*35S::ProLFY-WL4*, *35S::ProLFY-L5* and *35S::ProLFY-D1*) to determine functional homology to *AtLFY* . Col-0 plants were also transformed with *35S::AtLFY* and an empty vector control (pEarlyGate*ΔccdB*). No *35S::ProLFY-L5* Col-0 transgenic plants were, however, recovered during screening.

Phenotypic and morphological characteristics were documented for T_2 plants. The following *lfy-2* transgenic lines had the highest seed yields: *35S::ProLFY_WL4* lines 41 and 44; *35S::ProLFY_L5* lines 24 and 42 and *35S::ProLFY_D1* lines 1 and 8. These

transgenic lines were used to investigate the segregation of the transgene in the T_2 generation. Segregation ratios, determined by the number of T_2 plants containing the transgene compared to those without, were much higher for transgenics in the Col-0 compared to the *lfy-2* background (Table 4.3). Ratios much higher than those expected from Mendelian genetics (3:1) were concluded to be due to the insertion of more than one copy of the transgene.

Genotyping of the *lfy-2* T_1 lines that were chosen for further analyses confirmed that they were in the *lfy-2* background. Genotyping was performed by dCAPS outlined in Fig. 4.9 A. A size difference between the unrestricted (UR) PCR amplified fragment and *Bam*HI restricted fragment could be seen in the Col-0 wild type, but not for *lfy-2* or any of the *lfy-2* transgenic lines (Fig. 4.9 B).

Table 4.3. Analysis of transgene segregation, days to bolting and number of rosette leaves at bolting in the T_2 populations of Col-0 (wild type) and *lfy-2 ProLFY* transgenic plants.

	Transformant	Transgene present:absent		Days to bolting	Rosette leaf number at bolting
		Number	Ratio		
Col-0				27.3 ± 1.7 **be**	6.7 ± 0.8 **a**
	35S::A. thaliana LFY-1	43:6	7:1	27.0 ± 1.9 **b**	6.7 ± 0.9 **a**
	empty vector-3	50:1	50:1	27.5 ± 2.5 **bcd**	6.8 ± 0.9 **a**
35S::ProLFY→	*-WL4-1*	46:3	15:1	26.8 ± 2.2 **b**	6.6 ± 0.9 **a**
	-WL4-3	45:2	22.5:1	28.3 ± 2.2 **de**	7.0 ± 0.6 **a**
	-D1-1	44:3	15:1	29.8 ± 3.3 **a**	7.5 ± 0.9 **b**
	-D1-2	45:3	15:1	28.2 ± 1.6 **ce**	7.4 ± 0.8 **b**
lfy-2				29.1 ± 2.6 **a**	8.6 ± 1.0 **a**
35S::ProLFY→	*-WL4*-41	37:10	4:1	24.9 ± 2 **b**	6.3 ± 0.7 **bc**
	-WL4-44	42:6	7:1	24.7 ± 1.7 **b**	6.1 ± 0.5 **c**
	-L5-24	40:9	4:1	28.2 ± 3.1 **a**	6.9 ± 0.9 **d**
	-L5-42	42:9	5:1	28.1 ± 3.5 **a**	7.4 ± 1.0 **e**
	-D1-1	38:11	3:1	25.5 ± 1.4 **b**	6.6 ± 0.8 **bd**
	-D1-8	33:12	3:1	26.9 ± 1.2 **c**	6.9 ± 0.6 **d**

Data are means ± SDev. Dissimilar letters after data indicate significant differences (P<0.05) determined by ANOVA followed by *post hoc* Fisher LSD test.

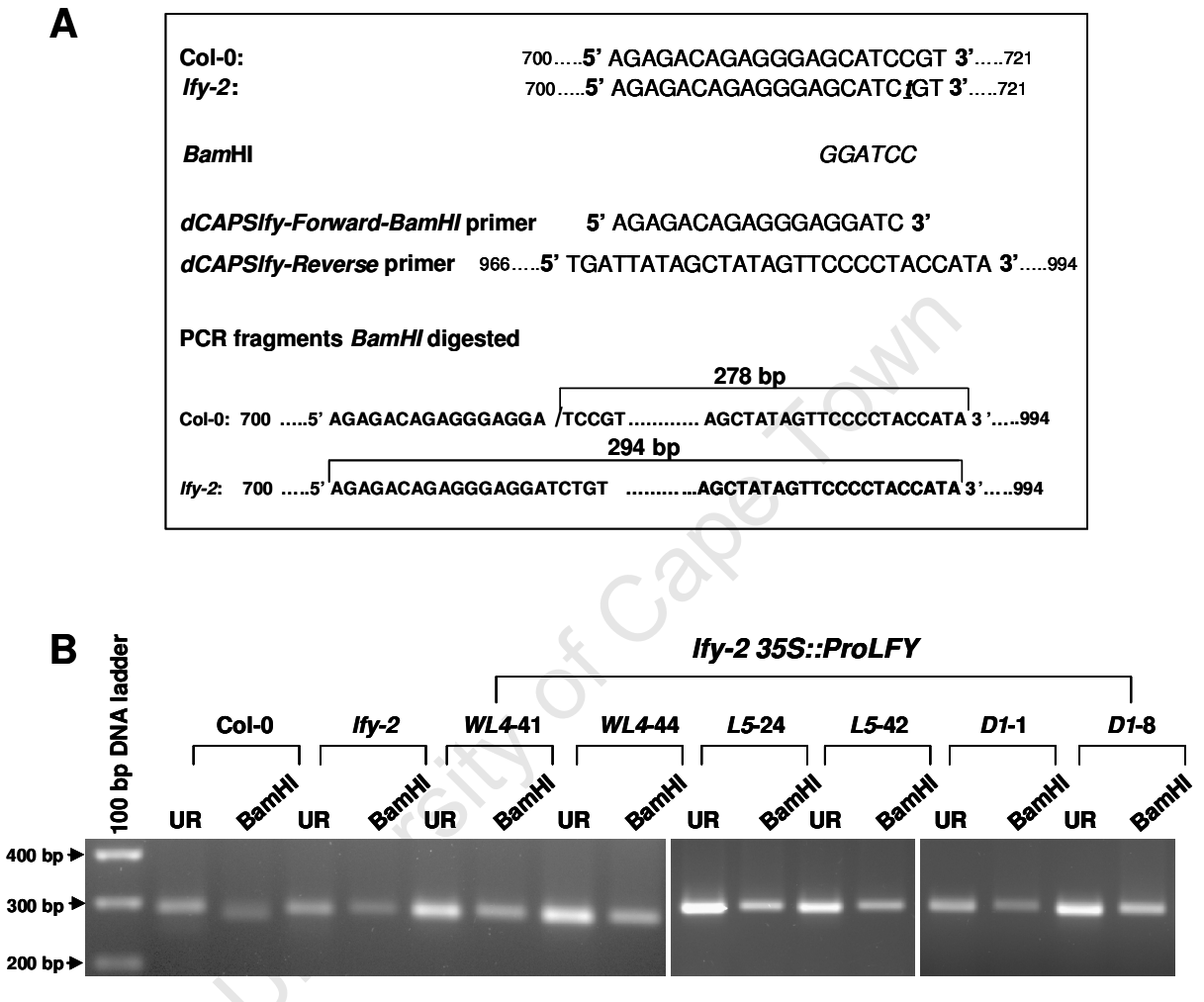


Figure 4.9. Genotyping of *A. thaliana lfy-2* mutant plants by derived cleaved amplified polymorphic sequence (dCAPS) analysis. A. Diagrammatic representation of the dCAPS technique used, showing primer sequences. **B**. Gel electrophoresis of dCAPS products amplified from wild-type (Col-0), *lfy-2* and the six *lfy-2 35S::ProLFY* T_1 lines used for further analysis. Both the unrestricted (UR) and *Bam*HI restricted samples are shown.

Col-0 *35S::ProLFY-D1*-1 plants flowered significantly (P<0.05) later (29.8 ± 3.3 d) than Col-0 plants (27.3 ± 1.7 d) and both *35S::ProLFY-D1* transgenic lines, 1 and 8, had significantly (P<0.05) more rosette leaves at bolting when compared to Col-0 plants (Table 4.3). When compared to wild-type Col-0, none of the other *ProLFY* transgenic lines showed any significant differences in flowering time. However, flower morphology was greatly affected by the presence of 35S::*ProLFY* and *35S::AtLFY*. Many of the *35S*-transgenic Col-0 lines showed fused sepals and petals such as the flowers from *35S::ProLFY-D1*-8 (Fig. 4.10 D), whereas *35S::ProLFY-WL4*-3 and *35S::AtLFY*-1 transgenics developed an abnormal number of floral organs (Fig. 4.10 E and F). The empty vector control did not show any abnormal floral morphology (Fig. 4.10 G) or difference in flowering time when compared to untransformed Col-0 plants (Table 4.3).

lfy-2 plants transformed with 35S::*ProLFY-WL4* (lines 41 and 44) and 35S::*ProLFY-D1* (lines 1 and 8), flowered earlier and with fewer rosette leaves than the parental *lfy-2* mutant plants (Table 4.3). *lfy-2 35S::ProLFY-L5* plants did not flower significantly earlier in terms of days to flowering, but had fewer rosette leaves at bolting than *lfy-2* mutants (Table 4.3). *lfy-2 35S::ProLFY-D1* plants developed a greater number of large leaves (Fig. 4.10 D), whereas *lfy-2 35S::ProLFY-L5* plants developed smaller leaves than *lfy-2* plants (Fig. 4.11 C). Both *lfy-2* 35S::*ProLFY-L5* and *lfy-2* 35S::*ProLFY-D1* transgenic plants developed abnormal flowers with fused floral organs (Fig. 4.10 H and I). *lfy-2* 35S::*ProLFY-WL4* plants developed flowers and inflorescences with a wild-type phenotype (Fig. 4.10 J and Fig. 4.11 B) and had a much greater seed yield than *lfy-2* plants or any of the other *lfy-2 35S::ProLFY* lines (Fig. 4.12 B).

The seed yield from *lfy-2* mutant plants were mostly lower than 30 mg, whereas Col-O plants on average produced close to 50 mg seed per plant (Fig. 4.8; Fig. 4.12 A). All T_1 *lfy-2* transgenic lines had similar seed yields to untransformed *lfy-2* mutant plants (Fig. 4.12 A, B, C and D). T_2 lines however showed different distributions of seed yields, with the two transgenic *lfy-2 35S::ProLFY-WL4* lines showing the greatest rescue of *lfy-2* fertility (Fig. 4.12 B).

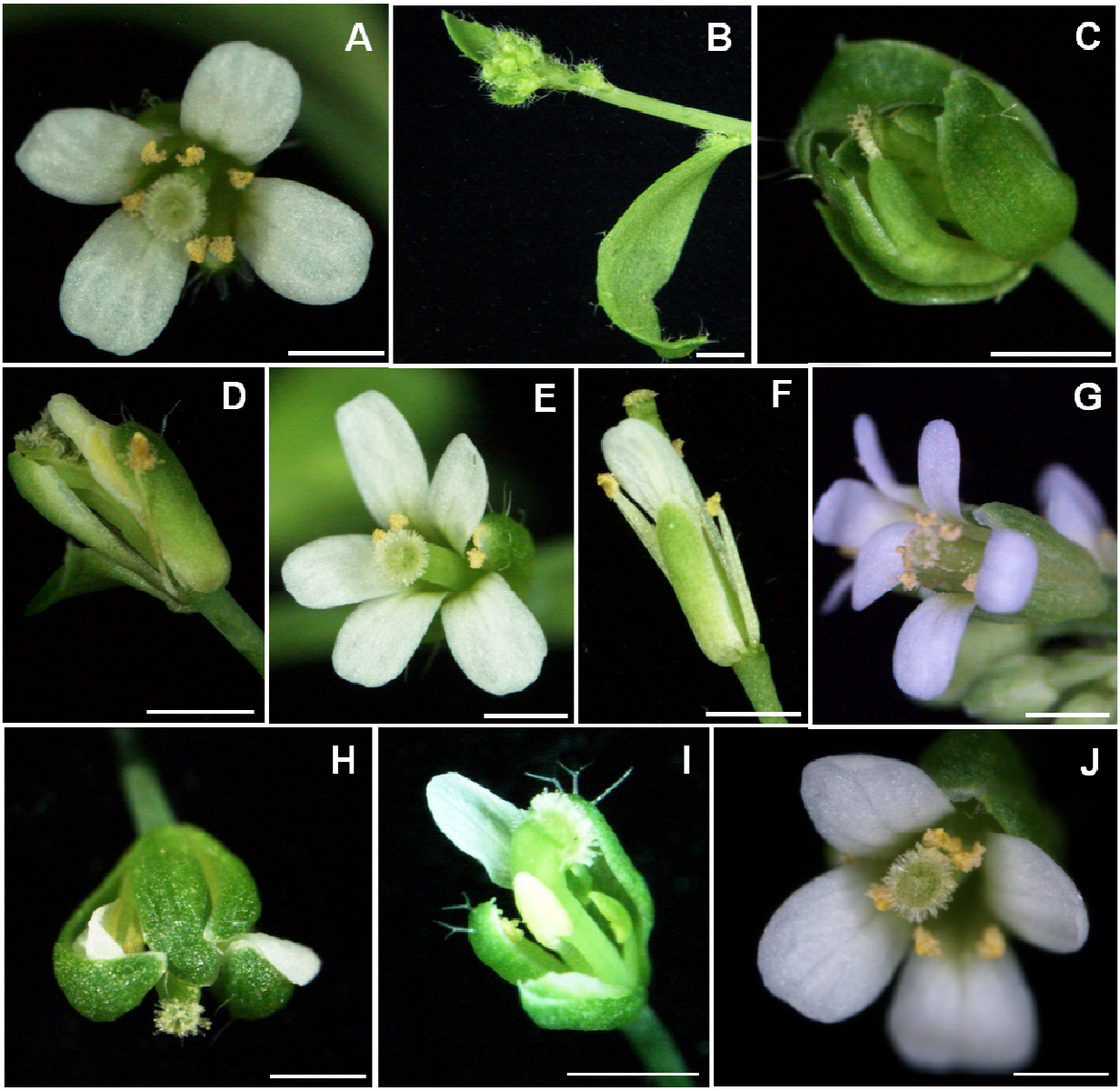


Figure 4.10. Morphological characteristics of flowers from Col-0 and *lfy-2 A. thaliana 35S::ProLFY* transgenic plants. A. Col-0 wild type. **B**. *lfy-2* inflorescence. **C** *lfy-2* flower. **D**. Col-0 *35S::ProLFY-D1*-1 **E**. Col-0 *35S::ProLFY-WL4*-1. **F**. Col-0 *35S::A. thaliana LFY*-1. **G**. Col-0 empty vector-3. **H**. *lfy-2 35S::ProLFY-L5*-24 **I**. *lfy-2 35S::ProLFY-D1*-8. **J**. *lfy-2 35S::ProLFY-WL4*-44. Pictures were acquired with a Nikon stereoscopic zoom microscope SMZ1500 (Nikon Inc., New York, USA). White lines represent scale bars (1mm).

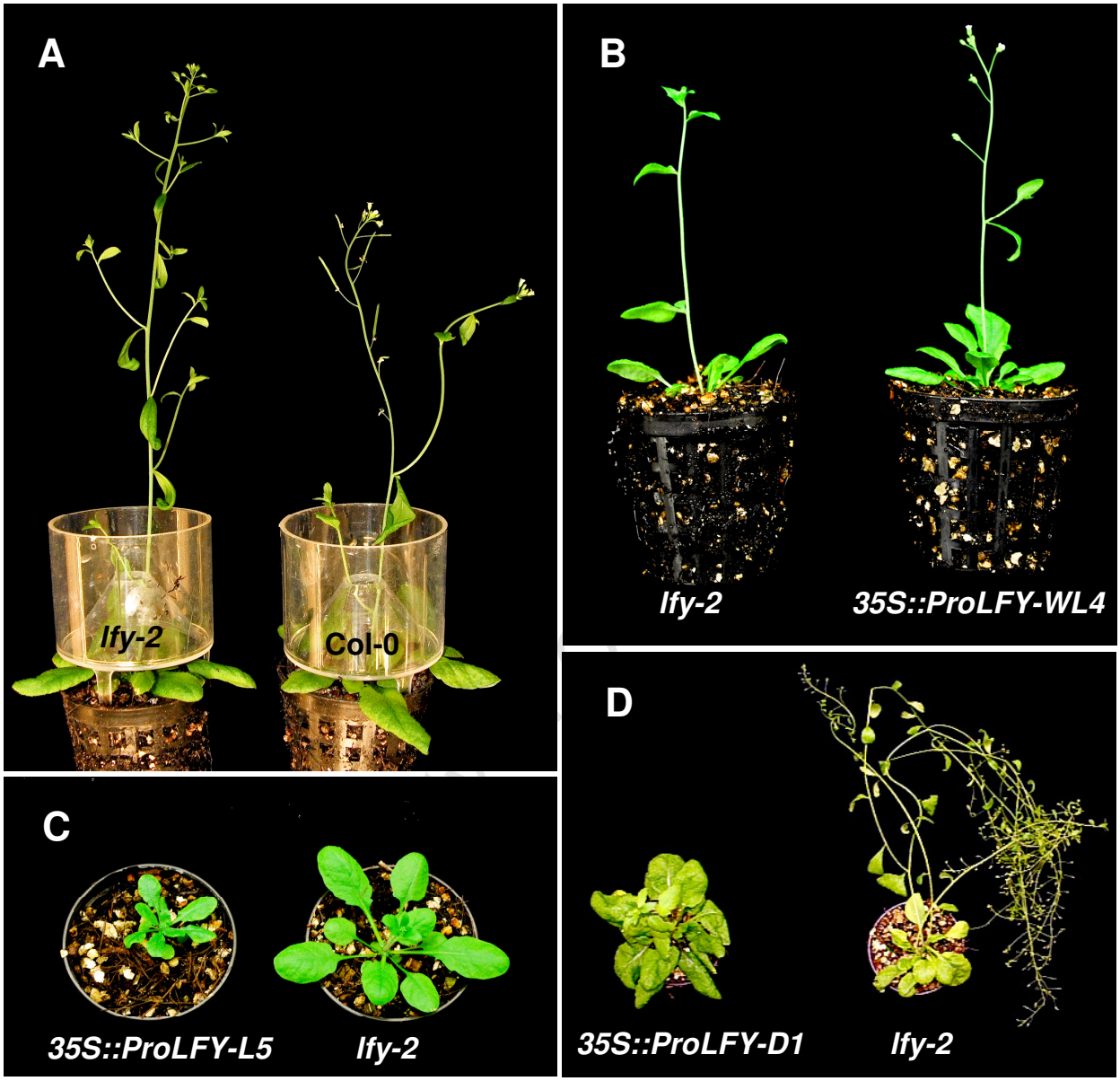


Figure 4.11. Phenotypic differences between Col-0, *lfy-2* mutant and *lfy-2 ProLFY* transgenic *A. thaliana* plants. A. *lfy-2* and Col-0. **B**. *lfy-2* mutant and *lfy-2 35S::ProLFY-WL4*. **C**. *lfy-2 35S::ProLFY-L5* and *lfy-2*. **D**. *lfy-2 35S::ProLFY-D1* and *lfy-2*.

4.4.3. ProLFY-WL4 *expression in* Protea *hybrid 'Carnival' meristematic and leaf tissue*

As *35S::ProLFY-WL4* transgenic *A. thaliana lfy-2* plants showed wild-type flowering characteristics and rescued seed yields, the expression levels of *ProLFY-WL4* were measured in leaf and meristematic tissues of *Protea* hybrid 'Carnival'. The *WL4* paralogue of *ProLFY* was preferentially amplified over the other three paralogues: *FB3*, *D1* and *L5* present in the 'Carnival' genome (Fig. 4.13 A). *ProLFY-WL4* expression levels, normalised to the constitutively expressed gene *eIF4A*, were significantly ($P<0.05$) higher in 'onset dormancy' meristems compared to any of the other stages of vegetative development. However, the highest expression ($P<0.05$) was in florally determined meristems (Fig. 4.13 B). No amplification was detected in the negative or non-reverse transcribed samples.

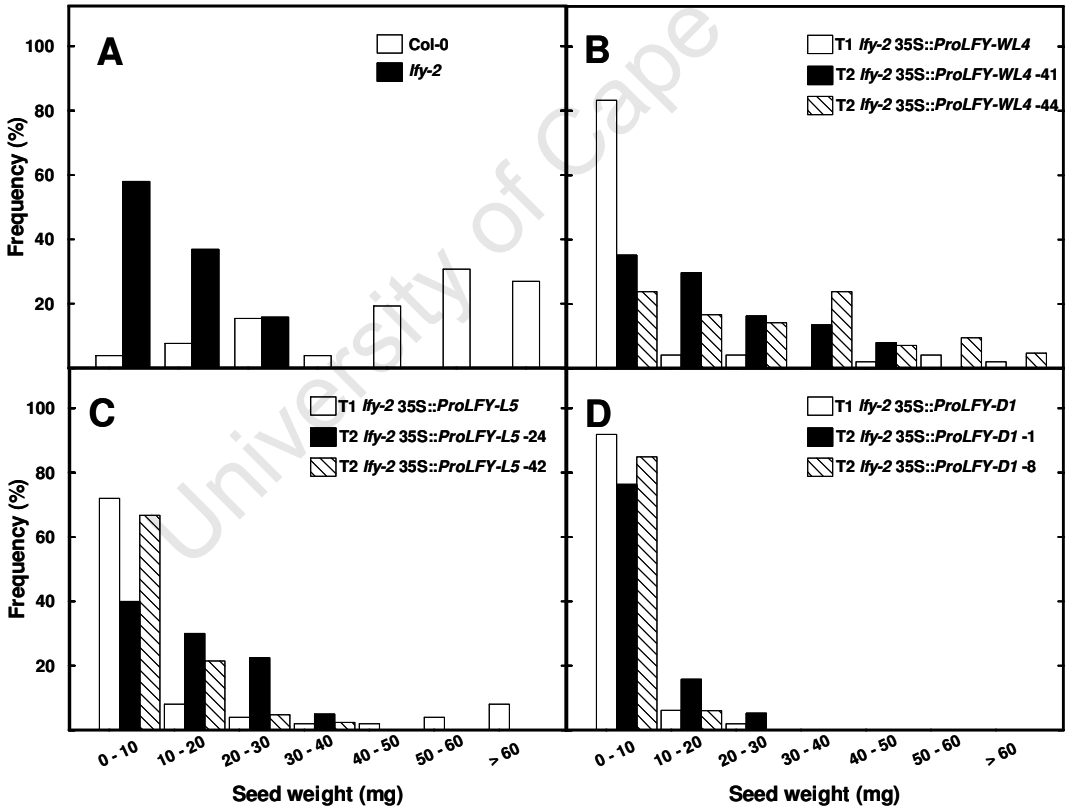


Figure 4.12. Seed weights of *A. thaliana* Col-0, *lfy-2* and *lfy-2 35S::ProLFY* transgenic plants expressed as the frequency (% of total plants) that produced either: 0-10; 10-20; 20-30; 30-40; 40-50; 50-60 or greater than 60 mg seed. A. Wild-type Col-0 and *lfy-2* mutants. **B**. T_1 *lfy-2 35S::ProLFY-WL4* and T_2 lines 41 and 44. **C**. T_1 *lfy-2 35S::ProLFY-L5* and T_2 lines 24 and 42. **D**. T_1 *lfy-2 35S::ProLFY-D1* and T_2 lines 1 and 8.

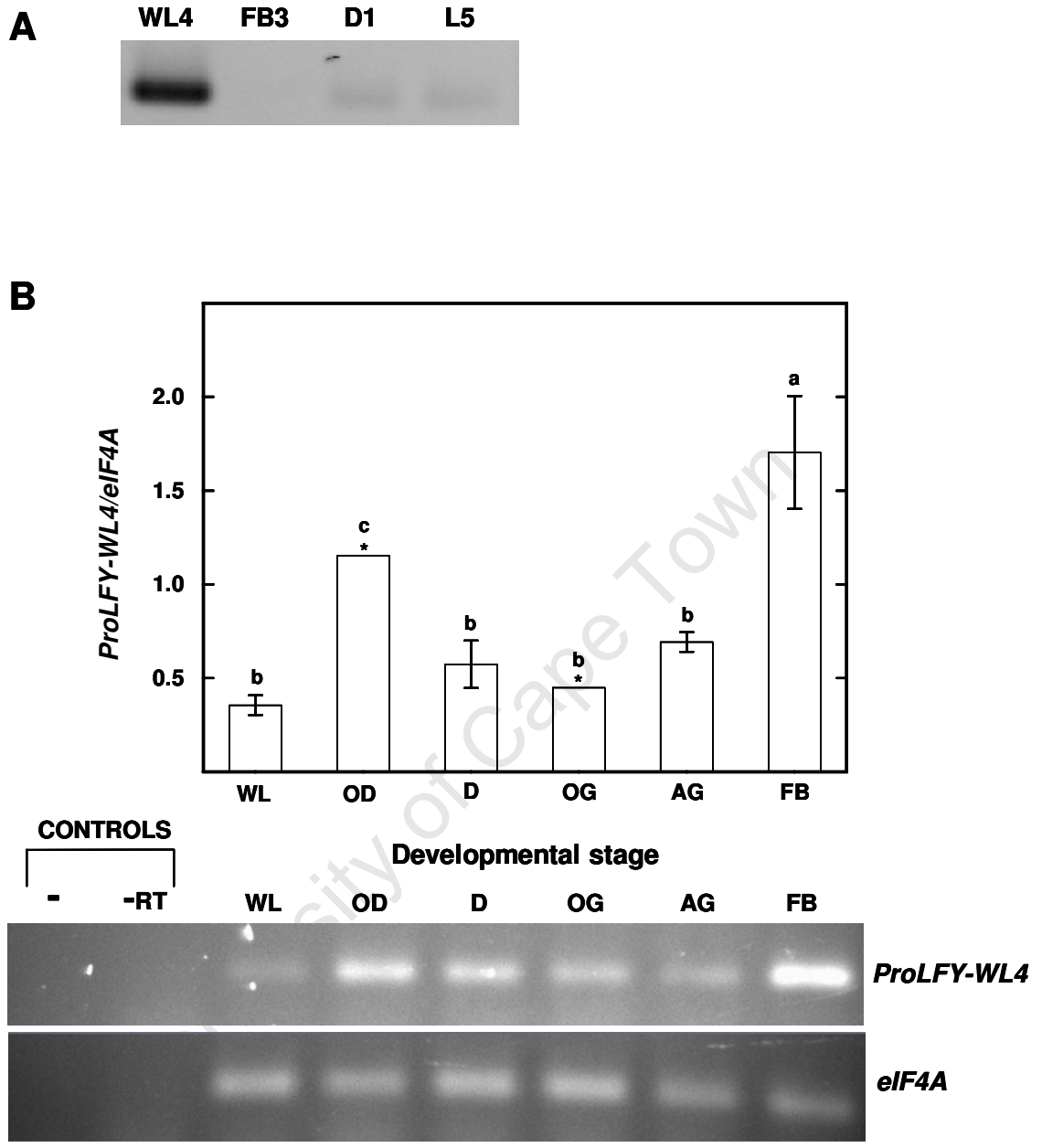


Figure 4.13. Semi-quantitative PCR expression profiling of *ProLFY-WL4* in meristematic tissue at various developmental stages of *Protea* cultivar 'Carnival'. A. PCR amplification of the *WL4* paralogue from 10 pg of plasmid containing *WL4*, *FB3*, *D1* or *L5*. **B**. Expression of *ProLFY-WL4* normalised to *eIF4A* in 'Carnival' meristematic tissue: WL (wavy leaf), OD (onset dormancy), D (dormant), OG (onset growth), AG (active growing) and FB (floral). Corresponding gels, including negative (-) and no reverse transcriptase (-RT) controls, are shown. Asterisks (*) indicate samples for which only two biological replicates were available. Amplification of all samples was performed in triplicate. Bars represent means ± SE. Dissimilar letters above bars indicates statistically significant differences ($P<0.05$) determined by ANOVA followed by *post hoc* Fisher LSD tests.

4.5. Discussion

In this study, four paralogous *ProLFY* genes with homology to *LFY* were isolated from the genome of a commercial *Protea* cultivar 'Carnival'. The nucleotide and deduced amino acid sequences of these paralogous genes shared high percentage sequence similarities (97-99%). There is no evidence to suggest that paralogous *ProLFY* genes in 'Carnival' have been reduced to pseudogenes, unlike *ELF2* in *Eucalyptus globulus* (Southerton et al., 1998) and as suggested for a second *StLFY* copy in *S. tetraptera* (Song et al., 2008). This is based on the fact that the *ProLFY* paralogues are all expressed, their GC contents are mostly similar and no aberrant or unique sequence motifs or premature stop codons are present in the sequences (Fig. 4.3; Grimm and Denk, 2008). From heterologous expression studies in *A. thaliana*, *ProLFY-WL4* isolated from 'wavy leaf' meristems (Fig. 3.6) was found to be functionally equivalent to *AtLFY* as it could rescue the abnormal floral morphology and fertility of *lfy-2* mutant plants in the T_2 population. Mutant *lfy* plants only develop a few 'normal' flowers on axillary meristems (Weigel *et al*., 1992) and therefore have a much reduced seed yield (Fig. 4.8). The insertion of transgene *35S::ProLFY-WL4* in *A. thaliana lfy-2* plants also resulted in earlier flowering measured as the number of days to bolting and the number of rosette leaves at bolting (Table 4.3). The lack of similar early flowering phenotypes in the *35S*::*ProLFY-WL4* Col-0 transgenic plants was probably a consequence of transgene silencing because of the integration of multiple transgene copies (Table 4.3). Gene silencing through multiple insertions may also explain the delayed flowering of *35S::ProLFY-D1* and lack of early flowering of *35S::AtLFY* transgenics. Similar multiple gene integrations and resulting gene silencing have been reported in *Pinus strobus* (Tang *et al*., 2007). The transgenic studies together with the increase in *ProLFY-WL4* expression during floral morphogenesis of *Protea* indicated that *ProLFY-WL4* may be functionally equivalent to *AtLFY*, fulfilling the role of a meristem identity gene in *Protea*.

ProLFY-WL4 levels were low during vegetative development and were significantly ($P<0.05$) up-regulated in florally determined meristems (Fig. 4.13). This expression pattern is consistent with that of *LFY* in *A. thaliana* (Weigel et al., 1992) and *FLORICAULA* in snapdragon (*Antirhinum majus*; Coen et al., 1990). *ProLFY-WL4* expression was also similar to that of *LFY* orthologues from woody perennial species such as *StLFY*, the *LFY* orthologue from *Sophora tetraptera* (Song et al., 2008). Expression of *LFY* orthologues from kiwifruit (*ALF*; Walton et al., 2001) and *M. excels*a

(*MEL*; Sreekantan et al., 2004) followed bimodal patterns of expression corresponding to high levels during floral initiation, lower levels during winter dormant phases and up-regulation during spring when floral differentiation occur. 'Carnival' growth and floral development follow a similar sequence of events: meristems enter a dormant phase during the winter period and develop inflorescences during spring after a short period of vegetative growth in autumn. An increase in *ProLFY-WL4* expression in 'Carnival' meristems was observed at the onset of dormancy when meristems were preparing for the dormant period. In a previous study, which followed the temporal expression pattern of *ProLFY* from before dormancy up till floral structures were visible also showed a similar bimodal pattern of expression (Smart, 2005; MSc dissertation, University of Stellenbosch). This expression pattern is similar to that of *ALF* and *MEL* and may suggest that the floral transition is made prior to meristems entering a dormant state in 'Carnival'. However, as a new growth flush is formed during autumn it has been proposed that flowering in 'Carnival' is only initiated during the extension of this vegetative flush (Gerber et al., 2001a). The timing of floral initiation in 'Carnival' warrants further investigation including a more detailed expression profile over meristem development. The increase in *ProLFY-WL4* expression at the onset of dormancy may also suggest that *ProLFY-WL4* plays a role in preparing the meristem for dormancy. During bud-set, meristems develop protective 'bud-scales' which are small, hairy, grey 'leaf-like' structures (Fig. 3.6 F). It is possible that *ProLFY-WL4* may be involved in the formation of these structures.

The presence of *ProLFY-WL4* transcript during the vegetative stages was not surprising, as *LFY* is expressed in leaves and vegetative primordia of *A. thaliana* (Blázques *et al*., 1997; Weigel *et al*., 1992). The other paralogous *ProLFY* genes were also expressed in leaves and meristematic tissues at all stages of development when analysed by DGGE (Fig. 4.7). As the amplification of *ProLFY* paralogues from cDNA samples preceding DGGE was not performed quantitatively it is difficult to comment on the expression levels of these genes. However, the intensities of the electrophoresed bands which represented *ProLFY-WL4* transcript after DGGE was higher in the florally determined meristem compared to the other cDNA samples (Fig. 4.7). This suggested that *ProLFY-WL4* is involved in floral transition, which was confirmed by the rescue of *lfy-2* mutants ectopically expressing *ProLFY-WL4*, while different functions are proposed for the other paralogues based on results from this study. Transgenic *lfy-2*

plants expressing *35S::ProLFY-L5* and *35S::ProLFY-D1* developed abnormal leaves, suggesting that these paralogues may have a role in leaf development. Work in pea (*Pisum sativa*; Champagne *et al.*, 2007) and *Medicago truncatula* (Wang H *et al.*, 2008) showed that these two members of the legume family (Fabaceae) do not express the *KNOTTED1-like* (*KNOX1*) gene required for leaf development. The role of regulating compound leaf development in these species is instead fulfilled by *LFY* orthologues named *UNIFOLIATA* (*UNI*) in both legumes. It may be possible that even in species which express *KNOX1*, *LFY* maintains a role in regulating leaf complexity, as was suggested for tomato (Molinero-Rosales et al., 1999). A possible role for *LFY* orthologues in leaf development of woody perennial systems is also likely, as *LFY* orthologues were expressed in leaf primordia of various woody perennial species including: two *Eucalyptus* species, *E. globulus* (Southerton et al., 1998) and *E. grandis* (Dornelas et al., 2004), poplar (*P. trichocarpa*; Rottmann et al., 2000) and kiwifruit (*Actinidia deliciosa*; Walton et al., 2001). Similarly, *ALF1* and *ALF2*, two *LFY* orthologues from apple (*Malus x domestica*) were expressed in leaf stipules (Wada et al., 2007). *LFY* may even act through the same pathway as *KNOX1*, involving *NO APICAL MERISTEM* (*NAM*) and *CUP-SHAPED COTYLEDON3* (CUC3), to facilitate leaf development as suggested by Blein *et al.*, 2008 and 2010. The role of *LFY* during early leaf development may be through its capacity to stimulate meristematic growth as suggested by Moyroud et al. (2010). This may explain the abnormal leaf phenotypes observed in this and other heterologous expression studies of *LFY* orthologues (Peña *et al.*, 2001). The diverse effects of ectopic expression of *LFY* orthologues suggest that their expression needs to be tightly controlled in plants such as *Protea*, expressing multiple *LFY* genes, to ensure normal reproductive and vegetative development.

Multiple copies of *ProLFY* were also present in the genomes of 'Sylvia' (*P. eximia* x *P. susannae*; Fig. C3 B), *P. repens* (Fig. C3 C) and 'Safari Sunset' (*Leucadendron laureolum* x *Lcd. salignum*; Fig. C3 D) when analysed by Southern hybridisation (shown in Appendix C). Unfortunately even after various attempts Southerns on the gDNA of *P. compacta* and *P. neriifolia*, the parental species of 'Carnival', did not yield clear results (data not shown). As the naturally occurring species *P. repens* contains more than one *ProLFY* copy in its genome it is likely that the parental species of 'Carnival' also do, although this would need to be confirmed. Paleopolyploidy, resulting from ancient polyploidy events, may have contributed to the multicopy nature of *ProLFY* in

the Proteaceae. This duplication event may have occurred before the divergence of Proteaceae and Platanus from the ancient plant order Proteales (Carpenter et al., 2005; von Balthazar and Schönenberger, 2009), as *P. racemosa* also contains multiple *ParaLFY* copies within its genome (Grimm and Denk, 2010). Alternatively, 'Carnival' could have retained biparental *ProLFY* copies obtained during hybridization resulting in polyploidy of the 'Carnival' genome and the presence of multiple homoeologous *ProLFY* alleles. The presence of homoeologous genes may result in the differential expression or silencing of genes (Adams and Wendel, 2005) as suggested for natural and synthetic tetraploid cotton (*Gossypium*; Adams et al., 2003) and hexaploid wheat (*Triticum*; Bottley et al., 2006). Polyploid plant species such as the natural allotetraploid *Nicotiana tabacum*, a hybrid of the diploid *N. sylvestris* and *N. tomentosiformis* have two *LFY* copies, *NFL1* and *NFL2* (Kelly *et al.*, 1995), indicating that chromosomal duplications may lead to multiple *LFY* copies in plant genomes. Similarly, domesticated apple (*Malus* x *domestica*) which has a polyploid genome as the Maloid family members have been reported to be polyploid (Harris *et al.*, 2002) has three copies of the *LFY* gene (Wada *et al.*, 2007). It is suggested that many plants are polyploid (Masterson, 1994) and both diploids and triploids were identified in populations of the Proteaceae species *Grevillea repens* in Australia (Holmes *et al.*, 2009). The polyploidy of 'Carnival' and its parental species, however, warrants further investigation and could easily be determined by cytogenetic studies such as those done by Félix and Guerra (2000) on orchids (spp. from the Cymbidioid phylad), Lynch et al. (1998) on *Lomatia tasmanica* and Rye (1979) on spp. of Myrtaceae. The additional *ProLFY* copies in the *Protea* genome may have diverged to fulfil different functions in reproductive or vegetative development of *Protea* as suggested for apple (Wada et al., 2007).

The arrangement of *Protea* flowers in the inflorescence, or capitula, are significantly different from those of simple flowers such as *A. thaliana*. As floral complexity is determined by the expression of homeotic, floral organ identity genes, under transcriptional control of LFY-like proteins (Hong *et al.*, 2003; Lohmann *et al.*, 2001; William *et al.*, 2004), *ProLFY* genes may be involved in controlling *Protea* inflorescence architecture. Involucral bract formation in two Asteraceae, gerbera (*Gerbera hybrida*) and *Dendrathema grandiflorum*, which have similar floral architectures to *Protea* is presumed to be controlled by orthologues of *APETALA1* (*AP1*; Ruokolainen *et al.*, 2010; Shchennikova *et al.*, 2004; Yu *et al.*, 1999). *AP1* in *A. thaliana* is integral in the

formation of sepals and petals (Yanofsky, 2001; Weigel and Meyerowitz, 1993) and *AP1* expression is transcriptionally controlled by LFY (Benlloch et al., 2011; Hamès et al., 2008; Parcy et al., 1998). *AP1* orthologues expressed under control of *Pro*LFY may also be involved in involucral bract formation of *Protea*. Roles for *ProLFY* genes in inflorescence development may be elucidated by investigation of their spatial and temporal expression from either cDNA or by *in situ* hybridisation in meristems at various stages of development. Alternatively, a method used by Song et al. (2008) to determine expression of homeotic genes in the different whorls of *S. tetraptera* flowers, which involved dissecting flowers into their various parts, may be used to study spatial *ProLFY* expression. Careful qRT-PCR amplification from cDNA generated from individually dissected structures may then be performed. As their nucleotide sequences share a high degree of homology (98.8 - 99.5%) designing primers/probed within the coding regions of these genes to differentiate their expression patterns may prove challenging. In this study, primers were successfully designed that preferentially amplify *ProLFY_WL4*, but a small degree of amplification was still observed when *ProLFY_D1* and *ProLFY_L5* clones were used as templates. Designing primers/probes to regulatory regions of *ProLFY* paralogues may be a better option as greater sequence dissimilarity is expected within these regions. In citrus, probes to the 5'UTR regions of three paralogous *FT* genes with high levels of sequence similarity were successfully designed to differentiate between them during expression studies (Nishikawa et al., 2007). However, these methods would require further investigation to ascertain their feasibility.

'Carnival' expresses multiple copies of *ProLFY*, however, these may not all function as transcription factors. Although none of the amino acid substitutions in the deduced amino acid sequences of the *Pro*LFY amino acid sequences corresponded to amino acid residues previously identified as essential for DNA binding (Fig. 4.5; Maizel et al., 2005; Hamès et al., 2008), their ability to bind to DNA would need to be established in DNA binding assays. Altered amino acid residues in close proximity to those identified as 'core' to functionality may result in a conformational change of the protein and subsequent reduced or altered activity. Deduced amino acid sequences of the paralogous *ProLFY* genes showed very high sequence similarity, especially in the C-terminal region involved in DNA binding (Fig. 4.4; Maizel *et al.*, 2005; Hamès *et al.*, 2008). However, they elicited different responses when heterologously expressed in *A. thaliana*. These differences may be attributed to amino acid differences localised to the

N-terminal regions of *Pro*LFY proteins, a region involved in transcriptional activation (Maizel *et al*., 2005; Hamès *et al*., 2008). Partial truncations of the LFY N-terminal region in *A. thaliana* was found to compromise protein dimerisation and the DNA binding efficiency, thus indicating that this region may also play a crucial role in protein function (Maizel *et al*., 2005; Siriwardana and Lamb, 2012 in press). This may suggest that other LFY targets, such as those proposed to be involved in leaf development (*NAM* and *CUC3*; Blein *et al*., 2008 and 2010), may not be dependent on the same amino acid residues for regulation. *Pro*LFY targets in *Protea* may also be regulated differently, and involve different interacting partners, from those in *A. thaliana* and therefore ectopic expression in *A. thaliana* may not provide a true reflection of *Pro*LFY function in *Protea*.

In 'Carnival', regulatory sequences in *ProLFY* promoter regions or the presence of other genes involved in *ProLFY* regulation may play key roles in controlling the spatial and temporal expression of the different *ProLFY* paralogues. During sequencing of the initial 34 clones, one *ProLFY* clone was identified that maintained the first intron sequence. The second intron sequence was absent in the nucleotide sequence, thus excluding DNA contamination as a possible reason for this observation. Retention of the first intron results in the presence of a premature stop codon and the proposed truncated peptide, 140 amino acids in length, would lack the conserved C-terminal DNA binding domain. Alternative splicing of this intron has also been reported in *A. majus* (Coen *et al*., 1990) and *A. thaliana* (Weigel *et al*., 1992), but these did not result in truncated proteins. During amplification of cDNA fragments derived from 'Carnival' meristematic tissue that spanned the first intron, the presence of the intron was also observed (data not shown). Differences in the levels of amplification of this alternatively spliced variant (intron containing fragment) compared to the archetypical variant from different staged meristems was also observed. This may suggest an important level of control during post-transcriptional processing of *ProLFY* pre-mRNA, but more experimental work will be required to confirm these findings.

4.5.1. Conclusion

Heterologous expression studies in *A. thaliana* suggested that *ProLFY* paralogues may function as meristem identity genes with roles in both floral and vegetative development. However, the multi-copy nature of *ProLFY* could also contribute to the complexity of the

Protea inflorescence. In *Protea*, *Pro*LFY proteins may be interacting with different homeotic genes and binding sites to regulate their transcription and ultimately floral organ formation. Further work on the interaction of *Pro*LFY proteins with homeotic genes and the transcriptional control of *ProLFY* paralogues in *Protea* by promoter analyses will be required to ascertain the role of these genes in inflorescence development. Identification of genes that may control *ProLFY* expression, including further investigation into the alternative splicing of the first intron, and identification of down-stream targets of *Pro*LFY may also provide valuable information towards understanding reproductive development of *Protea*. This information may also help elucidate factors that affect flowering times and inflorescence development of *Proteas.*

CHAPTER 5: OVERVIEW AND DISCUSSION

The variability in flowering times between *Proteas* suggests that numerous factors influence the initiation of flowering across this genus. In this study, three factors related to flowering of *Protea* were investigated: 1) the carbon required for inflorescence development, 2) seasonal induction of flowering through the floral integer *ProFT* and 3) subsequent floral development under control of the meristem identity gene *ProLFY*. This discussion proposes how these factors, and possible 'interplay' between them, may be involved in floral initiation and development in *Protea*, using 'Carnival' as an example. The development of an inflorescence-bearing shoot is discussed from the formation of the first seasonal flush until subsequent floral initiation on the fourth growth flush. Other factors that may influence the timing of flowering in *Proteas* such as genetic variation and resource requirements, subjects for future studies, are also discussed. And finally, a discussion of the management of these crop plants based on results from this study is presented.

Proposed model for growth and flowering of 'Carnival'

Inflorescence development in *Protea* was shown to be a carbon expensive venture (Table 2.1; Fig. 2.3) and therefore, ideally, the timing of flowering needs to coincide with favourable conditions for photosynthesis to ensure a constant carbon supply during inflorescence development. To achieve this, plants need to be able to anticipate when favourable conditions will be occurring so that flowering is initiated and completed during these periods. Plants utilise an endogenous time keeper, the circadian clock, which through interactions and complex cross-talk with members of the floral inductive pathways controls flowering in response to seasonal change (Ibáñez *et al*., 2010).

Common practice in 'Carnival' cultivation is to prune plants biennially during the winter months. This ensures synchronised shoot growth and maximises the number of flowers at harvest (Gerber *et al*., 1995). Re-sprouting from pruned stems, termed bearers, is initiated from auxiliary buds early in spring, and the first growth flush extends and matures during this season (Fig. 5.1 A and B). The carbohydrates required for the formation of these shoots and leaves, are presumably provided from stored reserves due to the absence of photosynthetically active source leaves.

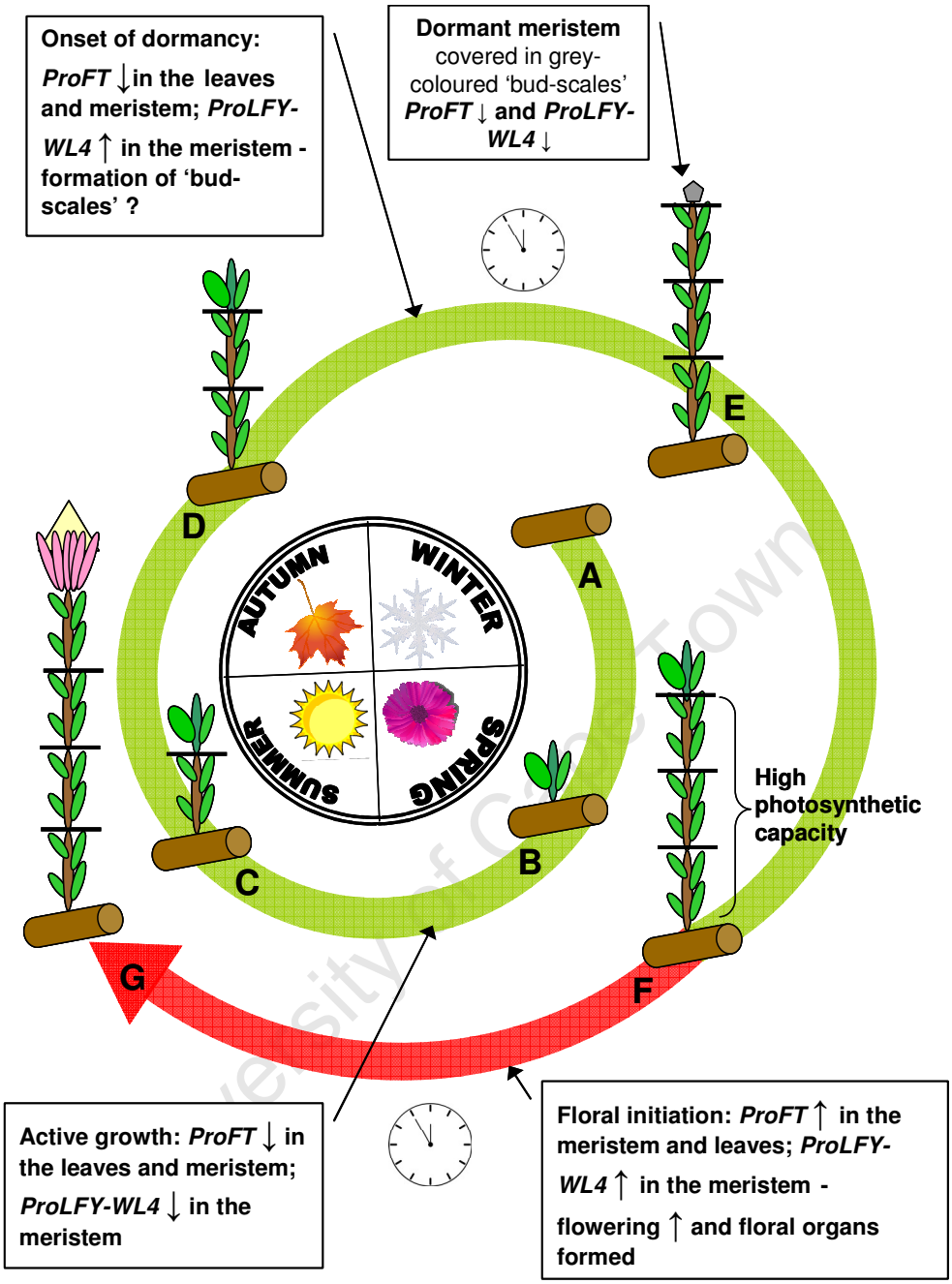


Figure 5.1. Proposed model of *Protea* hybrid 'Carnival' (*P. compacta* x *P. neriifolia*) growth and development. A-G refers to developmental stages mentioned in text. Floral initiation is proposed to be dependent on by both endogenous factors such as carbon availability and assimilatory capacity, as well as environmental factors such as day-length. Development is discussed in relation to the floral inducer, *ProFT*, and the meristem identity gene, *ProLFY* in text. The clocks indicate phase transitions which are controlled by the circadian clock. ↑ indicates increase and ↓ indicates decrease.

Although carbon storage in 'Carnival' has not been investigated, other *Proteas* such as *P. cynaroides* are known to store carbon reserves in lignotubers to facilitate re-growth after the destruction of above ground tissue by fire (Stock *et al.*, 1992; Rebelo, 2001). After the maturation of the spring flush the shoot develops two more seasonal flushes in summer and autumn (Fig. 5.1 C and D), before the shortening of day-length signals the approaching winter conditions. During this period of active growth the expression levels of the floral inducer, *ProFT*, were low in both the leaves and meristem (Fig. 4.5), while expression of the meristem identity gene *ProLFY-WL4* remained low in the meristem (Fig. 4.11 B). The shoot develops a greater photosynthetic capacity as every seasonal growth flush matures and is able to contribute photosynthate to the next developing structures.

Shortening day-length and reduced temperature at the onset of winter is perceived by the circadian clock (Ibáñez *et al.*, 2010), and the meristem initiates bud set to prepare it for dormancy. The transition to dormancy in woody perennial plants is thought to be facilitated through the *CO*/*FT* module (Böhlenius *et al.*, 2006; Gyllenstrand *et al.*, 2007; Holliday *et al.*, 2008; Horvath *et al.*, 2008). In 'Carnival', *ProFT* expression was low at this stage of development, similar to the expression levels reported for *FT* orthologues in leafy spurge (*Euphorbia esula*) buds during the onset of dormancy (Horvath *et al.*, 2008). As the meristem prepared for dormancy there was an increase in the expression levels of *ProLFY-WL4* (Fig. 4.11 B), suggesting that *ProLFY-WL4* may play a role in the formation of 'leaf-like' 'bud-scales' that protect the SAM during the winter period (Fig. 5.1 E). 'Bud scales' have a similar appearance to sepals and *ProLFY-WL4* may therefore interact with genes orthologous to *APETALA1* and *APETALA2* to facilitate their formation. *ProLFY-WL4* induced early flowering and 'rescued' the reduced fertility of the *lfy-2* mutant when heterologously expressed in *A. thaliana* (Fig 4.8 J; Fig. 4.9 B; Fig. 4.10 B; Table 4.2), suggesting that it functioned as a meristem identity gene in 'Carnival'. During the onset of dormancy, *ProFT* expression was low (Fig. 3.5) and therefore the observed up-regulation of *ProLFY-WL4* expression was presumably not as a result of induction through the normal floral inductive pathway which includes *FT*. Alternatively, expression of *ProLFY-WL4* at this developmental stage may be regulated by *Protea* orthologues of genes such as *LUMINIDEPENDENS* (Aukerman *et al.*, 1999), *CLAVATA1* (Clark *et al.*, 1993) or *WUSCHEL* (Laux *et al.*, 1996) that also regulate *LFY* expression in *A. thaliana*. As the primordia of the following growth flush are already pre-

formed in the meristem during dormancy (Gerber *et al*., 2001a), *ProLFY-WL4* expression is unlikely to induce flowering at this developmental stage. The meristem remains in a dormant state until the onset of spring at which time growth resumes.

After a sufficient period of cold exposure (Perry, 1971; Rohde and Bhalerao, 2007) as the day-length and temperature starts to increase during spring, dormancy is released and the pre-formed spring flush extends (Fig. 5 F). In poplar (*Populus tremula* x *Populus tremuloides*) the release of dormancy has been found to be under control of the circadian clock (Ibáñez *et al*., 2010). At this time, 'Carnival' shoots have a large photosynthetic capacity carrying three mature growth flushes with photosynthetically active source leaves. The increased photoperiod during the extension of the spring flush is perceived as the floral inductive signal in the leaves, presumably through the circadian clock and its interaction with a *CO* orthologue (Schaffer *et al*., 1998; Wang and Tobin, 1998; Green *et al*., 2002). Evidence that a CO orthologue in 'Carnival' may be mediating the increase in *ProFT* expression during lengthening photoperiodic, is that the peak in expression *ca*. 3 h after dusk is similar to that of *FT* up-regulated by light-stabilised CO protein in *A. thaliana* (Jackson, 2009). The formation of floral organs in the SAM coincides with high *ProLFY-WL4* expression, possibly up-regulated by *Pro*FT and its interacting partners. The initiation of flowering during the spring ensures that the shoot has a high photosynthetic capacity and conditions are suitable for carbon assimilation, thus guaranteeing a continual carbon supply throughout inflorescence development.

Although seasonal flowering is observed in 'Carnival', this does not apply to all *Proteas*. Species and hybrids of this genus display very diverse flowering times and some may even flower year-round (Gerber *et al*., 2001c). This suggests that factors other than photoperiod may also be involved in floral initiation of *Proteas*.

Other factors that may influence flowering time of *Protea*

To ensure successful pollination, seed production and thus reproductive success; plants need to flower at a suitable time for the environment that they inhabit. As plants have co-evolved with their pollinators, the timing of flowering is often correlated to periods when pollinators are most active (Mosquin, 1971; Ollerton and Diaz, 1999; Forrest and Thomson, 2009; Sandring and Ågren, 2009). Species which are contrained to time their

flowering to coincide with pollinator activity seem to have a narrow flowering window, and the variation that does occur will simply be slightly earlier or later in the same season (Hurlbert, 1970; Berjano *et al.*, 2009). Many of these 'tightly' fixed flowering species also occur in more extreme latitudes whereas Mediterranean climates, in which *Protea* grow, offer more suitable climatic conditions to support a broader range of flowering periods. *Proteas*, such as those cultivated as cut-flowers, carry solitary inflorescences on long stems and are mostly bird pollinated (Coetzee and Littlejohn, 2001). As birds will feed on nectar throughout the year (Cronk and Ojeda, 2008) pollinator activity would be less seasonal than for other pollination types. Flowering may therefore rather depend on the availability of other resources, such as nutrients and water, required for seed production.

The distribution of *Protea* spp. is predominantly confined to the Cape Floristic Region (CFR) of South Africa spanning 87892 km^2 of the southernmost tip of Africa (Cowling and Heijnis, 2001). Some species inhabit only a very narrow and clearly defined area, while others are broadly distributed across this area (Rebelo, 2001). Using data collated from the Protea Atlas Project (Rebelo, 2001) we found a significant Pearson's correlation ($r=0.69$; $r^2=0.48$; $P<0.001$; $n=66$) between the duration of flowering (in months) and the distribution range (the area in km^2) occupied by the species. This suggested, that species able to adapt to a broader environment may be able to do so because their flowering times are not as constrained as in the more narrowly distributed species. There is a significant east-west gradient of rainfall seasonality and soil composition across the CFR (Campbell and Werger, 1988). While the soils in the east are less nutrient poor than that of the west, the east has lower rainfall in the winter months than the west. These factors may contribute to the spatial distribution of *Protea* spp. with different flowering strategies.

Most Proteaceae inhabit nutrient poor soils (Cowling and Holmes, 1992; Groom and Lamont, 2010), yet large quantities of nutrients are loaded into seeds to ensure reproductive success (Kuo *et al.*, 1982; Stock *et al.*, 1990; Witkowsky and Lamont, 1996; Henery and Westoby, 2001; Groom and Lamont, 2010). The uptake and transport of nutrients by mass flow and diffusion in plants depends on water availability (for examples see Cramer *et al.*, 2009; Šimůnek and Hopmans, 2009). *Protea* spp. may therefore time flowering so that seed filling occurs during the period with the highest

rainfall. This seems to be true for *P. repens* and *P. neriifolia* with broad distributions across the CFR. Flowering time in the east coincides with the spring and summer rainfall months (September to March) and in the west flowering occurs during the autumn and winter rainfall months (May to October; Rebelo, 2001). The timing of flowering in plants has also been correlated to water availability in other species (Sivaraj and Krishnamurthy, 1989; Walker *et al.*, 1995; Prieto *et al.*, 2008). As water is also required for carbon assimilation (Chaves, 1991; DaMatta *et al.*, 2002; Medrano *et al.*, 2003), water availability may also influence the carbon available for inflorescence development. A link between carbon assimilation and nutrient availability has also been established in many plant species (Milewski, 1983; Karlsson, 1994; DaMatta *et al.*, 2002; Kanai *et al.*, 2007). *Protea* spp. may therefore initiate flowering through the integration of various signals, and the ability of these plants to respond to the various flowering stimuli may determine their distribution and flowering time.

The ability of plants to respond to different flowering stimuli is dependent on their genetics. Variation in flowering times between species within the same genera, as observed for *Protea*, has been widely reported (Hurlbert, 1970; Koornneef *et al.*, 2004; Zhao *et al.*, 2009). Most angiosperms (70%) have undergone polyploidy during their evolutionary histories (Masterson, 1994). This is believed to be the greatest contributing factor to speciation in angiosperms (reviewed by Soltis, 2009). Polyploidy leads to multiple copies of homologous genes, including flowering-related genes, within the genomes of plants. These multiple copies may follow any of the following routes: 1) retain the same function as the homologous gene, 2) diverge and acquire new function or, 3) lose their functions and become pseudogenes (Axelsson *et al.*, 2001). Polyploidy has not been established for *Protea* but in another Proteaceae genus, *Grevillea*, naturally occurring polyploid species have been reported (Holmes *et al.*, 2009). The multi-copy nature of *ProLFY* in 'Carnival', 'Sylvia' and *P. repens* also suggests that *Proteas* may be polyploids. This may explain why *Proteas* have such diverse flowering times, as polyploidy and hybridisation would presumably result in multiple quantitative trait loci (QTLs) for flowering related genes.

In fast-cycling plants such as *Brassica* spp., progeny from compatible parental species allowed to self fertilise, known as recombinant inbred lines (RILs), are often generated to identify QTLs that are important in the flowering response (Osborn *et al.*, 1997;

Koornneef *et al.*, 2004). A large variation in flowering time was observed between RILs generated from crossing early flowering male parent plants to late flowering female parent plants of three different *Brassica* spp. (Axelsson *et al.*, 2001). In the study by Axelsson *et al*. (2001) and an earlier study by Bohuon *et al.* (1998) the QTL responsible for the flowering time observed mapped close to *CO*, which encodes a protein critical for photoperiodic responsive flowering. A similar broad flowering response was observed for RILs generated from two parental *A. thaliana* accessions, Cvi from the Cape Verde Islands and a European accession Landsberg *erecta* (L*er*), which display very differing flowering times under various environmental conditions. The flowering times of the RILs could however, be synchronised to a much narrower period after a vernalisation treatment (Alonso-Blanco *et al.*, 1998). This suggested that more than one QTL is involved in the flowering response of the parental accessions and that the segregation of these QTLs in RILs results in a diverse flowering response. Most plants will harbour several QTLs involved in flowering time. Based on the diversity of flowering time across the *Protea* genus, this seems to be true for *Protea* as well. Different combinations of these QTLs in hybrid cultivars may have resulted in the diversity of flowering times observed across the progeny.

Management of *Protea* production - suggestions from this study

The *Protea* floriculture industry in South African is more than 50 years old, but *Protea* flowers are still only produced as a minor crop. The potential for expansion of this industry hinges on a constant supply of high quality flowers to the international market. To date, most efforts aimed at increasing productivity have been focused on breeding programs, which may take up to 10 years before the release of a cultivar (Reinten and Coetzee, 2002), as well as the refinement of cultivation practices on already available cultivars. Although breeding programs have succeeded in the development of cultivars with aesthetically pleasing flowers, very little is known about the genetic and physiological limitations to flowering time in these hybrids. As discussed above, nutrients and water may play a role in flowering time of wild grown *Protea* spp. but these may not be determining factors for flowering in a cultivated setting, as the plants are often irrigated and fertilised (e.g. Hawkins *et al.*, 2007).

Commercially attractive *Protea* flowers with traits suitable for cut-flower production are selected by breeders from progeny generated after hybridisation. As suggested above,

Protea may harbour many QTLs that influence flowering time across the genus. Within the same hybrid population an array of flowering responses may therefore be present. All-year-round flowering, a desired flowering trait, was co-selected with the aesthetically pleasing 'Sylvia' flower. Unfortunately, together with its parental species *P. eximia* that also displays year-round flowering, it suffers from a post harvest problem known as 'leaf blackening' (Bieleski *et al.*, 1992). The blackening of leaves during transport reduces the value of the cut-flower and has been correlated to low carbohydrate levels in cut, inflorescence-bearing shoots (Bieleski *et al.*, 1992; Jones *et al.*, 1995). In this study, the carbon cost of inflorescence development was assessed in a number of *Proteas*, including 'Sylvia'. 'Sylvia' plants were found to be carbon limited, presumably due to the all-year-round development of large, carbon-expensive inflorescences. Reducing the carbon demand during the flowering period by either removing vegetatively developing shoots or some floral shoots may increase the carbon status of the cut flowers. This may prevent leaf blackening of cut shoots during storage, a practice that can also be applied to other cultivated species and hybrids that suffer from leaf-blackening.

Application of 6-benzyladenine (BA) has been suggested as a tool for manipulating flowering time of 'Carnival' (Hoffman *et al.*, 2009). BA, at a concentration of 500 mg ml^{-1}, 'painted' on the apical meristems at the developmental stage termed 'buds set' (between Fig. 5.1 D and E) caused an 'out-of-season' winter flush to develop. These manipulated shoots subsequently developed inflorescences two months earlier than untreated shoots. BA application has broadly been practiced in horticulture and floriculture to increase fruit size and induce flowering (Wismer *et al.*, 1995; Stern and Fleishman, 2003; Blanchard and Runkle, 2008; Ishimori *et al.*, 2009). The response observed after BA treatment may be a consequence of its ability to facilitate the movement of photosynthate to developing sink tissues (Quilan and Weaver, 1969; Ogawa and King, 1979; Corbesier *et al.*, 2003). At the time of BA application proposed by Hoffman *et al.* (2009) the leaf primordia of the next seasonal growth flush are already preformed in the apical meristem of 'Carnival' (Gerber *et al.*, 2001b). Rather than directly inducing flowering it is therefore more likely that BA acts to prevent the onset of dormancy, and therefore advance subsequent vegetative flush formation. A similar 'out-of-season' flushing response and subsequent early flowering was observed in this study due to warmer weather in the winter of 2007. This may have been as a consequence of more favourable carbon assimilatory conditions during the onset of dormancy. Growers

need to be very cautious when applying BA. Inducing too many shoots to 'flush' will place a tremendous carbon demand on shoots during the winter period when photosynthetic productivity is low, resulting in an unfavourable cost-benefit for the plant. This may have adverse effects on growth and flowering in the next season as well as the quality of the cut-flowers produced. Selecting a few suitable shoots for BA treatment with sufficient leaf area to support inflorescence development photosynthetically, even under the low light conditions experienced in winter, may be key to the development of good quality inflorescences for the cut-flower market. This will also ensure that sufficient carbon is available for continued vegetative growth and support of the next season's crop.

Conclusion

Currently, research information on *Protea* is mostly limited to conference proceedings and popular articles. Very few studies have been published as 'peer-reviewed' scientific articles. The *Protea* industry can only reach its full potential through a better understanding of physiological and molecular factors that influence flowering. These findings can then be integrated with breeding and cultivation efforts to increase the productivity of the *Protea* industry. Furthermore, research into flowering times of wild-growing *Protea* spp. may provide information on genetic resources available for cultivation and help advise management towards the conservation of these unique species in a changing environment. This study has opened the door to demystifying the induction of flowering in *Protea*.

Appendix A: Generally used techniques

General laboratory chemicals

All chemicals used in this study were of AR grade or molecular biology quality and purity. Reagents and chemicals were prepared with either distilled or millipore quality (resistance, 10 MΩ-cm) water as appropriate.

Microbial strains and general microbiological technique

Sterile technique was followed for all microbiological work.

Escherichia coli *DH5α cells, transformation and culturing conditions*

Escherichia coli DH5α cells were used for routine transformations. *E. coli* competent cells were prepared with the calcium chloride method and transformed as described by Sambrook and Russell (2006). *E. coli* DH5α cells transformed with the pJET1.2 (Fermentas) vector harbouring fragments of interest were cultured either in liquid Luria-Bertani (LB) medium or on LB solid media containing 1.5% (w/v) agar both supplemented with ampicillin (100 µg ml^{-1}) at 37℃.

Agrobacterium tumefaciens *cells, transformation and culturing conditions*

Agrobacterium tumefaciens strain GV3101 (Holsters *et al.*, 1980) was used for *Arabidopsis thaliana* floral-dip transformation (Clough and Bent, 1998). To prepare competent *A. tumefaciens* cells, 2 ml of a 10 ml overnight culture grown at 30℃ in LB supplemented with 0.5 g ml^{-1} $MgSO_4$, 100 µg ml^{-1} rifampicin and 15 µg ml^{-1} gentamycin were used to inoculate 50 ml LB containing 0.5 g ml^{-2} $MgSO_4$ but no antibiotics. Cultures were incubated with agitation at 30℃ until their optical density at 600 nm were between 0.5 and 0.6 (7 - 8 h) and thereafter incubated on ice for 10 min. Cells were transferred to 50 ml Beckman centrifuge tubes and centrifuged at 3 500 xg for 5 min at 4℃. Pelleted cells were re-suspended in 1 ml 20 mM $CaCl_2$ and the cell suspension aliquoted in 200 µl volumes into Eppendorf tubes. Tubes were flash frozen in liquid nitrogen and stored at -80℃ until required for transformation.

Two µg plasmid (pEarlyGate201*ΔccdB* harbouring *ProLFY* paralogues, *AtLFY* or the empty vector) was added to 200 µl cells and allowed to defrost on ice. Cells were refrozen for 5 min in liquid nitrogen and thawed in a 37℃ waterbath for 5 min. One ml

LB was added to the recovering cells and cells incubated at 28℃ for 2-4 h. Cells were pelleted by centrifuging at 3 500 xg for 5 min and plated on LB media supplemented with 100 µg ml^{-1} rifampicin, 15 µg ml^{-1} gentamycin and 50 µg ml^{-1} kanamycin and cultured at 30℃ for selection of transformants.

Kits for gel- and plasmid purification and PCR reaction clean-up

Kits used in this study were purchased from the following companies:

Qiagen, Hilden, Germany

PeqLab, Erlangen, Germany

BioFlux, Tokyo, Japan

Vacutec, Johannesburg, South Africa

Invitec, Berlin, Germany

Appendix B: Supplementary information to chapter 3

Optimisation of cycle number for semi-quantitative PCR analyses of ProFT

The cycle number representing the linear phase of PCR amplification of *ProFT* and the reference gene *eIF4A* was determined using the PCR conditions described in 3.3.5. cDNA derived from the 22:00 leaf sample from October, found to have the highest *ProFT* expression in preliminary analyses, was used as template in the PCR. Tubes were removed from the thermal cycler at 30, 32, 33, 34, 35, 36, 37 and 38 cycles for *ProFT* and 28, 30, 32, 33, 34, 35, 36 and 38 cycles for *eIF4A*. The final 7 min extension step at 72°C was performed in a waterbath.

PCR products were electrophoresed, stained with ethidium bromide and visualised as described in 3.3.5. The intensities of bands were determined using Quantity One 1-D imaging and analysis software (version 4.6.3, Bio-Rad). Fig. B1 shows the intensities of the PCR bands plotted against cycle number and representative agarose gels are shown below. All reactions were performed in duplicate and data depict the average of the two replicates. Cycle numbers chosen for the respective amplification of *ProFT* and *eIF4A* were 35 (Fig. B1 A) and 33 (Fig. B1 B) and are indicated by dashed lines on the Fig. To confirm the suitability of these cycle numbers for semi-qPCR, PCRs with these cycle numbers were performed with increasing volumes of template cDNA. Trendlines fitted to data from these PCRs showed a near linear increase in band intensity with increasing template, confirming the suitability of these cycle numbers for semi-qPCR (Fig. B2 A and B)

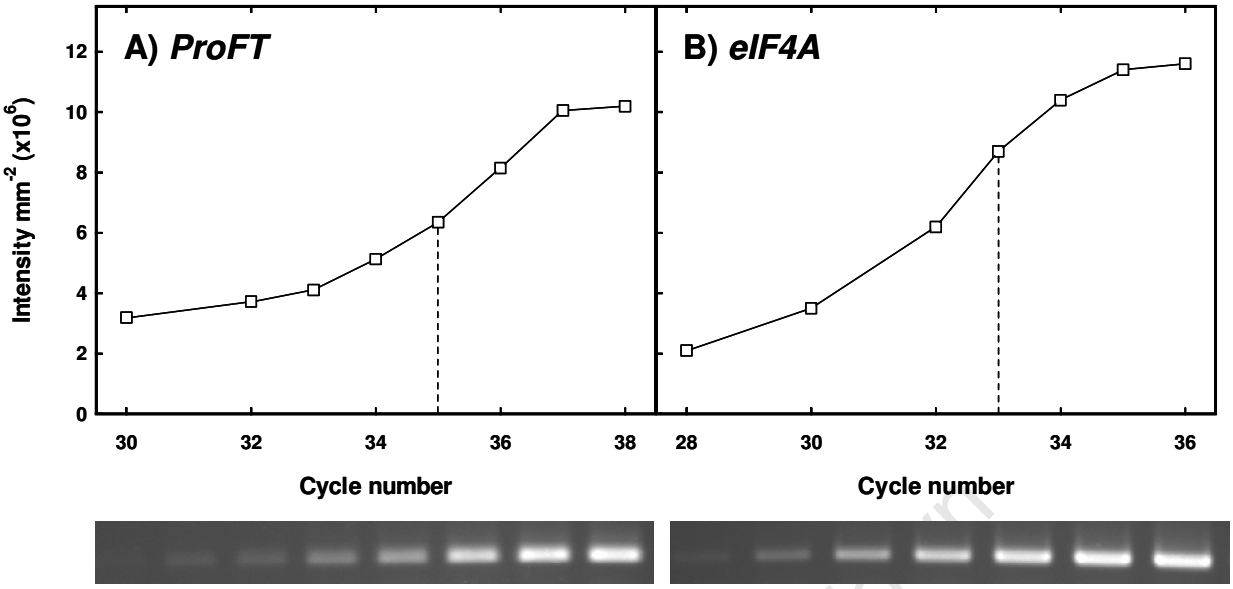


Figure B1. Cycle number optimisation of *ProFT* (A) and *eIF4A* (B) for semi-quantitative PCR. Tubes were removed from the thermal cycler at the indicated cycle numbers and the final extension at 72°C was performed in a waterbath. Dashed lines indicate the cycle number identified for semi-quantitative

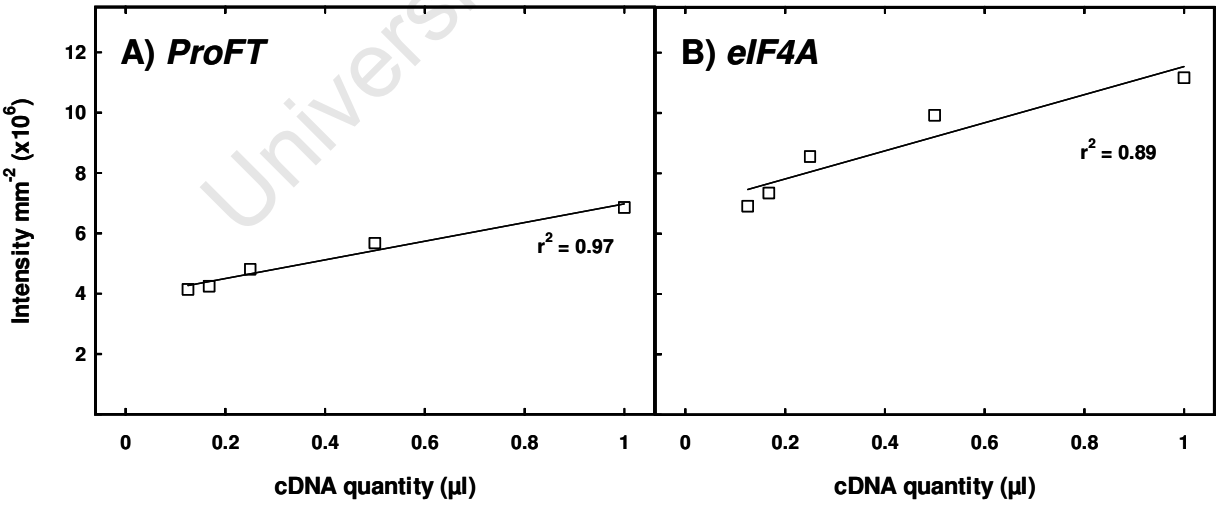


Figure B2. Increasing cDNA volumes at the cycle numbers chosen for semi-quantitative PCR of *ProFT* (A) and *eIF4A* (B).

Quantitative real-time PCR optimisation

All quantitative real-time PCR (qRT-PCR) reactions were performed in duplicate and the variation between duplicate samples was 0.33 ± 0.07% for the reference gene (*eIF4A*) and 1.22 ± 0.32% for the gene of interest *ProFT*. Average qRT-PCR efficiencies were calculated using LinRegPCR (Ramakers *et al.*, 2003). Fig. B3 shows a melt curve of *ProFT* and *eIF4A* amplified from a mixture of cDNA samples. The other lines indicate the absence of amplification in the 'no reverse transcriptase' and 'no template' controls for each primer set. qRT-PCR the diurnal expression of *ProFT* in the leaf samples were performed and analysed as described in 3.3.5. Results are shown in Fig. B4.

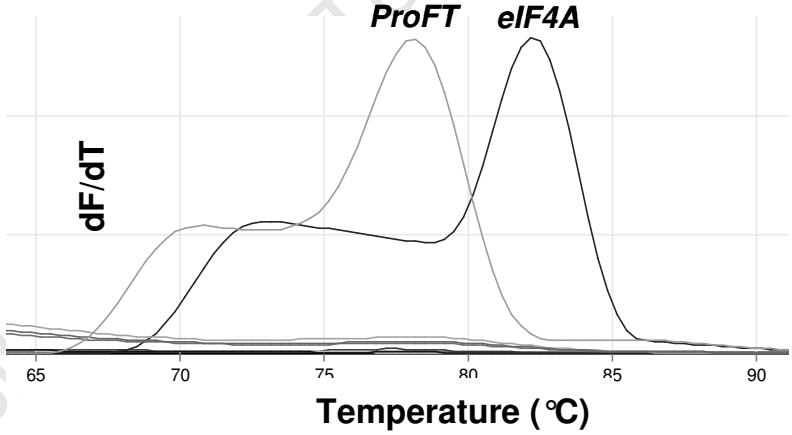


Figure B3. Melt curves of *ProFT* and *eIF4A* products. The lines without products indicate the lack of amplification in the negative and non-reverse transcribed controls.

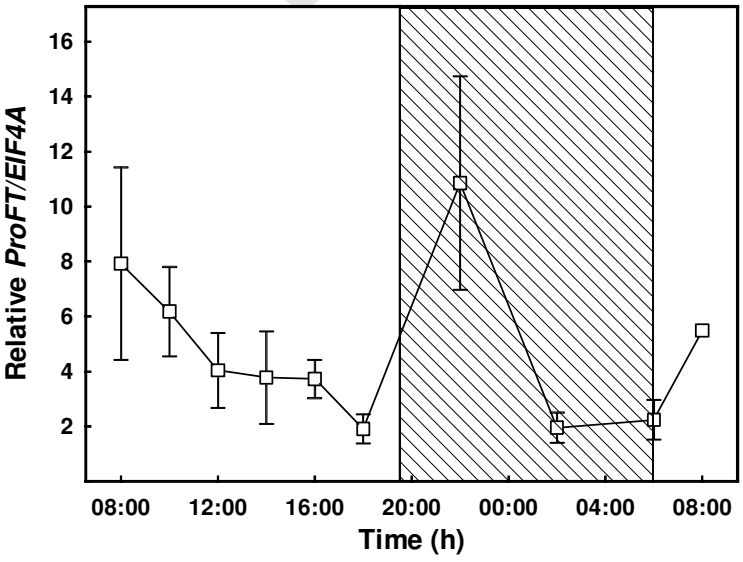


Figure B4. Quantitative real-time PCR of *ProFT* diurnal expression in leaves during October. Hashed areas of the graph indicate the dark period based on time of sunrise and sunset for the date of sampling. *eIF4A* was used as reference gene for normalisation.

Appendix C: Supplementary information to chapter 4

***Optimisation of cycle number for semi-quantitative PCR analyses of* ProLFY-WL4**

The cycle number, representing the linear phase of PCR amplification, suitable for semi-qPCR was determined using PCR conditions described in 4.3.11. For the gene of interest, *ProLFY-WL4*, FB (floral meristem) cDNA was used as template and for the reference gene, *eIF4A*, a mixture of cDNA samples derived from meristem tissue was used. For amplification of *ProLFY-WL4*, tubes were removed from the PCR cycler at 25, 28, 30, 32, 34, 36, 38 and 40 cycles and for *eIF4A* tubes were removed at cycle numbers 22, 25, 27, 29, 31, 33, 35 and 37. The final 7 min extension at 72°C was performed in a waterbath. PCR products were electrophoresed, stained with ethidium bromide, visualised and analysed as described in 4.3.11. Fig. C1 shows the intensities of the PCR bands on the agarose gels for *ProLFY-WL4* (A) and *eIF4A* (B) respectively, and the cycle number chosen for semi-quantitative PCR is indicated. All reactions were performed in duplicate and data is the average of the two replicates. The suitability of these cycle numbers for each product was confirmed as the intensity of product staining with increasing volumes of template cDNA was linear (Fig. C2 A and B).

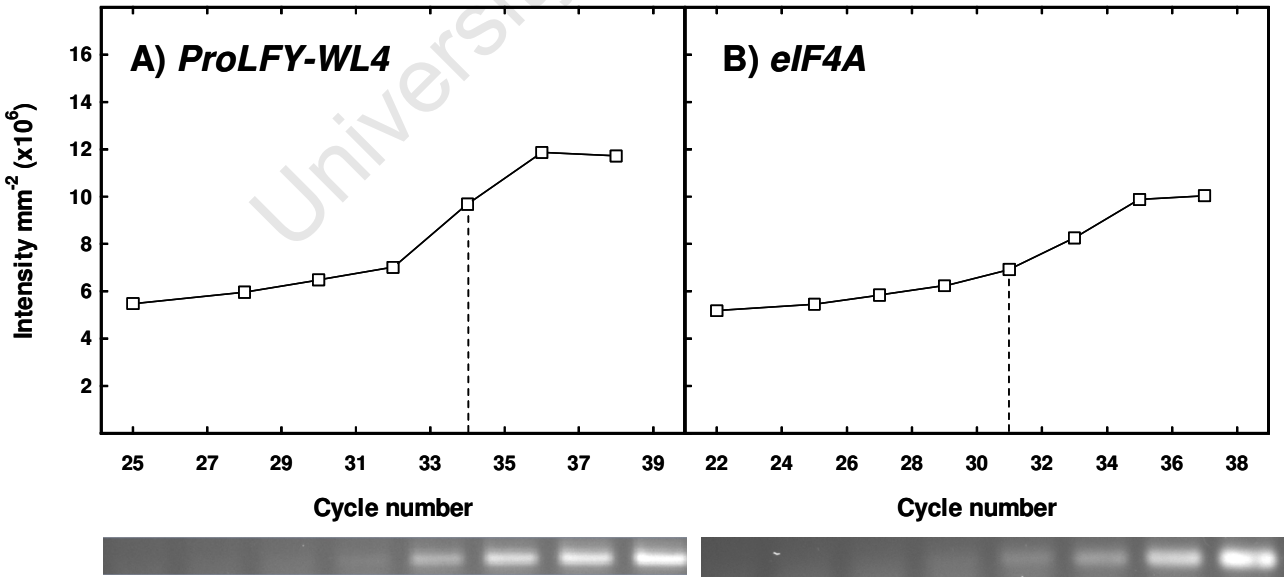


Figure C1. Cycle number optimisation of semi-quantitative PCR of *ProLFY-WL4* (A) and *eIF4A* (B). Dashed lines indicate the cycle number chosen from expression analyses.

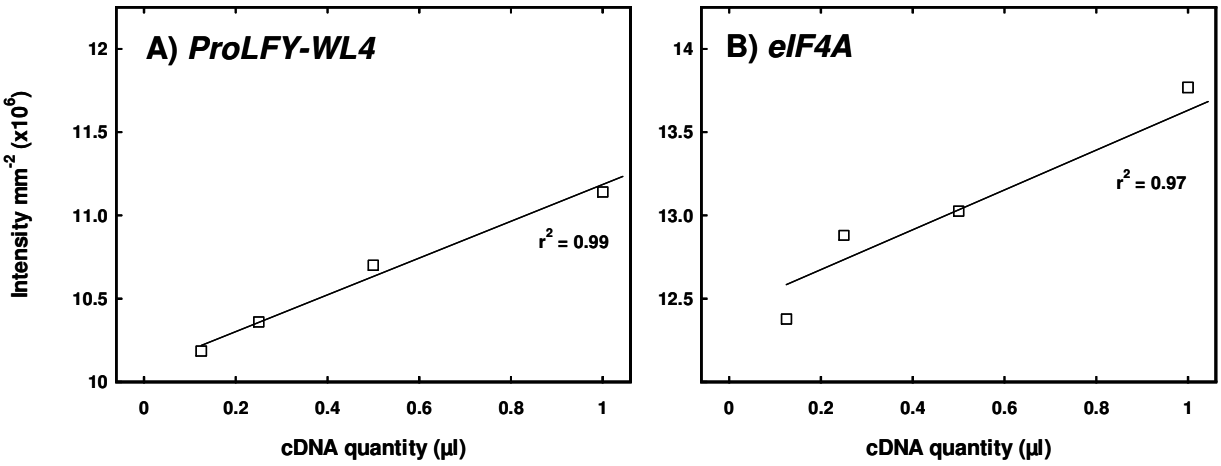


Figure C2. Increasing cDNA volumes at the cycle numbers chosen for semi-quantitative PCR of *ProLFY-WL4* (A) and *eIF4A* (B). cDNA derived from florally determined meristems (FB) were used as template for the optimisation of *ProLFY-WL4* whereas a mixture of cDNA from meristematic tissue was used for optimisation of *eIF4A*.

Southern analysis of* A. thaliana*, 'Sylvia',* P. repens *and 'Safari sunset'

Genomic DNA, 10 µg, from *A. thaliana*, 'Sylvia' (*P. eximia* x *P. susannae*), *P. repens* and 'Safari sunset' (*Leucadendron laureolum* x *Lcd. Salignum*) was restricted with *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I, *Xba*I and *Xho*I. Electrophoresis, denaturing, neutralising and blotting of restricted DNA was performed as described in 4.3.3. Hybridisation was performed with the 450 bp *ProLFY* fragment shown in Fig. 4.1 and labelled as described in 4.3.3. Hybridisation, washing and detection conditions were as given in 4.3.3.

Southern analysis confirmed the presence of one *LFY* copy in the *A. thaliana* genome (Fig. C3 A). Only restrictions from *Eco*RI and *Eco*RV yielded clear hybridisation bands and are indicated with arrows on the figure. Multiple hybridisation bands were visible on Fig. C3 showing 'Sylvia' (B), *P. repens* (C) and 'Safari sunset' (D) blots, suggesting the presence of multiple *ProLFY* copies.

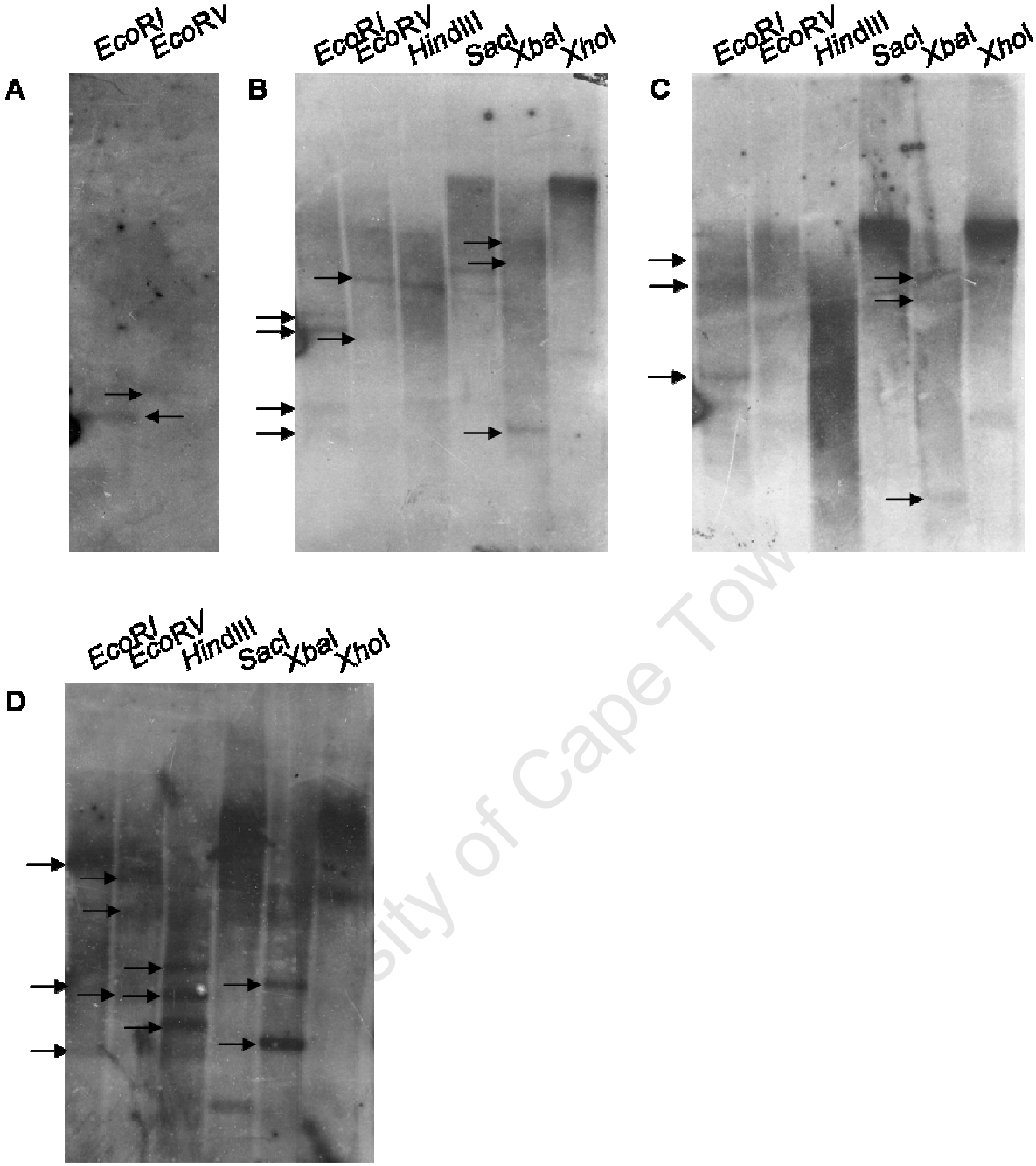


Figure C3. Southern hybridisation of gDNA from *A. thaliana* (A), *Protea* cultivar 'Sylvia' (B), *P. repens* (C) and *Leucadendron* cultivar 'Safari sunset' (D). Genomic DNA was restricted with either *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I, *Xba*I or *Xho*I**.** Restricted gDNA, immobilised on a N^+ hybond membrane (Amersham), was probed with the radioactively labelled (α-^{32}P[dCTP]) 450 bp sequence of *ProLFY* previously amplified from floral meristematic cDNA (Fig. 4.1). Hybridisation was visualised by exposing the probed membrane to x-ray film at -80℃ for 7 days. Arrows indicate bands of interest.

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