# PHYSIOLOGY OF FLORAL INDUCTION IN *PROTEA* spp

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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 at any university for a degree.

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# SUMMARY

The aim of this study was to elucidate the control of flowering in *Protea* spp. The main factor that makes studying flowering in this diverse genus so challenging is the fact that most *Protea* spp. and their commercial hybrids have very dissimilar flowering times. The carbon input into floral organ formation and support is expensive as flowers from *Protea* spp are arranged in a very large 'flowerhead' (50 mm by 130 mm for 'Carnival') that can take up to two months to develop fully. Therefore the carbon needed for structural formation, metabolic respiration and the sugar-rich nectar production make these structures extremely expensive to form and maintain. *Protea* is a sclerophyllous, woody perennial shrub with a seasonal flush growth habit. The leathery leaves (source tissue) produce most of the carbon needed for support and growth of the new leaves, roots and flowers (sink tissue). In the case of expensive structures, such as the inflorescences, remobilization from stored reserves, probably from underground storage systems, can be observed for structural development and maintenance. At all times the flush subtending the apical meristem or florally developing bud provides the largest proportion of carbon for support of the heterotrophic structures. *Protea* apical meristems stay dormant during the winter months, but BA (benzyl adenine, 6-benzylaminopurine) application to the apical meristem of the Protea hybrid 'Carnival' has shown to be effective in the release of dormancy and subsequently shift flowering two months earlier than the natural harvesting time. BA is thought to shift source/sink relationships by stimulating the remobilization of carbon to the resting meristem. Although no direct evidence was found for this in our assay, possible reasons for a weak assay are discussed. This study combined physiological research with the use of molecular tools. An homologue of the *Arabidopsis thaliana* meristem identity gene, *LEAFY,* was identified in Proteaceae. *PROFL* (***PRO****TEA* ***F****LORICAULA* ***L****EAFY*) is expressed in both vegetative and reproductive meristems as well as leaves. *PROFL* expression in leaves may have an inhibitory effect on vegetative growth, as the expression was high at the same time as the expression in the apical meristem increased marking the transition to reproductive growth. In perennial species such as *Protea,* the availability of carbon is thought to be the main factor controlling floral development. Possible mechanisms of control may be through the direct control of meristem identity genes such as *PROFL* through sugar signaling. BA did not have a direct effect on *PROFL* expression although the expression pattern was one month in advance when compared to the natural system. *PROFL* expression seems to be consistent with that found for other woody perennial species and would therefore be a convenient marker for floral transition.

# OPSOMMING

Die doel van hierdie studie was om die inisiëring van blomvorming in *Protea* spp. te ondersoek. Die verskil in blomtyd tussen *Protea* spp. en hul kommersieel ontwikkelde hibriede maak die studie van hierdie genus ‘n groot uitdaging. Die groot hoeveelheid koolstof wat benodig word vir blomvorming in Protea is hoofsaaklik as gevolg van die grootte (50 mm by 130 mm vir ‘Carnival’) van die blomkop waarin individuele blomme geranskik is. Hierdie blomkoppe kan tot 2 maande neem om volwassenheid te bereik. Die koolstof benodig vir strukturele ontwikkeling, metaboliese respirasie en produksie van suiker-ryke nektar maak die vorming van hierdie structure ongelooflik duur. *Protea* is ‘n bladhoudende, houtagtige bos met ‘n seisoenale groeipatroon. Die leeragtige blare voorsien die grootste hoeveelheid koolstof vir die ontwikkelende blare, wortels en blomme. Koolstof vir die ontwikkeling en ondersteuning van die groot stukture soos die blomkoppe word gedeeltelik deur die huidige fotosinfaat voorsien en bewyse vir die remobilisasie van gestoorde koolstof, heel waarskynlik vanaf ondergrondse stukture, is gevind. Die blare van die stemsegment wat die apikale meristeem of ontwikkelende blom dra, voorsien altyd die grootse hoeveelheid koolhidrate aan die ontwikkelende struktuur. Die apikale meristeme van *Protea* bly dormant gedurende die winter maande, maar applikasie van BA (bensieladenien, 6-bensielaminopurien) aan die apikale meristeme van die *Protea* hibried ‘Carnival’ verbreek dormansie en die blomtyd van hierdie gemanipuleerde plante is daarom twee maande vroeër as die natuurlike oestyd. Daar word gespekuleer dat BA applikasie aan die apikale meristeem die hoeveelheid koolstof wat na die dormante meristeem gestuur word verhoog wat dan die dormansie verbreek. Hierdie studie beproef ongelukkig hierdie hipotese swak en redes hiervoor word bespreek. In hierdie studie word fisiologiese analises met molekulêre studies gekombineer. ‘n Meristeem identiteits gene wat homologie wys met *LEAFY (LFY)* in *Arabidopsis thaliana* (Arabidopsis), *PROFL* (***PRO****TEA* ***F****LORICAULA* ***L****EAFY*), is in Proteaceae geïdentifiseer. *PROFL* word uitgedruk in reproduktiewe meristeme so wel as die vegetatiewe meristeme en blare. *PROFL* uitdrukking in blare mag dalk ‘n inhiberende effek hê op die vorming van nuwe blare, omdat die uitdrukking hoog was op die selfde tyd as wat blominisiëring plaasgevind het in die apikale meristeem. Die transisie tot reproduktiewe groei word gekenmerk deur ‘n verhoging in *PROFL* uitdrukking in die apikale meristeem. In meerjarige plante soos *Protea* spp word daar verwag dat die teenwoordigheid van voldoende koolstof die oorskakeling na reproduktiewe groei inisieer. Dit mag wees deur die direkte aksie van suikers met gene soos *PROFL* wat die finale skakel na reproduktiewe groei beheer. Alhoewel BA applikasie geen direkte effek gehad het op *PROFL* uitdrukking nie, was die blomtyd met twee maande vervroeg. *PROFL* uitdrukking was vergelykbaar met die uitdrukking van *LFY* homoloë

in ander houtagtige, meerjarige plante en kan gebruik word as ‘n merker vir blominisiëring in *Protea* spp.

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# List of Abbreviations

| | |
|---|---|
| $\delta^{13}C$ | carbon isotopic ratio |
| °C | degrees centigrade |
| $^{14}C$ | radio-labeled carbon |
| $^{14}CO_2$ | radio-labeled carbon dioxide |
| ABA | abscisic acid |
| *AG* | *AGAMOUS* |
| *AFL1* | *APPLE FLORICAULA LEAFY1* |
| *AFL2* | *APPLE FLORICAULA LEAFY2* |
| ANOVA | analysis of variance |
| *AP1* | *APETALA1* |
| *AP2* | *APETALA2* |
| BA | benzyl adenine, 6-benzylaminopurine |
| *ca.* | *'circa'* / approximately |
| *CAL* | *CAULIFLOWER* |
| *CO* | *CONSTANS* |
| *FLO* | *FLORICAULA* |
| FW | fresh weight |
| GA | gibberelic acid |
| IAA | indole acetic acid |
| *LFY* | *LEAFY* |
| LSD | least significant difference |
| mRNA | messenger ribonucleic acid |
| P | probability |

| | |
|---|---|
| PCR | polymerase chain reaction |
| ppm | parts per million |
| *PROFL* | *PROTEA FLORICAULA LEAFY* |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SE | standard error |
| spp | species |
| *TFL1* | *TERMINAL FLOWER 1* |
| *TFL2* | *TERMINAL FLOWER 2* |
| UFO | *UNUSUAL FLORAL ORGANS* |

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# CHAPTER 1

# GENERAL INTRODUCTION

South Africa, and particularly the Cape Floristic Region, has a highly diverse indigenous flora of which a large number are highly sought after for use as cut flowers. With the many indigenous genera of Proteaceae in particular, having great cultivation potential. One of the most important of these genera are the members of *Protea*. *Protea* cultivation has developed into a very successful agro-industry in South Africa. The large attractive inflorescence with typical "warm" colours that occur within the South African Proteaceae genera are probably due to the fact that birds, which are the main pollinators of *Proteaceae*, are sensitive to these colours (Brits, 1984). Bird pollination, especially the Cape Sugarbird (*Promerops cafer*), is probably also responsible for the characteristic that inflorescences are borne prominently, solitarily and terminally on flowering branches. This is the prime reason why, although Australia is home to more *Protea* genera, the South African Proteaceae as a group are the most attractive of the family and have outstanding potential as cut flowers.

*Proteas* naturally occur in the southwestern and southern Cape, of South Africa, and are one of the most underdeveloped natural resources in South Africa. South Africa has the world's sixth largest floral biodiversity, with the Cape Floral Region being home to over 7 700 plant species. According to market survey estimates, only one tenth of the potentially R1.7 billion South African indigenous cut flower market is currently being utilised (www.sagric.net). The fynbos flower industry includes both fresh (60%) and dried (40%) flower markets, with cut flowers being the mainstay of the industry. Initially most Proteaceous material was collected from the natural habitats, thus endangering the biodiversity and survival of this fynbos. A survey by Wessels et al. (1997) indicated that more than 80% of *Proteas* sold as cut flowers, were derived from cultivated plantations. About 70% of all fresh cut flowers produced in South Africa are exported to markets in Europe, although America and the Far East are also supplied.

One of the most important factors that determines the economic success of *Protea* export from South Africa is the timing of flower production to coincide with the demand in Europe. Currently the demand season in Europe coincides with the off-production season in South Africa and competitors that cultivate *Proteas*, including Australia, New Zealand and Zimbabwe in the Southern Hemisphere and

Hawaii, Israel and Portugal-Madeira in the Northern Hemisphere, are competing for this lucrative market.

The hypotheses and aims of this study are threefold. Firstly we hypothesise that the intrinsic floral signal in *Protea*, and other woody perennials, will ultimately be the internal carbohydrate status of the multicomponent plant system. Perennial plants like *Protea* spp would therefore flower only when there is sufficient carbohydrates available to sustain the floral organ, young leaves, vegetative meristems and roots. Secondly, hormonal manipulation of flowering by the synthetic cytokinin, BA (benzyl adenine, 6-benzylaminopropane) influences flowering in *Protea* spp through alteration of carbohydrate allocation patterns which elicits signalling pathways and ultimately triggers expression of flowering related genes such as *LEAFY* and *FLORICAULA*. Thirdly, although flowering in perennials such as *Protea* might not be as tightly controlled as in the case of annuals, we hypothesise that the expression and control of genes involved in the flowering pathway will be comparable between annuals and perennial *Protea* spp. Floral initiation and control has been extensively studied in simple model species such as *Arabidopsis* and *Antirrhinum.* Homologues of flowering genes such as *LEAFY* and *FLORICAULA*, responsible for the switch between vegetative and reproductive growth have, however, only been identified in a few woody perennial species (Table 2.2).

We tested these hypotheses by using $^{13}C$ natural isotope enrichment and $^{14}CO_2$ labelling to investigate the translocation of carbohydrates both from vegetative shoots to surrounding shoots as well as from the different seasonal flushes to the apical meristems and other flushes. The photosynthetic capacity of shoots carrying developing flowers was measured as well as the respiration rate of these flowers to indicate source and sink strength during flower development. Secondly, $^{14}CO_2$ labelling was used to determine whether BA application increases the carbon flux to the treated apical meristems. A search for a *LEAFY* homolog in Proteaceae was undertaken, and its expression was followed through the flowering season of the commercially grown Protea hybrid ‘Carnival’. The expression pattern of the *LEAFY* homologue, *PROFL*, in the Protea hybrid ‘Carnival’ was also followed after BA treatment.

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www.sagric.net

# CHAPTER 2

# LITERATURE REVIEW

## 2.1. Introduction

Plants have had to adapt to greatly changing environments through evolutionary time. The control of flowering is thought to be a powerful, adaptive mechanism that is likely to be under considerable evolutionary pressure. Great diversity and response to environmental stimuli between plant species, as well as the multiple steps in the flowering process indicate that an enormous variety of control mechanisms are likely. One of the factors that makes flowering such a complex system to study is that, unlike in the case of animals where the fate of a cell is determined and cannot be reversed, a floral meristem may revert back to vegetative growth before the floral organs are formed. It is still uncertain exactly when during floral development the fate of the meristem is irreversibly determined as being reproductive. Research in this field still has to elucidate most of the mechanisms involved in the floral transition. Another remarkable difference between plant and animal development is that animals complete most of their development before beginning maturation and aging whereas plant development and maturation take place concurrently in many plants. The developmental program of a plant must ultimately be controlled by a mechanism that is sensitive to environmental stimuli, but controlled by endogenous mechanisms.

The concept of a single flower-inducing signal, dubbed “florigen” (Chailakhyan, 1936; Lang, 1952; Evans, 1971; Colasanti and Sundaresan, 2000), which was accepted as the ultimate model for the control of flowering several decades ago, has long since been replaced with alternate hypotheses. The concept postulated the existence of a single specific hormonal floral promoter that was conserved over species, and had sole responsibility for flowering (Chailakhyan, 1936; Lang, 1952). It was later accompanied by an “antiflorigen” concept that claimed a single specific hormonal inhibitor was responsible for the inhibition of flowering and had to be overridden by “florigen” for the initiation of flowering (Evans, 1971; Colasanti and Sundaresan, 2000). In plants shown to produce both the florigen and antiflorigen substances, floral evocation would be a result of the balance shifting in favour of florigen. These theories imply that all the alternate pathways act through a common mechanism, and since no single promoter or inhibitory substance universal in all plants has yet been found, it is proposed that unknown hormones might be responsible for this function. Supportive evidence for the

existence of these compounds is largely derived from grafting experiments which are limited by the compatibility of the plants selected for grafting. It is therefore impossible to determine if incompatible species also show the same effects. One of the criticisms of earlier work is that it was only done on plants that were considered to be absolutely photoperiodic, therefore not taking into account most day neutral and facultatively photoperiodic plants. Alternatives to the 'florigen' model like the 'nutrient diversion model' postulates a shift in the source to sink relationship of the plant that favours the translocation of assimilates to the apical meristem during floral induction (Sachs and Hakett, 1983; Bernier, 1988). Anything that prevents the movement of assimilates to the shoot apex may function as floral inhibitors. A model of multifactorial control that proposes the involvement of several factors (promoters and inhibitors) in the control of floral initiation was proposed by Bernier (1988). These factors would only be able to affect floral initiation if a suitable quantity of assimilates were available for meristem transition.

*Arabidopsis thaliana* (*Arabidopsis*) has been widely used as a model species to study flowering. Physiological and genetic components of flowering in *Arabidopsis* are already well characterised, but its suitability as a model system for the study of flowering in woody perennials is still largely undetermined. Perennial plants such as *Protea* spp are multi-component systems that have to continue to support roots, shoots, leaves and vegetative meristems as well as floral organs, once they have formed, and these components should be seen as independent demands from the floral organ. Resource allocation in annuals differs in that they only need to support the floral organ until seed production, after which reserves are diminished and the whole plant dies.

### 2.2. Biology of *Protea* spp

The genus *Protea* is known for their great variety of shapes and sizes, and was named after the greek god *Proteus* who could change his shape at will. They range in size from small prostrate shrubs to large trees. All are evergreen, woody perennials with leathery leaves, suited to withstand periods of hot, dry weather. Regeneration takes place by either sprouting from the lignotuber or by release of achenes from infructescences maintained on the plant (Coetzee and Littlejohn, 2001). *Protea* stems develop in spurts of growth, termed "flushes". The number of flushes, which range from one to two per season, are influenced by the environmental conditions and the species. The inflorescence is borne terminally on a shoot generally consisting of two or more growth flushes in most cultivated *Proteas*.

*Proteas* generally grow on nutrient poor soils (Rebelo, 1995). Unlike most plant families which will form symbiotic associations with soil-living fungi to assist with the uptake of water and nutrient under nutrient deficient circumstances, *Protea* spp do not generally form mycorrhizal associations, but instead form clusters of lateral roots up to 200 mm long made up of thousands of very fine short lived rootlets. These carbon expensive structures are termed "proteoid" or "cluster" roots and they are almost twice as efficient at absorbing nutrients and water as normal roots because of the increased exploitation of soil volume (Rebelo, 1995). These structures require large amounts of carbon for construction and functioning. *Proteas* are sclerophyllous and are generally thought to be constrained by nutrition, rather than carbon availability. This implies that carbohydrates are readily available which is important for the construction of the floral structures.

### *2.2.1. Morphology of flowers*

The involucral bracts provide the main floral display. The individual flowers develop spirally from the outer edge of the involucral receptacle (Coetzee and Littlejohn, 2001). These individual flowers are grouped in a flowerhead that is surrounded or enclosed by the involucre or whorl of bracts, forming the well-known cup shape of the flower (Vogts, 1958). *Proteas* do not have separate sepals and petals, but instead have a single set of four segments (tepals), which make up the perianth. As the bud opens the perianth separates to expose the style. The male organs are also distinctive, as the anthers are joined directly to the base of the perianth limb. Another unusual feature is that they shed their pollen onto the topmost portion of the style just before the flowers open (Rebelo, 1995). Female organs of *Protea* spp consist of an ovary, a style and a stigma. The ovary is superior and in all southern African species it contains one ovule which develops into a small dry fruit that contains one seed. The style is often elaborately shaped (Rebelo, 1995). In some species four minute nectaries are situated at the base of the ovary that secrete nectar to attract pollinators. Protea hybrids such as 'Carnival' form very large floral structures with diameters of up to 50 mm and heights of 130 mm. These structures take up to three weeks to develop from a morphologically identified floral bud to a mature flower (personal observations).

**Table 2.1**. Production times of commercially grown *Protea* species and hybrids in relation to the period of optimum marketing, timed with the European market, shown within the shaded area. The benefits of lengthening the flowering times of these *Proteas* are therefore obvious. 'Lady Di', 'Pink Velvet', 'Sheila' and 'Susara' are *P. magnifica* hybrids. 'Pink ice', 'Pink Duke', 'Carnival' and 'Brenda' are *P. compacta* hybrids, whereas 'Sylvia' and 'Cardinal' are *P. eximia* hybrids

| | Flowering time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Protea* species/hybrid | Jun | Jul | Aug | Sep | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May |
| P. compacta | * | * | * | * | | | | | | | * | * |
| P. cynaroides | * | * | * | * | * | * | * | * | * | * | * | * |
| P. eximia | | * | * | * | * | * | * | | | | | |
| P. grandiceps | | | | * | * | * | * | * | | | | |
| P. lacticolor | * | | | | | | | | * | * | * | * |
| P. magnifica | | * | * | * | * | * | * | * | | | | |
| P. mundii | * | * | * | * | | | | * | * | * | * | * |
| P. nana | | * | * | * | * | | | | | | | |
| P. neriifolia | * | * | * | * | * | * | | | * | * | * | * |
| P. pityphylla | * | * | * | * | | | | | | | | * |
| P. repens | * | * | * | * | * | * | * | * | * | * | * | * |
| P. scolymocephala | | * | * | * | * | | | | | | | |
| 'Lady Di' | * | * | * | * | | | | | | | | |
| 'Pink Velvet' | * | * | | | | | | | | | * | * |
| 'Sheila' | * | * | * | * | | | | | | | | |
| 'Susara' | * | * | * | * | | | | | | * | * | * |
| 'Pink Ice' | | | | | | | * | * | * | * | * | * |
| 'Pink Duke' | * | * | * | | | | | | | | | |
| 'Carnival' | | | | | | | | * | * | * | * | * |
| 'Brenda' | * | * | | | | | | | | | | * |
| 'Sylvia' | * | * | * | * | * | * | * | * | * | * | * | * |
| 'Cardinal' | * | | | * | * | * | * | * | * | * | * | * |

### *2.2.2. Physiology of Protea flowering*

Protea species grown in the Southern Hemisphere flower naturally during months from autumn to spring. The optimum marketing period in Europe is mid-spring to mid-summer, a time when very few southern African Proteaceae flower (Table 2.1). This has resulted in studies aimed at elucidating how flowering is initiated in *Protea* and how it can be manipulated, as most of the physiological processes in *Protea* are still largely unknown.

In some species of *Banksia*, a Proteaceae native to western Australia, flowers are initiated on shoots in their second year of growth, although it has been reported that some shoots produce an inflorescence in the first year (Fuss et al., 1992). The ability of a shoot to flower seems to be influenced by both the age and size of the shoot. In the study on *Banksia* it was observed that in all cases the flowering shoots were thicker and longer than the non-flowering ones (Fuss et al., 1992). Shoots that had more than one growth flush in one growing period also did not tend to produce a flower. Flushes arise in succession

from a distal axillary bud, with the flushes exhibiting strong apical dominance during active growth. Lateral buds of florally determined shoots seem to become more pronounced, from which lateral shoots can be seen after floral formation. Similar patterns are observed during flowering in *Protea*. A spring flush must be subtended by at least two or three previous flushes for flower initiation to take place in the commercial *Protea* hybrid Carnival (*P. compacta* x *P. neriifolia*) (Greenfield et al., 1993). In the hybrid 'Sylvia' (*P.eximia* x *P. sussanae*) shoots of more than 2 flushes can produce a flower any time during the year. A minimum diameter of the flush subtending the inflorescence, a possible requirement for flowering, has not been determined for any of the *Protea*. A sufficient shoot diameter is important for support of the large flower that can weigh up to 80 grams (Fig. 3.6). Defoliation studies by Gerber et al. (2002), on 'Carnival', showed that the presence of mature leaves are essential for flower initiation and these leaves must be retained on the shoot until 6 to 7 weeks before spring bud break. Conditions prevailing during winter, whether environmental or endogenous factors, appear to be conducive to flowering in 'Carnival'. Leaf removal studies can be used to test for a commitment to persistent floral stimulus production in the leaves (Hemple et al., 2000). Early defoliation prevented flowering in the Carnival study. Gerber et al. (2002) proposed several reasons for this: 1) defoliation may have removed the source of photoassimilate and that this resulted in a decrease in available carbohydrates making the plant unable to support the formation of a floral organ; 2) removal of leaves resulted in a weaker growth of new leaves during the spring flush preceding flowering, and thereby reducing the carbohydrate status of the plant; 3) leaves may be needed in the winter to perceive the floral "signal".

The ability of the shoot to support the floral structure by supplying continuous carbohydrates and mechanical support for a floral meristem would seem to be a logical indication of when the apical meristem would be able to act on the floral signal. Pruning studies by Gerber et al. (1993) and Hettasch et al. (1997) seem to support this argument. Pruning of the plant during the early spring months results in no flowering in the following spring, probably due to limited leaf area and therefore a reduced source strength as well as the fact that most of the available carbohydrates would have been invested in structural growth. Inflorescences are initiated on the spring and summer flushes of the following year, resulting in peak flowering during February as opposed to normal peak flowering during April. Harvesting is therefore better timed with the demand for flowers and longer stems caused by the additional growth flushes also increase marketability. Growers already practice pruning of *Proteas* to increase harvesting at optimum marketing times extensively.

## 2.3. Events leading to floral development

Floral development is characterised by changing of the biochemical processes that determine vegetative growth. The most obvious of these are the anatomical changes the meristem undergoes to develop the floral organs. It is however the intraplant changes that signal these macroscopic changes. In woody perennial plants, a shift in the source-sink relationships that benefit the developing bud might be the determining factor of floral induction (Jackson and Sweet, 1972).

### *2.3.1. Meristem competence*

Floral evocation, the events occurring in the apex that commit it to flowering, involves major transformation of the shoot apex. Evocation seems to be characterised in different types of plants by an increase in respiration and therefore an increased energy metabolism (Bernier, 1988). Not all shoot meristems can react to conditions that otherwise are known to promote flowering; the meristem needs to be competent to react. Juvenile meristems are generally believed to be incompetent to respond to the floral stimulus. This may mean that the floral stimulus is not produced in these plants, that the stimulus does not reach the meristem or that inhibitors are present that prevent flowering. Competence is demonstrated if a cell or group of cells exposed to a developmental signal responds in the expected manner (McDaniel et al., 1992). In most cases it is impossible to know which factors including time, node number, leaf area, distance from roots or other subtle changes are critical for reaching competency for flowering (Bernier, 1988). It is quite straightforward to establish if a cell or group of cells is competent to respond to a given developmental signal if the signal is known and available. This is not the case for the floral signal(s), as it has yet not been successfully defined in any plant.

### *2.3.2. Meristematic changes*

Meristematic tissue is made up of thin-walled, tightly packed living cells that undergo frequent divisions. The main functions of the apical meristem are firstly to initiate the formation of lateral organs and structures and secondly to perpetuate itself by maintaining a population of undifferentiated cells that remains uncommitted to a specific program (Colasanti and Sundaresan, 2000). In the process of forming an inflorescence the phyllotactic pattern of the leaves as well as the growth pattern of the stem may change (McDaniel et al., 1992). In floral initiation the whole meristem seems to acquire a new developmental state, which is clearly not a single-step process. Great interspecies variation exists between the sequences of initiation of floral organs.

An elongation of internodes often precedes flowering. One of the most characteristic signs of floral development is the precocious development of axillary buds. These precocious axillaries grow out to form flowers, spikelets or inflorescence branches. The growth of axillary components is presumably related to a loss of apical dominance and thus to changes in the factors (mainly auxins, cytokinins and nutrients) that are known to be involved in the correlation between apical and axillary buds (Bernier, 1988). Exogenous application of cytokinins stimulates the release of apical and auxiliary buds from dormancy (Li and Bangerth, 2003). At transition to flowering the apex undergoes remarkable changes in size and shape. These changes are observed in both formation of a single flower and inflorescence. Before and during this change in shape, termed "doming" in many species, the meristem undergoes a sharp increase in mitosis and also a change in the amount and distribution of endoplasmic reticulum (ER). One apparent critical event of the transition to flowering is the elimination of the pattern characteristic of the vegetative state. This is essentially achieved by the increase in meristem size (Bernier, 1988). Gradually the cells in the central core of the apex become highly vacuolate, in contrast to the smaller cell layers that form the outer covering or mantle. After further growth the proportional size of the central parenchymous core increases and floret primordia are formed at the periphery of the inflorescence apex. When a flower is formed, the floral organs (sepals, petals, stamen and carpels) are initiated in a phyllotactic pattern that is normally different from that of leaf phyllotaxy, and internode growth between those floral organs is usually minimal or nonexistent (McDaniel et al., 1992). The formation of floret primordia continues until they cover the whole surface of the meristem. During this time the apex has continued to increase in size.

The number of each type of floral organ is often considered as a fixed character in the flowers of many species, but they may be altered by exposure to various treatments. A *TFL1* (*TERMINAL FLOWER 1-1*) mutation causes early flowering and limits the development of the normally indeterminate inflorescence by promoting the formation of a terminal floral meristem. The *tfl1-1* terminal flower is morphologically abnormal and lacks complete whorls of sepals and petals (Shannon and Meeks-Wagner, 1991). Analyses of pleiotropic mutations like tfl1-1 may elucidate the relationship between meristematic structure and activity during development.

*2.3.3. Carbohydrate levels*

Increases in sucrose, ATP, invertase activity, mitochondrion number and energy charge with the initiation of flowering have been recorded for *Sinapsis* (Bodson, 1977 and 1985). Soluble sugar content in both the leaves and buds increased soon after floral initiation while starch levels in the buds

increased later. This may be interpreted as increased energy availability to support the meristematic changes occurring during floral transition. A study of the sugar metabolism in the flowering buds of two pear cultivars, showed an increase in the activity of most sugar metabolising enzymes (Ito et al., 2002). This could especially be seen for soluble acid invertase and NAD-dependent sorbitol dehydrogenase that is responsible for the catabolism of the main sugars transported in pear namely sucrose and sorbitol. Because floral buds mainly function as metabolic sinks rather than storage sinks, it is likely that the products of these enzymes are needed as metabolic substrates. Cytochrome P450 mono-oxygenase has been found to be specifically expressed in flower buds of *Zea mays* and *Petunia* (Imaishi and Ohkawa, 2002). Cytochrome P450 mono-oxygenases play an important role in the metabolism of fatty acids and other secondary metabolites in higher plants. Carbohydrates are required to supply energy for pollen germination, pollen-tube growth and also nectar production in floral organs. Import of sugars for nectar production in *Protea* spp. seems to be regulated by invertase activity with the final sugar concentrations in *P.repens* nearly 20% (w/w) (Nicholson, 2002). The expression of sugar transporters has been reported in several of these metabolically active floral organs (Williams *et al*., 2000). All the above mentioned compounds and the presence of enzymes responsible for their metabolism indicates the large demand for a sufficient energy supply needed for the development and maintenance of floral organs until the reproductive cycle has successfully been completed.

### *2.3.4. Floral repression*

A signal from the roots of *Nicotiana tabacum* L. seems to maintain vegetative growth in young seedlings (McDaniel, 1996). This might be a floral inhibitor produced in the roots. The repression of flowering may be critically important for timing of flowering with pollinators and other environmentally favourable factors like sufficient water and nutrient availability. Mutants such as *Embryonic flower* (*emf*) have been identified in a variety of plants and are characterized by the absence of vegetative rosette growth, with direct inflorescence and flower development from embryo or callus (Sung et al., 1992; Bai and Sung, 1995; Yang et al., 1995). It has been suggested that *EMF* genes are active in early embryogenesis specifying vegetative development, and therefore repressing flowering. The loss of vegetative growth in these mutants suggests that *EMF* gene expression inhibits the production of the floral stimulator (Yang et al, 1995; Pidkowich et al., 1999). *EMF* may negatively influence the expression of the floral meristem identity genes (*LFY, AP1* and *AG*) as they are expressed early in germination, and continue repression until the plant reaches adult phase (Amasino, 1996; Levy and Dean, 1998). Some gene products that promote flowering may in turn directly or indirectly act in

repressing *EMF* function. An *EMF* mutant study by Yang et al (1995) shows that the primary roles of *EMF1* and *EMF2*, two loci present on chromosome 5 of *Arabidopsis*, are to specify the vegetative rosette versus inflorescence development. These proposed roles of *EMF* genes suggest that continual expression of these genes throughout the development of the plant may lead to a plant that is never competent to form a flower.

The rate of floral morphogenesis may be dependent upon the duration as well as the intensity of the influx of a floral stimulus. If the floral signal is produced in the leaves we would expect to see an increase in the movement of signals from the leaves to the apex. A recent study by Gisel et al. (2002) however indicates a reduction in the symplastic movement of 8-hydroxypyrene 1,3,6 trisulfonic acid, a symplastic tracer, during floral induction in *Arabidopsis*. Varying long day treatments were used to induce flowering. In all conditions inductive to flowering, a reduction in the movement of the symplastic tracer was observed. A possible explanation for this phenomenon is that it reduces the movement of a floral inhibitor, also produced in the leaves, from moving to the apex.

*2.3.5. Environmental factors inducive to floral induction*

Three distinct inductive conditions can be identified in a range of plants. These environmental factors might not act alone and plants may be dependent on a combination of conditions to ultimately induce flowering. Plants that flower in response to these stimuli have an advantage to time flower development with maximum pollinator activity and carbohydrate availability from photosynthesis. Some plants have a specific day-length requirement before flowering will be induced. Flowering usually occurs most rapidly with continuous inductive treatments, but some flowering responses can be obtained with inductive periods far too short to permit any flower formation (Lang, 1952). Even within species the range of adaptive response can be extremely broad. The ability of leaves to respond to these photoperiodic conditions is thought to be closely related to the juvenile adult transition, as not all leaves are able to respond inductively (McDaniel et al., 1992). This has been supported by grafting experiments (Bernier et al., 1993), which indicate that the stimulus is perceived by the upper part of the epidermis of leaves and relayed to the shoot apex (Lang, 1952). Shading studies on 'Red Sunset', a *Leucospermum* hybrid and qualitative short day plant, caused a decrease in the carbohydrate content of both shoots and leaves and reduced the decapitated shoot responsiveness to inductive conditions (Jacobs and Minnaar, 1980). This is thought to be because of insufficient carbohydrates for floral induction. Previous shading studies on *Leucospermum* aimed at slowing down flower development and

therefore increasing the marketable time of the 'pincushion' inflorescences, showed a decrease in flower quality and size.

Plants respond differently to cold treatments in terms of vegetative and reproductive growth. The effect of vernalisation appears with some delay, and frequently after the causative action is no longer operative. During the actual cold treatment the growth of the plant may be completely suspended. Propagation of *Leucospermum* cv. 'Red Sunset' *in vitro* from multinodal shoot segments showed that bud break is increased when the cultured tissue was subjected to three weeks of cold treatment at 5°C (Rugge et al., 1989). This indicates that vernalisation might be conducive to vegetative growth in this Proteaceae species. Studies done on fruit-bearing, woody perennials such as mango and lychee have found that the release of dormancy may precede floral induction with a relatively short period in which induction can occur (Batten and McConchie, 1995).

With time, most plants will flower autonomously. The only requirement being that the environmental conditions are conducive to growth (Bernier, 1988). It has been proposed that the autonomous pathway measures the internal status of the plant. That is its current age, juvenile or mature, number of leaves able to provide the plant with sufficient carbohydrates for survival and support of a floral meristem and also, if the shoot has sufficient mechanical strength to support the flower. The autonomous model is based on the fact that very few *Arabidopsis* mutants exist that never flower. All environmental requirements for floral induction can therefore theoretically be overridden by the internal status of the plant.

*2.3.6. Hormonal control of flowering*

The gibberellin class plant hormones comprises of several forms of the hormone. A gibberellic acid (GA) pathway has been identified as a separate floral induction pathway in *Arabidopsis*. It is required for early flowering in *Arabidopsis,* but not all plants have been shown to respond in the same way to GA treatment (Colasanti and Sundaresan, 2000). Application of GA shortens the juvenile phase in several plants leading to early flowering (Brooking and Cohen, 2002; Serres and McCown, 1994). It has therefore been suggested that juvenility can be related to low levels of endogenous gibberellin. Studies on two tobacco varieties with different photoperiodic responses indicate that the biosynthesis of gibberellin is responsible for floral induction only in the case of long-day plants (Grigorieva and Kucherov, 1971). This might indicate that GA synthesis has light-dependant steps. In the day-neutral perennial, *Citrus sinensis,* treatment of potentially flowering resting buds with gibberellins ($GA_3$), *in*

*vivo*, led to the partial or complete reversal to the vegetative state (Lord and Eckard, 1987). In these plants it was possible to divert 90% of the potentially flowering buds to vegetative shoots by applying $GA_3$ to the resting buds in the previous winter. When $GA_3$ was applied to buds of which the bud scales were parting and the apex was swelling and when the first sepal was being formed by the terminal meristem, reversal by $GA_3$ application was not as successful. It is thought that the irreversible transition to a flowering shoot must have been determined once the first set of sepals is produced. In *Citrus* spp, as in other woody perennials, hormonal levels possibly control the signalling pathways and growth of the plant. GAs decrease the amount of flowering and increase vegetative growth. This has also been found to be true for *Protea* after treatment with $GA_3$ (Hoffman, personal communication). Napier and Jacobs (1989) investigated the extent to which plants of 'Red Sunset', a *Leucospermum* hybrid, lose their responsiveness to floral induction when treated with growth regulators and exposed to low light intensities. *Leucospermum* R.Br. cv. Red Sunset is a qualitative short-day plant (Malan and Jacobs, 1989) which grows vegetatively during spring and summer. Reproductive development commences in autumn and flowerheads appear in winter. Growth regulators applied to all portions of decapitated shoots during inductive short days of autumn and early winter did not affect flowering, but $GA_3$ and ethephon, which releases ethylene, applications in mid-winter caused shoots to be less responsive to inductive short days. IAA (Indole Acetic Acid), BA (Benzyl adenine, 6-benzylaminopurine), ABA (Abscisic acid) and daminozide ([N-Dimethylamino]-succinamic acid, internodal elongation inhibitor) applied at the same time did not affect flowering.

Studying the effect of hormones on flower initiation and development is challenging. The application of a single hormone often affects many different plant processes and different hormones have been found to play similar roles within the plant, thus suggesting a complex interaction of hormone signalling in plants. One of the approaches to studying the role of hormones in relation to flowering is to monitor their endogenous levels during flower induction. A study by Chang et al. (1999) revealed that the endogenous cytokinin, which is synthesised in an unknown plant part, determines the formation of flower buds in plants with tuberose corms. Early floral initiation and flower developmental stages show increased tissue cytokinin levels. Application of synthetic cytokinin promotes the formation of flower primordia on tuberose. Chang et al. (1999) hypothesised that when a tuberose corm reaches an appropriate developmental stage, it produces a signal that is transported to the roots where it increases cytokinin production, and translocates it to the corm and leaves. Cytokinin levels also increase gradually and then peak when flowers are initiated in the *Protea* hydrid ‘Carnival’ (Hoffman, personal communication). The synthetic cytokinin, BA, exogenously applied to dormant buds from ‘Carnival’

causes early release from dormancy and ultimately produces flowers earlier than the unmanipulated flowering season. The response to cytokinin in plants differs significantly between species, as exogenously supplied cytokinin has been found to both inhibit and promote floral initiation in a variety of plant species.

### *2.3.7. Genes involved in flowering*

Changes in all RNA fractions and protein levels have been indicated as among the earliest events occurring in transitional meristems (Herdenberger et al., 1990). Two different types of genes are responsible for the initiation and completion of flowering; these are meristem identity and floral organ identity genes. Genes that positively effect the expression of organ identity genes are called meristem identity genes, because the absence of their activity usually causes partial or complete reversal of flowers into shoots (Weigel and Meyerowitz, 1994). A rather large gene family, the meristem identity gene family, has been identified and shown to be directly involved in the floral induction pathway. Most of the genes were identified by the study of late flowering mutants in model-system plants such as *Arabidopsis* and *Antirrhinum* spp. The use of transgenic plants to study the effect of over expression of these genes in various plants has also been helpful in building up a large body of knowledge as to the integrated functions of these genes in flower and plant development. Currently most models for the genetic control of flowering are based on studies of *Arabidopsis* and *Antirrhinum.* Although the models give a complete picture of the genetic control of flowering in these plants, genetic studies in more complex systems such as woody perennials have shown some of these genes to have more than one functional copy. The biggest problem with recent work is that for most of the studied plants, methods of cultivation and transformation are yet to be developed. Therefore, functional data on the expression and role of these genes in perennial plants are lacking.

Genes responsible for meristem transition and floral organ development are highly conserved in even distantly related dicotyledonous plant species (Yanofsky, 1995). Master floral controlling genes needed for normal development in a wide range of angiosperms include genes such as *LEAFY (LFY), APETALA 1 (AP1), AGAMOUS (AG), CAULIFLOWER (CAL), APETALA 2 (AP2)* and *UNUSUAL FLORAL ORGANS (UFO)* (Pidkowich et al., 1999). Of these *LFY* and *AP1* are believed to play primary roles in the floral transition, marking primordial meristematic cells for a floral fate. LFY in Arabidopsis has been shown to code for a transcription factor that controls developmental transition by inducing the expression of a second transcription activator *APETALA 1 (AP1)* as well as the expression of at least five other genes involved in the switch to reproductive growth (William et al., 2004). The

necessity for constitutive expression of *LFY* in *Arabidopsis* for floral morphogenesis in both the axillary and terminal meristems has been shown (Weigel and Nilsson, 1995). Mutant phenotypic analyses and expression studies indicate that an important function of *LFY* and its targets, *AP1* and *CAL*, is to enhance the expression of each other (Fig. 2.1) (Pidkowich et al., 1999). *UFO* appears to control a subset of the functions regulated by *LFY*. It has been suggested that *UFO* may lie downstream of *LFY* in the complex regulatory events that ultimately control flowering. A very limited amount of information is available on the expression of these genes in woody plants (Table 2.1). If the expression of a singular gene was sufficient to confer floral identity to a meristem, the embryonic expression of this gene should stop vegetative growth and produce a flower immediately upon germination. Interestingly the embryonic expression of either *LFY, AP1* or *AG* only results in an early flowering phenotype, and none of these are sufficient to result in the transformation of the embryonic meristem into a floral meristem (Pidkowich et al., 1999).

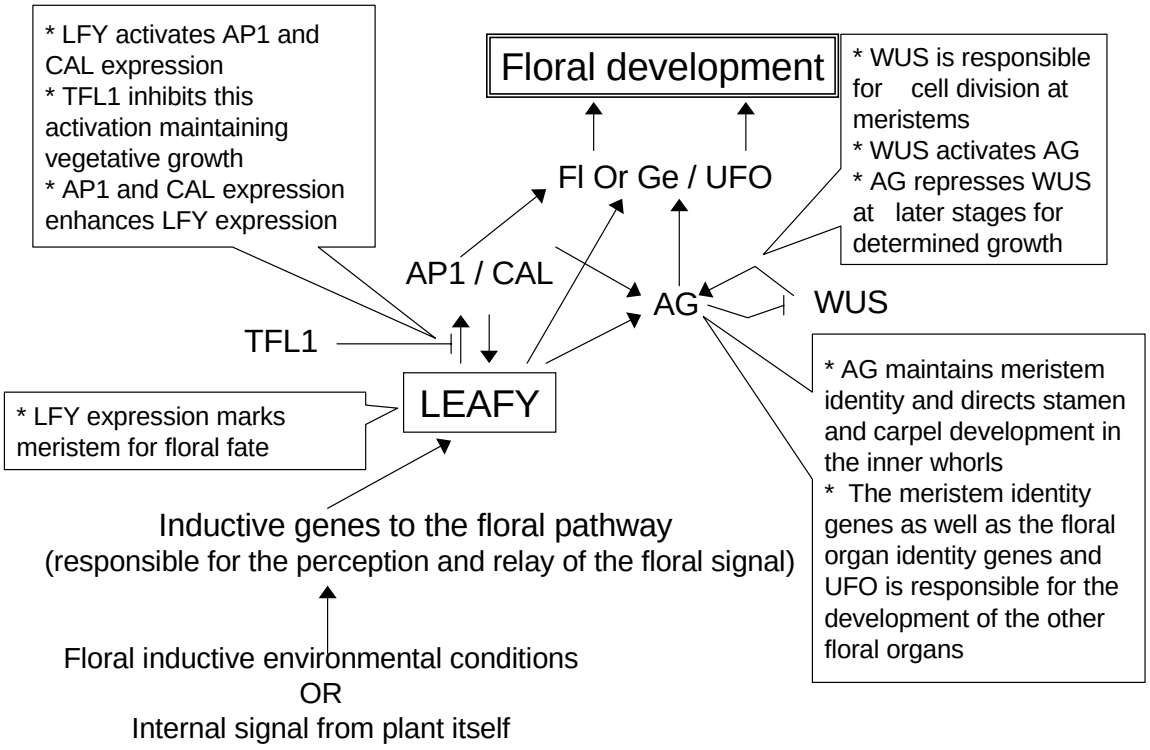


**Fig 2.1**. Diagrammatic representation of the events leading up to the expression of the master floral controlling meristem identity genes and their control. *LEAFY (LFY), APETALA 1 (AP1)* and *CAULIFLOWER (CAL)* have a functional relationship in which the expression of LFY is enhanced by the expression of *AP1* and *CAL* of which the expression is regulated by *LFY*. *TERMINAL FLOWER 1 (TFL1)* expression negatively affects the activation of *AP1* by *LFY*. *LFY*, *AP1* and *CAL* are responsible for the expression of *AGAMOUS (AG). AG* maintains meristem identity in the center of the floral primordium. Floral organ identity genes (Fl Or Ge) as well as the meristem identity genes mentioned above including *UNUSUAL FLORAL ORGANS (UFO*) is responsible for the development of floral organs. Modified from Pidkowich et al. (1999).

Recent phylogenic studies indicate that the lineage leading to flowering plants originally had two copies of the *LFY* gene, but that one copy was subsequently lost in flowering plants (Frohlich and Parker, 2000). These observations are made by comparing flowering plants with extant gymnosperms. *PTLF*, a homolog of *LFY* in the woody perennial, *Populus trichocarpa*, was overexpressed, resulting in developmental alterations, but not the early flowering phenotype that was expected (Rottman et al., 2000). When *LFY* was overexpressed in *Populus trichocarpa* similar results were obtained. The presence of negative regulatory factors that constrain *PTLF* function and that may be involved in juvenility has been proposed as a possible reason for these results.

**Table 2.2**. Woody perennial plant species in which *LEAFY* homologues have been identified

| Species name | Gene name | Plant organ | GenBank accession number (if submitted) | Authors |
|---|---|---|---|---|
| | | | | |
| *Vitus Vinefera* | VFL | flowers; vegetative shoot apices; tendrils | AF450278 | Carmona et al. (2002) |
| *Populus tomentosa* | | | AY211519 | Unpublished |
| *Populus trichocarpa* | PTLF | lateral floral meristems; bracts; vegetative meristems; young leaves | U93196 | Rottmann et al. (2000) |
| *Metrosideros excelsa* | | | AF007869 | Unpublished |
| *Pinus pinaster* | | | AL750947; AL750121 | Unpublished |
| *Pinus radiata* | NEEDLY | vegetative meristems; seed-cone buds; pollen-cone; needles | U76757 | Mouradov et al. (1997) |
| *Pinus radiata* | PRFLL | vegetative meristems; undifferentiated male cone primordia | U92008 | Mellerowicz et al. (1998) |
| *Platanus racemosa* | | | AF106842 | Frohlich and Parker (2000) |
| *Eucalyptus grandis* | EgLFY | floral meristems; petals; sepals; stamens; carpels; leaf primordia of adult trees | AY640313; AY640314 | Dornelas et al. (2004) |
| *Eucalyptus globulus* | ELF1 | young flower buds; sepals; petals; carpels; stamens | | Southerton et al. (1998) |
| | ELF2 | pseudo gene | | Southerton et al. (1998) |
| *Malus* x *domistica* Borkh. | AFL1 | floral meristems | AB056158 | Wada et al. (2002) |
| | AFL2 | Sepals; Stamens; Carpels; floral meristems; vegetative meristems; roots | AB056159 | Wada et al. (2002) |

Juvenile citrus seedlings were transformed with *Arabidopsis LFY* and *AP1* genes with the aim of accelerating their flowering time. *AP1* was as efficient as *LFY* in the initiation of flowers, and did not produce any severe developmental abnormalities (Peña et al., 2001). Ectopic expression of neither *AP1* nor *LFY* could cause the formation of a flower before the production of some lateral meristems. This indicates that the apical meristem stays vegetative for at least a short period and that *LFY* and *AP1* were not sufficient for the transition to a reproductive meristem during this time (Ma, 1998). Weigel and Nilsson (1995) generated transgenic *Arabidopsis* plants in which *LFY* was constitutively expressed, and was successful in converting vegetative shoots into flowers. Weigel and Nilsson (1995) tested the theory that *LFY* encodes a developmental switch that is both sufficient and necessary to convert shoot

meristems into floral meristems. This was tested in the perennial tree specie, *Populus* hybrid (*P. tremula x P. tremuloides*), in which transgenic *LFY* lines showed the same early flowering phenotype as the transgenic *Arabidopsis* plants. High sucrose levels within medium of *in vitro* cultured *Arabidopsis* negatively affected *LFY* expression (Ohto et al., 2001). The increased sucrose concentration seemed to extend the vegetative phase and delay flowering. The delay in *LFY* up-regulation may mean that the delay in flowering might be a direct or indirect consequence of the inhibition of *LFY* expression by sugars. This is however unlikely, and alternatively *SHORT INTEGUMENT (SIN1)* is proposed to mediate the negative effects observed in the presence of high sugar concentrations. Low sugar concentrations (1%[w/v]), however, induced flowering and might have a direct effect on *LFY* expression.

*AP1* and *AGAMOUS (AG)*, have been shown to be direct targets of *LFY* in *Arabidopsis*. Constitutive expression of *AP1* in transgenic *Arabidopsis* shows an early flowering phenotype (Mandel and Yanofsky, 1995). In these transgenic plants, the terminal meristem also prematurely terminated as a floral meristem. These results demonstrate that *AP1* alone is sufficient to convert inflorescence shoots to flowers. Mutations in the *AP1* gene significantly prolong the vegetative phase of *35S::LFY* (*LFY* gene under the control of the ubiquitous cauliflower mosaic virus 35S promoter) transgenic plants, but the phenotype of the *35S::AP1* plants was not significantly prolonged by mutations in *LFY.* This may indicate that *AP1* acts downstream of *LFY* to specify meristem identity. *AP1* homologues have also been identified in apple. When the homologous gene (*MdMADS5*) from apples was over expressed in *Arabidopsis* it caused early flowering (Kotoda et al., 2002). This indicates that the function of the apple homologue is similar to that of the *AP1* gene. Using *Arabidopsis LFY* and *AP1* genes would however not be an efficient way of producing early flowering transgenic lines of apple, because there seems to be a low transformation success rate in woody plants. It was suggested that the *MdMADS5* gene is involved in flower development after floral-bud differentiation, although the mechanism of flower-bud formation in apple might be different from that in *Arabidopsis*. This isolated case will however not be sufficient for the support of *Arabidopsis* as a model plant to study flowering in woody perennials.

*TERMINAL FLOWER 1* (*TFL1)* plays a pivotal role in maintaining the vegetative fates of meristems. Consistent with this function *TFL1* is expressed at low levels in the shoot before apex transition, and up-regulated in those meristems that will follow a vegetative growth pattern (Bradley *et al*., 1997). The proposal is that *TFL1* interferes with the ability of *LFY* and *AP1* to enhance each other's expression

(Pidkowich, 1999) or, prevents the movement of signals that affect *LFY* and *AP1* expression (Hemple et al., 2000). *Tfl* mutants flower early and both the apical and lateral meristems develop florally (Yanofsky, 1995). Another gene that is also involved in maintaining a vegetative meristem is *TERMINAL FLOWER 2* (Larsson et al., 1998); its method of action which is still largely unknown, will possibly provide greater insight as to how this floral inhibitory effect is achieved. Although its function is similar to *TFL1*, *tfl2* mutants are dwarfed in appearance, have reduced photoperiod sensitivity and have a more variable terminal floral structure. A mutation in the *CENTRORADIALIS* gene of snapdragon, homologue to the *Arabidopsis TFL1* gene, has shown sensitivity to environmental conditions in its response, with fewer flowers forming under floral inductive conditions (Cremer et al., 2001). This study supports the possibility of genetic reversion of a floral meristem back to vegetative growth because of environmental conditions, even after the terminal flower has been induced.

*CONSTANS (CO)* promotes flowering in *Arabidopsis* in response to day length (Samach et al., 2000) and greatly increases the capacity of the meristems to respond to *LFY* expression (Pidkowich et al., 1999). Previously it was thought that *CO* acts by targeting *LFY* directly, but later studies have shown that several genes are early targets of *CO*. Some of these target genes seem to be involved in proline and ethylene biosynthesis (*AtP5CS2* and *ACS10*). Ethylene has been shown to play a role in flower initiation in plants (Guzman and Ecker, 1990). Reduction in expression of AtP5CS2, which codes for an enzyme involved in the synthesis of a precursor of proline, showed no delay of flowering, but prevented elongation of the stem correlated with the appearance of the first floral buds (Nanjo et al., 1999). This phenotype could be corrected by exogenously supplying proline. Two other genes targeted by *CO* are *FLOWERING LOCUS T* (*FT)* and *SUPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1)*. *FT* and *SOC1* are regulated by a day length-dependent pathway (Samach et al., 2000) (Fig 2.2). *CO* is therefore a pivotal player in the floral initiation pathway, as it activates at least four early target genes with diverse biochemical functions that assist with floral transition. Additional copies of *CO* accelerate flowering time under both long and short days. Therefore it has been suggested that *CO* acts as a biological meter that measures exposure to light (Pidkowich et al., 1999). Expression studies of *CO* under varying light conditions may reveal more about this “photoperiodic-gene”. Other sister genes of *FT* have also been described in *Arabidopsis*. *FWA* and *FE* together with *FT* fall into the day-length-dependent class of flowering promoters (Fig 2.2). These genes seem to play roles that are partially redundant, yet independent of *LFY* in regulating the floral transition. It has been indicated that *FT* and *FWA* are involved in the activation of *CAL* (Pidkowich et al., 1999). The number of genes in the *CONSTANS-LIKE* family seems to be variable among plant species. Two *CONSTANS-LIKE* genes

were isolated from *Populus, PdCO1* and *PdCO2* (Yuceer et al., 2002). Expression of these genes was shown to increase in the leaf prior to inflorescence and floral meristem development. This is consistent with the view that leaf-derived signals stimulate floral initiation.

Floral evocation is controlled by a combination of external and endogenous signals, their perception and the expression of the meristem identity genes. Floral development, however, is regulated by floral organ identity genes after the floral transition has occurred. Floral organ identity genes are responsible for the development of the four floral organs (sepals, petals, stamens, and carpels) of the flower. The floral meristem produces a determinate number of organ primordia in a whorled arrangement. The perianth organs, sepals and petals, occupy the first and second whorls, whereas the reproductive organs, stamens and carpels, occupy the third and innermost fourth whorl (Yanofsky, 1995).

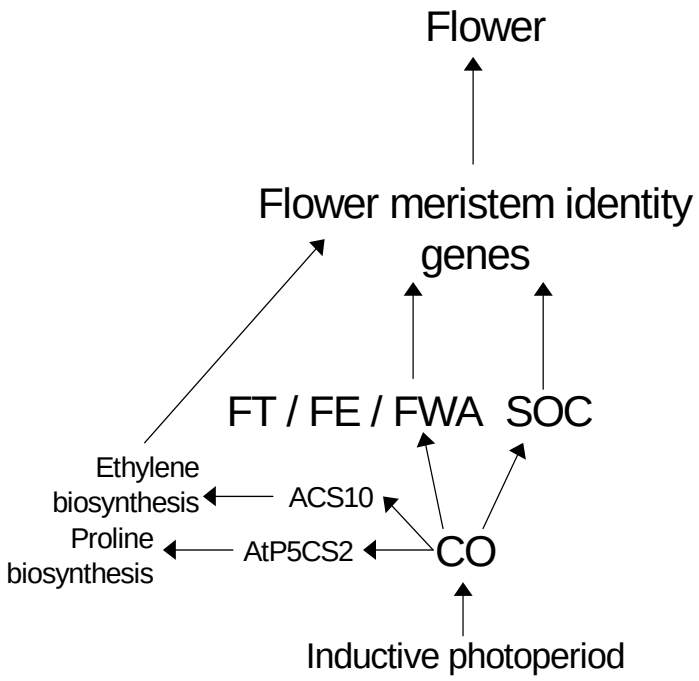


**Fig 2.2**. Interactions between the photoperiod pathway genes and the meristem identity genes leading to floral initiation under inductive conditions. Two early targets of *CONSTANS (CO)*, *ACS10* and *AtP5CS2*, have shown to be involved in the biosynthesis of ethylene and proline. Ethylene is involved in signal transduction and *AtP5CS2* encodes an enzyme that catalyses the synthesis of a precursor of the amino acid proline. *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC)* are early targets of *CO*. *FWA* and *FE* fall together with *FT* into the day-length-dependent class of flowering promoters. Modified from Samach et al. (1999)

Studies of homeotic mutants (mutants with a normal organ in a place where another type is typically found) has led to the organisation of organ identity genes into three classes A, B and C. These three classes of genes reflect three classes of organ identity function (Weigel and Meyerowitz, 1994). Loss

of A function causes transformation of first-whorl sepals into carpels, and of second-whorl petals into stamens. In loss of B function mutants, sepals replace second-whorl petals, and third-whorl stamens are transformed into carpels. Finally loss of C activity transforms third-whorl stamens into petals, and fourth-whorl carpels into sepals. The following *Arabidopsis* genes that are necessary for the A, B and C functions are; A function: *AP1* and *AP2*; B function: *AP3* and *PISTILLATA (PI)*; C function: *AG* (Yanofsky, 1995).

Recently Lohmann et al. (2001) found the homeotic gene *AG (AGAMOUS)* to have dual roles. The first is the known role of specifying organ fate and maintaining determinate growth within the floral shoot (Pidkowich et al., 1999), and the second is limitation of stem cell proliferation. A homeodomain protein *WUS (WUSCHEL)* was shown to cooperate with *LFY* to activate *AG*. *WUS* is responsible for keeping cell populations in both shoot and floral meristems actively dividing. *AG* represses *WUS* at later stages of floral development, thus creating a negative feedback loop that is required for the determinate growth of floral meristems. *HAG1* is believed to be a homologue of *AG* that has been identified in regenerated floral buds of *Hyacinthus orientalis* L. The phenotype of transgenic *Arabidopsis* in which *HAG1* was over expressed was similar to that of *AG* over expression (Li et al., 2002). The expression of *HAG1* in floral organ identity seems to be induced by low levels of hormones. High hormone concentrations cause the floral organs to only produce tepals, but when cultured on low hormone concentrations the flowers form stamens, carpels and tepals. mRNA expression studies show *HAG1* expression in the stamens and carpels but absent in the tepals. Several floral homeotic genes have additional functions apart from the specification of organ identity. AG has at least three different roles during early flower development. The most important of these is the provision of floral meristem determinacy. The regulation and function of these homeotic genes that control floral organ identity is still not completely understood (Weigel and Meyerowitz, 1994).

### *2.3.8. Reversion from phase transition*

Under certain environmental conditions shoot-like structures have been observed to form after floral organs had been developed, which means that floral determination is not an irreversible developmental condition. This phenomenon is known as “floral reversion”. Three phases have been identified in plant development: 1) vegetative; 2) inflorescence and 3) floral growth (Poethig, 1990). Upon the transition to flowering the apical meristem undergoes an abrupt transition into an inflorescence meristem. The inflorescence meristem is indeterminate and can seemingly give rise to an endless array of lateral meristems in a spiral arrangement. The inflorescence meristem develops into a floral meristem after

floral primordia are formed in place of leaf primordia (Yanofsky, 1995). The floral meristem produces a determinate number of organ primordia in a whorled arrangement. Floral meristems are determined and can not grow vegetative again. The possibility of a reversal from inflorescence back to vegetative phase implies that the genes and processes involved in the transition to flowering are required to both initiate and maintain reproductive development. *Leucodendron* reproductive terminal cuttings that were propagated after flower initiation had taken place showed reversion back to vegetative growth if florets had not yet formed (Ben-Jaacov et al., 1986). *Leucadendron* spp flower during early spring, and flower initiation is presumably established during the preceding winter. Of the cuttings rooted between end October to end December, those from December already showed floret development and did not undergo reversion back to vegetative growth. It is possible that the phase change to reproductive meristem in these cuttings had already been irreversibly determined.

### 2.4. Special constraints in perennials

Research to date has mainly focussed on herbaceous annuals without much attention to woody perennials. Flowering is not the final act of perennial plants, as perennials are polycarpic and some meristems continue vegetative growth. This may mean that the transition to reproductive growth might not be as tightly controlled as in the case of annuals, where the production of reproductive seed is the only way of survival. Flowering in annuals is induced by stresses such as nutrient deficiencies, drought and overcrowding (Levy and Dean, 1998) which produces seeds able to survive where the plant may not have. In the case of perennial plants, the necessity for an internal system exists that can counter the environmental factors that favour flowering in some meristems. Complex interactions between the different plant organs are essential to ensure that some of the meristems continue vegetative growth. Which meristems become flowers may be determined by a competence/juvenility mechanism (Battey and Tooke, 2002). It has been suggested that the undetermined meristems and leaves of perennials are less sensitive to senescence signals. For example, a detached leaf from an annual plant turns yellow much faster than one from a perennial plant (Thomas et al., 2000). Some resources are allocated to sexual reproduction by flowering and some to vegetative growth (Battey and Tooke, 2002). Conservation of vegetative meristems, by failing to impose determinacy on some meristems, is one of the keys to perennial growth. This separation of flowering from a senescence programme, as seen in annuals where death occurs after flowering, is the survival strategy of perennials. The fate of all meristems in perennial as well as annual plants is under the influence of a network of inter-organ long distance signaling. Plants need to be considered as multi-component systems with the meristem the target of the signal, the leaf the floral-signal generator and the plasmodesmata and vascular tissue the

signal conductors (Colasanti and Sundaresan, 2000). An important aspect of perennial growth is that it needs to keep most of its vegetative tissue alive even during flowering. This would mean that not all resources can be relayed to the flowering shoot, but rather need to be distributed in such a way as to sustain the other plant organs as well. Perennials have been neglected as models for the study of flowering because of their large sizes and slow regeneration times, which makes them more difficult to handle in large numbers. Most annuals initiate flowering in response to specific stimuli such as photoperiod or vernalisation, whereas flowering in woody plants is apparently less finely controlled (Jackson and Sweet, 1972). This is another reason why they are less popular for the study of flowering, because flowering is less reliably and repeatedly presented.

### 2.5. Future perspectives for elucidation of floral evocation

To date a fairly large amount of knowledge has been collected to aid in the unraveling of events leading up to flowering and the process itself. The biggest portion of knowledge was obtained from studies on annuals. The fact that flowering is not completely understood in *Arabidopsis*, emphasizes the complexity of this process. Continued studies on *Arabidopsis* will hopefully slowly piece together this complex flowering puzzle, but even then we have not yet started to understand the nature of flowering in perennials. Studies on *Arabidopsis* have led to the characterisation of many of the key genes involved in floral meristem identity. The identified genes have opened a number of doors to identify the roles of these genes with the help from a range of molecular tools. Genetic engineering may play two important roles in the study of flowering. Firstly, overexpressing or silencing of these genes with the use of transformation vectors may prove to be a useful tool in unravelling the gene functions. Secondly, manipulation of some of these genes may lead to early flowering phenotypes and other useful qualities that may be exploited at a commercial level. Recent studies in which this was attempted have proven to be successful in *Citrus sinensis* (Peña et al., 2001). One of these genes, *LFY*, is of particular interest because the transition to flowering in *Arabidopsis* seems to be modulated by *LFY* activity in the meristem (Battey and Tooke, 2002). *LFY* seems to be a multicopy gene in most perennials and the orthologues seem to have different functions in the plant. Analysis of the *LFY* promotor might shed light on the modes of activation of this florally important gene.

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# CHAPTER 3

## Carbon allocation for flowering in the *Protea* hybrids 'Carnival' and 'Sylvia'


### Abstract

The utilization of current photosynthate from subtending shoots for flowering was studied in the *Protea* hybrids 'Carnival' and 'Sylvia' using tracer studies with stable isotopes in 'Sylvia' and $^{14}CO_2$ labeling in 'Carnival'. The $\delta^{13}C$ values of vegetative shoots supplemented with $CO_2$ with a $\delta^{13}C$ value of -29.00‰ were not significantly different from shoots grown in ambient air (*ca.* $\delta^{13}C$= -10‰). The $\delta^{13}C$ values of leaves from flowering shoots harvested from plants on which a vegetative shoot was fed, were significantly lower than those of reference shoots. This indicates translocation from vegetative shoots to shoots supporting a large sink. The $\delta^{13}C$ values of the flowers were also significantly lower than those of the reference flowers. Natural $\delta^{13}C$ values of young leaves (-26‰) were more positive than those of older leaves (-27‰) with flowers having the highest $\delta^{13}C$ values (-23‰). High $\delta^{13}C$ values of flowers may indicate the utilization of stored reserves for flowering in *Protea* spp. Photosynthetic $^{14}CO_2$ labeling of the individual flushes on a three-flush shoot with a dormant apical meristem demonstrated translocation (3 to 5% of assimilated $^{14}C$ present after 24 h) from mostly the top two flushes, the Summer 1 and 2 flushes, to the other flushes and the apical meristem. The Summer 2 flush supplied the most carbohydrates to the resting meristem even when the flush was still young and soft with carbohydrate export increasing as it matured. Benzyl adenine (BA, 6-benzylaminopurine) application had little effect on the carbon import to the apical meristem. The photosynthetic capacity of 'Carnival' shoots with developing flowers showed the contribution of each seasonal flush to be significantly different, with the top (Spring 2004) flush carrying the developing flower having the greatest photosynthetic capacity. Whole shoot photosynthesis increased during inflorescence development until the small flower stage after which it stayed constant. The total respiration rates of the floral bud/tissue also increased significantly with increasing biomass until a small flower had developed, after which the respiration lowered. Since the flowers are large structures (*ca.* 80 g FW) a large amount of carbohydrates is needed for the structural development of flowers. When a small flower was present a large proportion of the tissue, comprising the showy bracts and petals, apparently became relatively metabolically inactive and thus less respiratory activity was required during further development. Daily respiration of the developing flowers used from 2 to 39% of carbon fixed by photosynthesis, depending on the size and developmental stage of the flower. This

indicates the large amount of carbon needed for floral development, but does not include the structural costs of the flowers or the costs for copious nectar production.

### 3.1. Introduction

Breeding of popular South African and Australian Proteaceae genera have lead to the development of a number of commercially cultivated hybrids. Most naturally occurring *Protea* spp. flower ('flower' is used colloquially here to mean the development of the inflorescence) during the winter months (Rebelo, 1995), whereas commercial hybrids have a very unusual flowering pattern with most differing significantly in flowering time. Some, such as *P. eximia* and the hybrid 'Sylvia' (*P. eximia x P. susannae*), flower throughout the year, with a peak flowering time during the warm summer months of January and February (personal observations). Others such as the hybrid 'Carnival' (*P. compacta x P. magnifica*) flower strictly during the late summer between February and May (Greenfield et al., 2001).

Protea exhibits a growth habit with seasonal flushes developing in all seasons except during winter when the apical meristem remains dormant. Floral manipulation in *Protea* has been managed exclusively through pruning (Gerber et al., 1995), but recent results from work by Hoffman (personal communication) on the manipulation of flowering time using cytokinin applications have proven successful. The application of the synthetic cytokinin, benzyl adenine (BA, 6-benzylaminopurine), results in release of the apical meristem from dormancy, when treated in autumn, with the shoot developing an out of season flush in winter (June to July). In most cases, unless some physiological limitations are evident, the shoot will proceed to initiate a flower on the winter flush that will develop during spring. This results in the production of flowers nearly two months prior to the natural flowering time for this hybrid. Involvement of cytokinins in the regulation of source/sink relationships has been suggested previously (Kuiper, 1993; Brenner and Cheikh, 1995; Leopold and Kawase, 1964; Roitsch and Ehneβ, 2000; Yang et al., 2003). BA has been shown to increase carbon import into sink tissue in both apple and pear, with an increase in fruit size and crop load reported after BA application (Stern and Flaishman, 2003; Stern and Flaishman, 2004). Endogenous cytokinin levels also increase in a range of woody perennials before flower initiation and is thought to have a positive effect on flowering (Hoffman, personal communication; Ramírez et al., 2004; Ulger et al., 2004). It has been reported that BA induces bud break in roses (Ohkawa, 1984), although, the precise mechanism of BA action is unknown. It may be speculated that cytokinin sensitive motifs exist in the promoters of key floral genes.

During the development of higher plants, heterotropic embryos develop into photosynthetically active source tissue and less active or inactive sink tissue (Roitsch and Ehneβ, 2000). The source tissue, usually mature leaves, is marked by a net export of carbohydrates that are imported and utilized by sink tissue such as flowers, roots and young leaves. In contrast to annual plants, some of the meristems of perennials have to remain vegetative to continue growth after flowering. Flowering plants produce and support floral organs until successful reproduction, and then the flower dies. Depending on the size of the floral structure as well as the duration of flowering, the carbon input into flowering may represent a substantial cost. Thus the source/sink relationships in perennials must constantly change to allow the development of these large sinks while supporting the less photosynthetically active vegetative structures. Carbohydrates are transported from source tissue in the form of soluble sugars via the phloem to the sink tissue. The stimulatory effects on flowering of fruit thinning may be a result of increased carbohydrate availability (McQueen et al., 2004; Tromp, 2000). Depletion of carbohydrates has often led to inhibition of flowering. It has been recognized that carbohydrates play a signaling role in many physiological processes including juvenile-to-adult transition, gene expression and flowering (reviewed in detail by Gibson, 2005). Evidence for the involvement of sugar in floral initiation, *in vitro*, has been found in *Arabidopsis thaliana* (*Arabidopsis*) (Ohto et al., 2001). Growing *Arabidopsis* on low sugar concentrations promotes flowering and is thought to have a stimulatory effect on floral-identity gene expression. These genes may be part of the pathway that senses carbohydrate availability in the sink tissue, thus allowing the plant to assess the internal carbohydrate status at any given time and initiate flowering at a time when carbon availability is sufficient.

Plants growing in fire-prone environments often store carbohydrates in large underground root systems. These storage carbohydrates are mobilised for regrowth after the aboveground structures have been destroyed. Evidence for this has been found in a large number of woody species and it has been suggested that these carbohydrates may also be mobilised during flowering (Langley et al, 2002). The fynbos habitat in which *Protea* spp. grow is prone to regular fires and one way of supporting regrowth after burning is by storing carbohydrates in large boles (lignotubers) or rootstocks. This regrowth strategy is used by *P. cynaroides* after fire (Rebelo, 1995). This and re-translocation from other surrounding shoots, may explain why the *Protea* hybrid ‘Carnival’ is able to continue growth after shoot defoliation, although flowering on these shoots is sometimes inhibited due to a lack of carbohydrate availability (Gerber et al., 2002; Hoffman, personal communication). The storage and transport compounds of *Protea* spp. are unknown. However, deciduous trees mainly use stored

carbohydrates in the form of starch during spring leaf regrowth (McQueen et al., 2004; Wong et al., 2003).

The aim of this study was to elucidate carbon distribution and redistribution during shoot development and flowering. Does the shoot alone supply the carbon for this expensive endeavor or do the surrounding shoots contribute? The effect of BA on source/sink interaction was also investigated. Tracer studies were used to determine translocation between source and sink tissue and gas-flux analysis was used to determine the photosynthetic capacity of the source leaves and respiratory costs of floral development.

## 3.2. Materials and Methods

### *3.2.1. Plant material*

Plants grown on a commercial plantation on the farm 'Protea Heights' in the Stellenbosch district (latitude 33°54'S; longitude 18°40'E, South Africa) were used for collection of the plant material. The hybrids 'Sylvia' and 'Carnival' were grown without irrigation or fertilization for 3 years. Plants were spaced 1 m in the row and 4 m between rows. The Stellenbosch climate is Mediterranean, with winter rainfall (600 to 700 mm per annum) and hot, dry summers (average maximum 22°C and average minimum 11°C per annum). Commercial practice for the cultivation of 'Carnival' involves pruning for biennial bearing (Gerber et al., 1995). Shoots of the same size and developmental stages were selected to serve as replicates for each experiment.

### *3.2.2. $^{14}CO_2$ labeling of seasonal flushes*

'Carnival' shoots with three flushes of which the apical meristem had entered a dormant state were selected in middle March 2004. Flushes were labeled by sealing one flush of each of five independent plants in a polythene bag, and repeating this for each of the other two flushes. $^{14}CO_2$ was released from 5 µCi sodium[$^{14}$C]bicarbonate (Amersham Biosciences, Buckinghamshire, UK) with a specific activity of 85 mCi per mmol by acidifying with 1.3 ml 20% lactic acid (v/v). The lactic acid was introduced with a syringe needle through the plastic bag and the hole sealed afterwards. Labeling occurred between 09:30 and 10:30 am. Flushes remained sealed in the bags for 2 h to allow the uptake of released $^{14}CO_2$, before the bags were removed. At this time two leaves from the labeled flush were taken to determine the total label that was assimilated from feeding. Leaves were weighed and cut into segments of 0.5 mm and stored at -20°C until analysis. Shoots were harvested after a 24 h period and leaves separated into different flushes and treated as above. The buds were analyzed separately.

*3.2.3. Effect of BA application on $^{14}$carbon distribution*

Shoots of similar size on 3-year old 'Carnival' plants were selected in March 2004. BA (Maxcel™, Valent biosciences corporation, USA) at a concentration of 500 ppm was applied to the dormant bud. BA application involved the 'painting' of the apical meristem resulting in thorough wetting with the BA solution. This was done at three different time points, two weeks apart starting on the third week of March. $^{14}$C labeling of the youngest (Summer 2) flush was performed 48 h after BA application and labeling was done as described above.

*3.2.4. Analysis of $^{14}$C*

Between 0.1 and 0.2 g of fresh leaf tissue was homogenized in liquid nitrogen and extracted in 3 ml 80% ethanol (v/v) for 2 h at 80°C. The extract was filtered through Whatman 1 filter paper and the filtrate washed with 80% ethanol to make up a volume of 25 ml. Preliminary experiments showed only trace amounts of $^{14}$C remained in the soluble fraction after the washing (data not shown). The insoluble fraction was dried on the filter paper for at least 24 h. $^{14}$C in both the soluble and insoluble fraction was counted in a TRI-CARB 2100 TR liquid scintillation analyzer (PerkinElmer Life Sciences, Boston, USA) using Ready Gel™ scintillation cocktail (Beckman Coulter TM, Cat No 68412-54-4) for the insoluble fraction and ULTIMA-FLO™ M scintillation cocktail (Packard Bioscience, Cat No 6013579) for the soluble fraction. For measurement of the insoluble fraction the dried filtrate was scraped from the filter paper and ground in a mortar and pestle. The resulting powder was placed in a scintillation vial, and Ready Gel™ and water added until the particulate matter was equally dispersed in the resulting gel to allow accurate counting of the material. Distribution of $^{14}$C was calculated as a ratio between the label in the measured flush and the total label exported from the leaves of the flush that received the label, taking into account the label that was lost to respiration and export to the parts that was not measured such as roots and stems. This was done for both the soluble and insoluble carbon fractions.

*3.2.5. Natural $\delta^{13}$C abundance in 'Sylvia'*

Flowering and vegetative shoots on 3-year old 'Sylvia' plants were harvested during middle December, and leaves divided into different flushes while flowers were measured separately. Harvested shoots included vegetative shoots with 2 or 3 flushes as well as flowering shoots with 3 and 4 flushes (Fig. 3.1). Samples were oven dried and milled in an M 20 IKA mill (IKA-Werke, Germany). Between

2.100 and 2.200 mg of each sample was weighed into an 8 by 5 mm tin capsule (Elemental Microanalysis Ltd, Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). Samples were combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{13}C$ value for the carbon gas released was determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to the CHN analyser by a Finnigan MAT Conflo control unit. Two in-house standards (Merck Gel and Nasturtium) were used to correct the samples for machine drift. The carbon isotope ratio of the samples was expressed in parts per thousand (‰) calculated using the following equation:

$$\delta^{13}C=[R_{sample}/R_{standard}-1] \times 1000$$

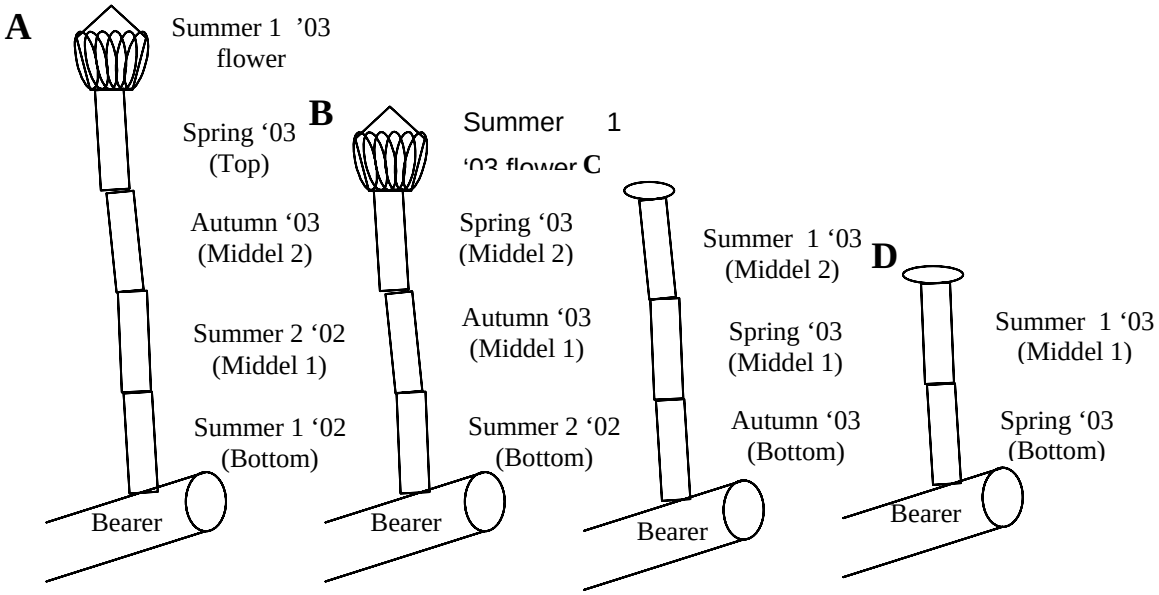


Figure 3.1. Diagrammatic representation of different shoots selected for natural $\delta^{13}C$ abundance measurements. Four flush flowering shoots (A), three flush flowering shoots (B), three flush vegetative shoots (C) and two flush vegetative shoots (D) were utilised.

*3.2.6. Enrichment of ambient air with fossil fuel derived $CO_2$*

Five three year old 'Sylvia' plants with vegetative and flowering shoots were selected. Vegetative shoots were sealed in polythene bags and enriched with $CO_2$ derived from fossil fuel combustion ($\delta^{13}C$ = –29.00‰) (Afrox, Cape Town, South Africa). $CO_2$ concentrations were maintained between 1500 and 2000 ppm $CO_2$ for one week, by mixing the flow from the fossil fuel $CO_2$ with atmospheric air using an air compressor. The $CO_2$ concentration was measured once a day, between 09:00 and 10:30

am, using an ADC Mk3 infrared gas analyser (The Analytical Development Co. LTD., Hoddeston, England). Additional to the fed shoots, shoots on the same bearer, shoots that had been shaded with 80% shade cloth and flowering shoots were selected on each plant and harvested after the one week period. Shoots allowed to grow in ambient air were also harvested and treated as described. Leaves were divided into different flushes and the flowers kept separately. Samples were oven dried and milled using an M 20 IKA mill (IKA-Werke, Germany). Analysis of $\delta^{13}C$ values was performed as described above.

*3.2.7. Photosynthetic capacity of source tissue*

Five year old 'Carnival' shoots with flowers of different developmental stages were selected on flower or developing floral bud, diameter and height (Table 1). Light intensity (LI-190SA quantum sensor, LI-COR Biosciences, Nebraska, USA) at each flush was measured by holding the light sensor parallel and against the stem midway down the flush. This was done for fifty shoots at midday. Photosynthesis was measured using a LI-6400 portable photosynthesis system (LI-COR Biosciences, Nebraska, USA) starting at 10:00 am and ending at 14:30 pm and the light intensity at each flush was set to the average measured intensity. The PAR settings for each flush were: top flush (Spring, 2004) carrying the flower, 1600 µmol $m^{-2}$ $min^{-1}$; Summer 2, 1600 µmol $m^{-2}$ $min^{-1}$; Summer 1, 1000 µmol $m^{-2}$ $min^{-1}$; Spring (2003), 250 µmol $m^{-2}$ $min^{-1}$. Five shoots of each developmental stage were harvested and the leaves divided into flushes. The leaf area of a whole flush was measured using a LI-3000 portable area meter (LI-COR, Lambda instruments corporation, USA). This was used to determine the contribution of the flush to total photosynthesis.

*3.2.8. Respiration of developing floral buds*

The respiration rate of the developing flowers was measured using the criteria for selection described in Table 1. Developing flowers were excised from the shoots, weighed, and small ones were placed in 160 ml cuvettes and larger ones in 260 ml cuvettes with one inlet and one outlet in the lid. Atmospheric air was pumped into the cuvettes from an area with stable $CO_2$ concentrations and the flow rate into each cuvette measured. Airflow from the cuvette was passed through an ADC LCA-2 infrared gas analyzer (The Analytical Development Co. LTD., Hoddesdon, England). The difference in $CO_2$ concentrations from ambient air and after addition from respiration was measured. Measurements were performed at room temperature that was maintained at 22°C.

*3.2.9. Statistical analyses*

Results were subjected to analysis of variance (ANOVA) to determine the significance of differences using the Statistical Package for Social Scientists version 11 (SPSS Inc., Chicago, Illinois). *Post hoc* LSD tests (95%) were conducted to determine the differences between different treatments.

## 3.3. Results

*3.3.1. $\delta^{13}C$ abundance in 'Sylvia'*

Increases in the $\delta^{13}C$ values were observed as the shoot matured and produced new flushes, with the pattern being most significant in the flowering shoots (Fig. 3.2). The $\delta^{13}C$ values of the different developing shoots followed the same pattern, except for the fourth flush of the flowering 4 flush shoot which deviated from the others and had a more negative value. Flowers had significantly lower $\delta^{13}C$ values than the leaf tissue.

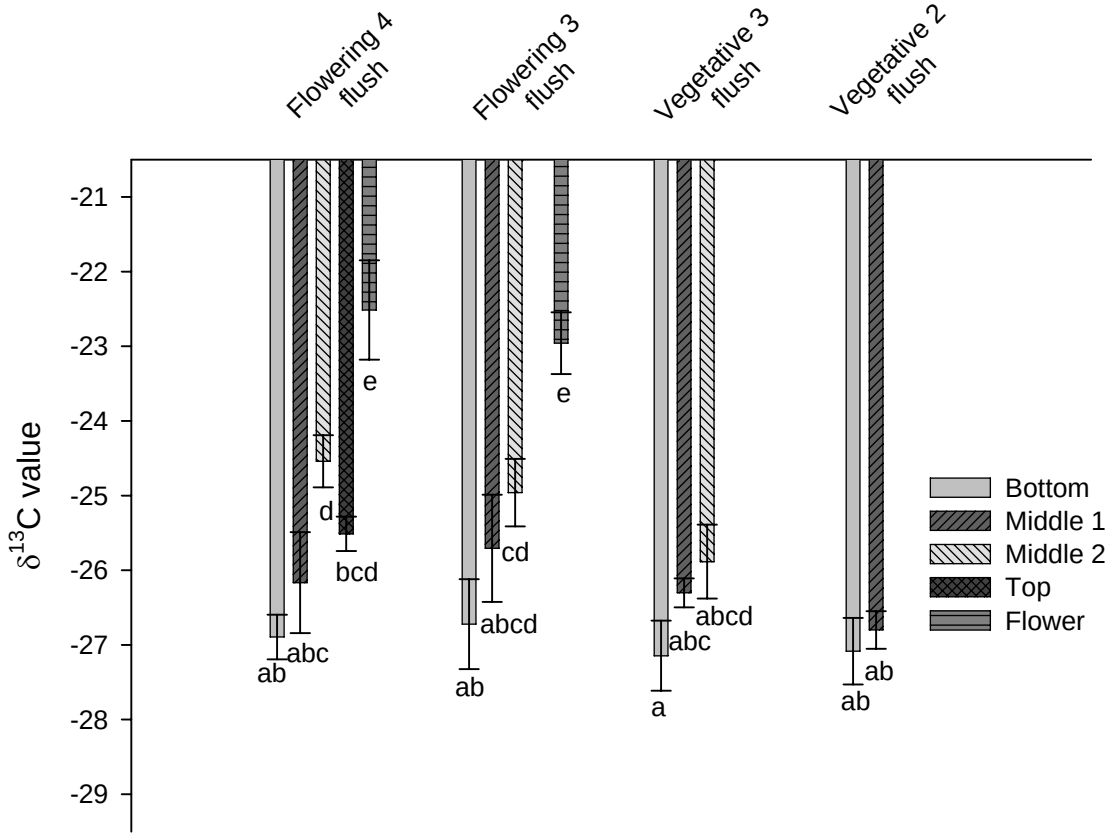


**Figure 3.2**. Natural $\delta^{13}C$ abundance in 'Sylvia' leaves from shoots at different developmental stages harvested in the middle of summer. Leaves from flushes were labeled from the bearer as described in Fig. 3.1. Letters below the SE bars indicate significant differences ($P<0.05$, ANOVA with *post hoc* LSD, n=5).

Enriching $CO_2$ surrounding a vegetative shoots with $CO_2$ resulting from fossil fuel combustion, resulted in a decrease in the $\delta^{13}C$ values of young leaves, although the change was not significant (Fig.3.3). Older leaves were less affected by the feeding than younger leaves. The $\delta^{13}C$ values of young and old leaves from reference shoots were significantly different and are comparable to the results from the natural isotopic fractionation (Fig. 3.2). Overall the strongest effect of fossil fuel $CO_2$ enrichment could be observed in the flowering shoot tissue, both in the young leaves and flowers. Flowering shoots from the same plants as the fossil fuel $CO_2$ enriched vegetative shoots showed the greatest difference in $\delta^{13}C$ values. The $\delta^{13}C$ values of the young leaves and flowers on these flowering shoots were more negative than those of the reference flowering shoots that were harvested from plants allowed to grow in ambient air.

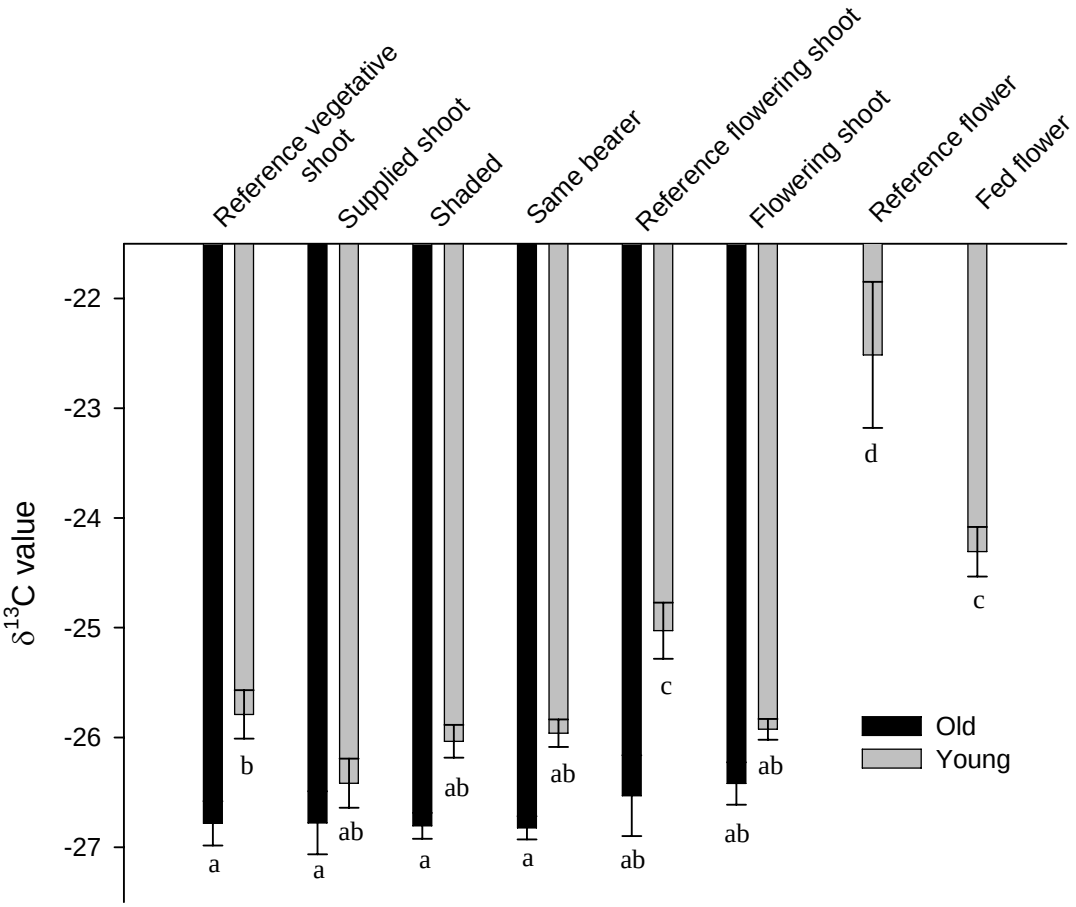


**Figure 3.3**. $\delta^{13}C$ abundance in 'Sylvia' (P. *eximia x P. susanneae*) leaves and flowers after enrichment of the ambient air surrounding a vegetative shoot ('Supplied shoot') with $CO_2$ resulting from fossil fuel combustion. Letters below the SE bars indicate significant differences ($P<0.05$, ANOVA with *post hoc* LSD, n=5).

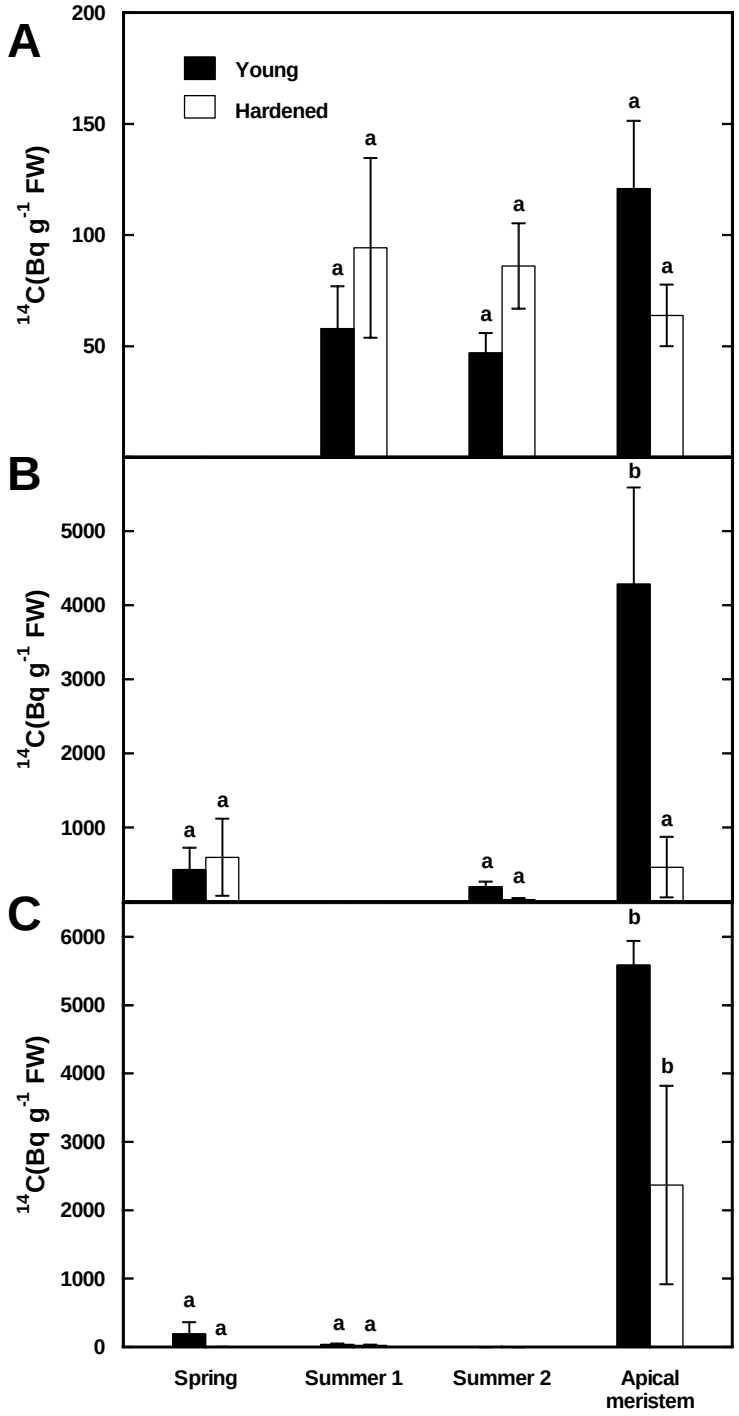


**Figure 3.4.** Carbon contribution of each seasonal flush expressed (oldest flush (Spring 2003), middle flush (Summer 1) and the youngest flush (Summer 2) as Bq per gram tissue remaining after 24 h. Total label was calculated for the label in each flush, including the labeled flush, as well as in the apical meristem. Labeling was done at two time points, when the Summer 2 flush was still young and after it had matured. Shoots consisted of three flushes that are named according to the season in which they were formed. Spring (**A**), Summer 1 (**B**) and Summer 2 (**C**) flushes were labeled. Letters above SE show significant differences between treatments. ANOVA, with *post hoc*, LSD test ($P<0.05$, $n=5$).

*3.3.2. Carbon distribution from seasonal flushes*

Flushes labeled with $^{14}CO_2$ at two distinct developmental stages indicated a shift in carbohydrate supply to the dormant apical meristem as the flush carrying the bud, the Summer 2 flush, matured (Fig. 3.4). The oldest leaves, from the Spring 2003 flush, exported the least carbon to any of the other two flushes and meristem. Even when the top, Summer 2, flush was still young (characterized by soft, light-green leaves and an un-wooded stem) it contributed the greatest amount of carbon to the resting meristem. The remaining bulk of carbon required for meristem survival was imported from the Summer 1 flush which carried mature leaves. After the Summer 2 flush had matured the carbon supply from the Summer 1 flush to the apical meristem decreased. There was no significant difference between the supply of carbon from the summer 2 flush between the young and mature stages. BA application did not alter the carbohydrate distribution from the summer 2 flush after $^{14}CO_2$ labeling (Fig. 3.5). This was when the summer 2 flush was still soft and young.

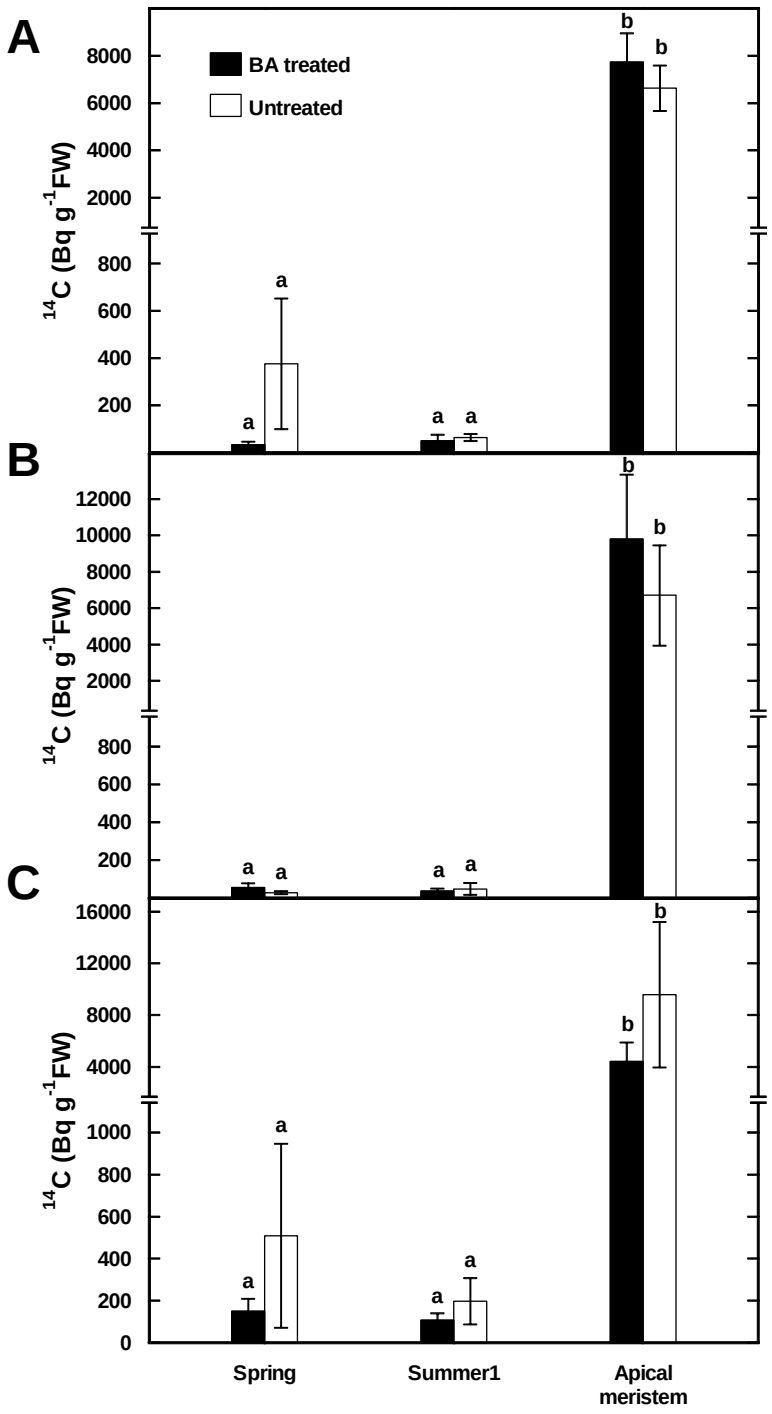


**Figure 3.5**. The effect of BA application on the distribution of $^{14}$C from the Summer 2 flush. 500 ppm BA was applied by complete wetting of the apical meristem 48 h before labeling. Flushes were labeled at three dates encompassing a period identified as being appropriate for BA elicited floral induction: A – 14/04/2004, B – 28/04/2004 and C – 5/05/2004. Letters above SE shows significant differences determined by ANOVA, with *post hoc*, LSD tests ($P<0.05$,n=5).

*3.3.3. Photosynthetic contribution of source leaves and the respiration rate of floral tissue*

The total photosynthetic contribution of shoots subtending the different developing flowers increased until the small flower stage after which it stayed constant (Fig. 3.6A). Respiration of floral buds and flowers increased as the flower developed, until it reached the small flower stage. The large floral structure, however, had significantly lower respiration than the small flower (Fig. 3.6B).

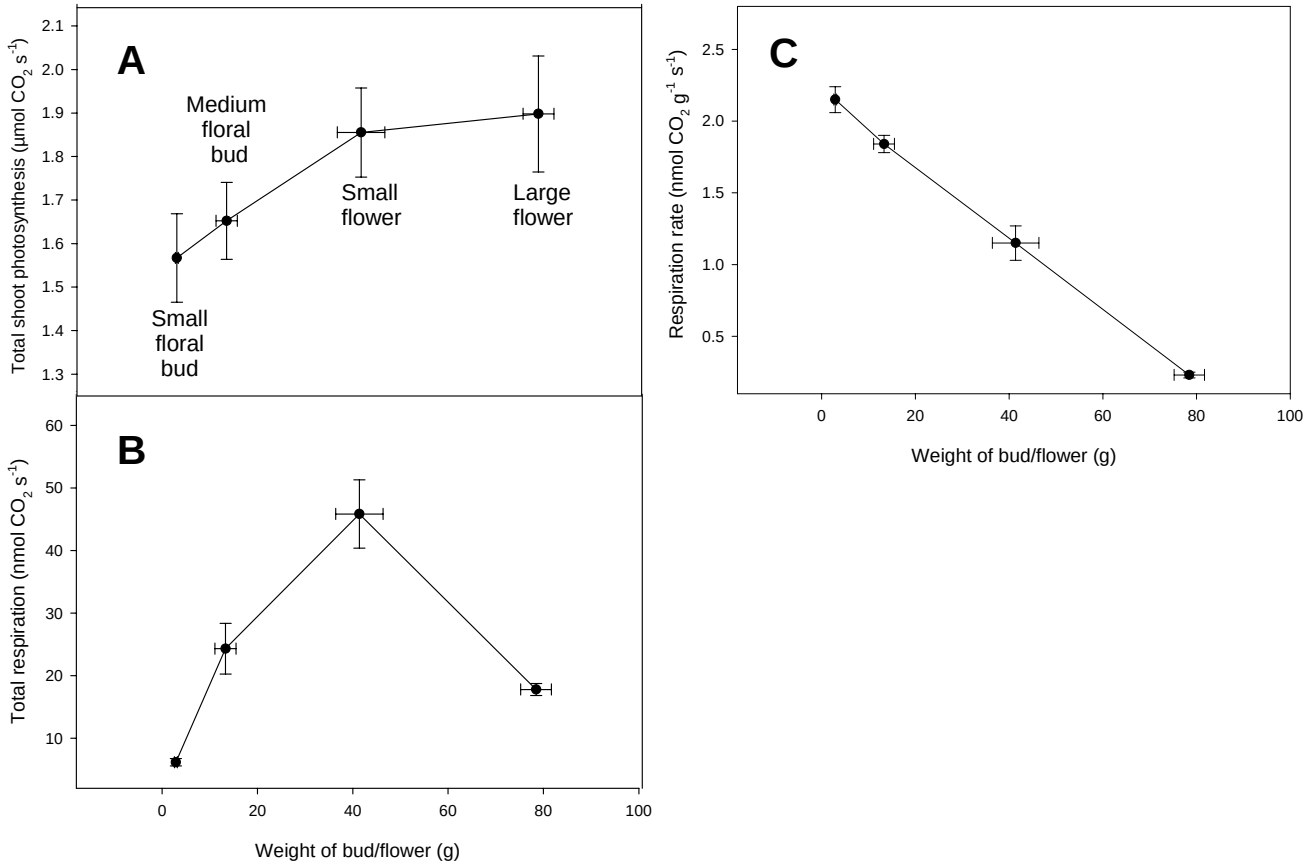


**Figure 3.6**. **A**. Photosynthetic capacity of leaves from shoots versus the weight of developing buds/flowers. **B**. Respiration of buds/flowers versus their weight. **C**. The specific respiration rate (µmol $CO_2$ $g^{-1}$ floral bud FW $s^{-1}$) versus the weight of developing floral tissue. The fresh weight corresponding to the developing stages was: small floral bud, 2.88 g; medium floral bud, 13.30 g; small flower, 41.48 g and large flower, 78.46 g.

The respiration rates of these metabolic sinks was, however, still *ca*. 50 fold lower than the total photosynthetic capacity of the source leaves. The percentage of the carbon fixed by photosynthesis on a daily basis (assuming 10 h effective photoperiod and constant light intensity and temperature) that was calculated to be required for the daily respiration rate (assuming constant temperature) of

buds/flowers; small floral bud, medium floral bud, small flower and large flower were 2, 12, 35 and 39%, respectively. Respiration rate in the sink tissue decreased as the weight of the tissue increased with development of the flower indicating reduced specific costs associated with mature floral structures (Fig. 3.6C). The percentage contribution of each of the seasonal flushes to total shoot photosynthesis was significantly different. The Spring 2004 flush that carried the developing flower had the greatest photosynthetic capacity, 45 to 55% (Fig. 3.7). Photosynthetic capacity decreased in the older flushes with the oldest flush, Spring 2003, contributing only 10% to total photosynthesis and the Summer 1 and Summer 2 flushes contributing 15 to 20% and 20 to 30%, respectively.

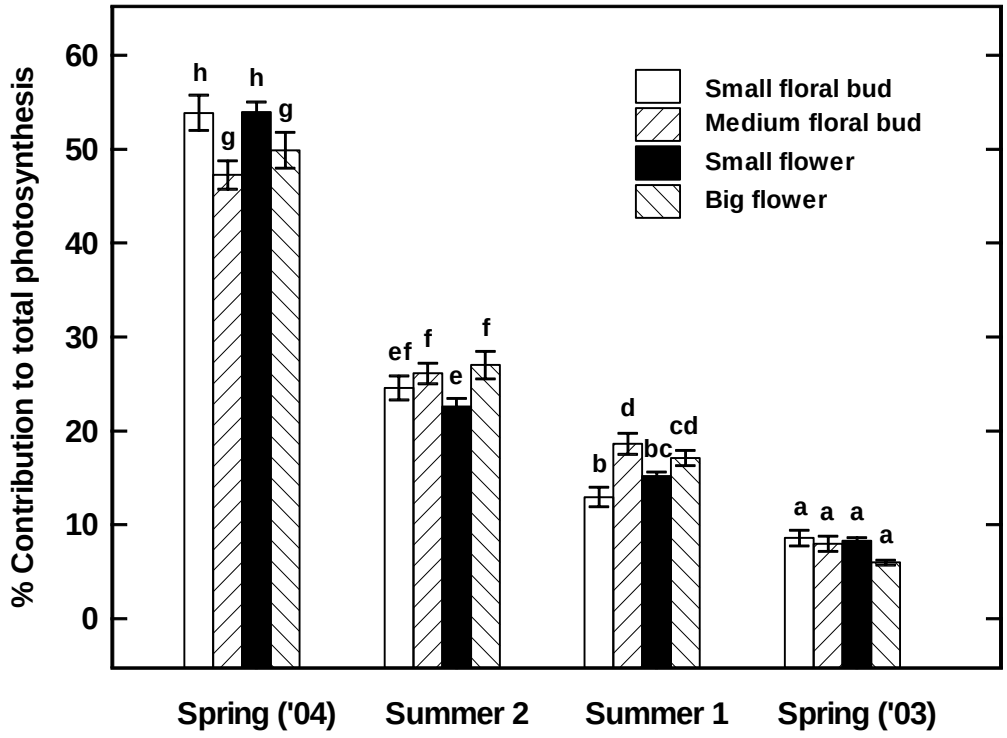


**Figure 3.7.** Percentage contribution of each seasonal flush to total photosynthesis for the four different developmental stages shown in Table 3.1. Different letters above SE bars indicate significant differences determined by ANOVA with *post hoc* LSD tests ($P<0.05$, $n=10$).

Light intensities differed significantly within the plant canopy (Table 3.1). The light intensity at the top two flushes, Spring 2004 and Summer 2, did not vary significantly and was 1606 and 1532 μmol $m^{-2}$ $s^{-1}$ respectively. Within the canopy the intensities were approximately 60% (Summer 2) and 16% (Spring 2003) of that received by the top two flushes. Only the Spring 2004 flush of the small floral bud shoot, with a leaf area close to 1000 $cm^2$, had a significantly different leaf area when compared to the other

developmental stages. The photosynthetic rate of this flush was, however; significantly lower than that of the other flushes. The stomatal conductance of the Spring 2004 flush of the big flower shoot was significantly higher than all the other developmental stages ($P<0.05$). This might explain the high contribution of the photosynthetic rate of this flush to the total available from the shoot.

**Table 3.1**. Characteristics of flowers or developing floral buds used for selection of experimental material and physiological characteristics of the shoots bearing the different stages.

| Morphological characteristic | Seasonal flush | Diameter | | Light Intensity | Leaf area | Photosynthesis rate | Stomatal Conductance |
|---|---|---|---|---|---|---|---|
| | | Width | Height | | | | |
| | | mm | mm | µmol $m^{-2}$ $s^{-1}$ | $cm^2$ | µmol $CO_2$ $m^{-2}$ $s^{-1}$ | µmol $H_2O$ $m^{-2}$ $s^{-1}$ |
| Small floral bud | Spring ('04) | 14-20 | 13-17 | 1606 ± 37 a | 976 ± 67 h | 9.2 ± 0.7 cdef | 0.09 ± 0.01 abc |
| | Summer 2 | | | 1532 ± 72 a | 365 ± 39 bcde | 11.1 ± 0.8 efgh | 0.18 ± 0.03 ef |
| | Summer 1 | | | 1005 ± 94 b | 288 ± 34 abcd | 7.4 ± 0.7 bc | 0.13 ± 0.02 bcdef |
| | Spring ('03) | | | 253 ± 46 c | 258 ± 25 ab | 5.4 ± 0.4 ab | 0.09 ± 0.02 abc |
| Medium floral bud | Spring ('04) | 20-35 | 25-55 | | 623 ± 23 f | 11.8 ± 0.8 ghi | 0.15 ± 0.02 cdef |
| | Summer 2 | | | | 405 ± 17 de | 10.0 ± 0.5 defg | 0.13 ± 0.02 bcdef |
| | Summer 1 | | | | 357 ± 32 bcde | 8.3 ± 0.7 cde | 0.10 ± 0.02 abcd |
| | Spring ('03) | | | | 262 ± 26 ab | 5.0 ± 0.7 a | 0.04 ± 0.01 a |
| Small flower | Spring ('04) | 35-45 | 60-90 | | 760 ± 91 g | 13.4 ± 0.9 l | 0.17 ± 0.03 cdef |
| | Summer 2 | | | | 411 ± 23 e | 10.5 ± 1.0 efgh | 0.13 ± 0.04 bcdef |
| | Summer 1 | | | | 364 ± 19 cde | 8.0 ± 0.7 cd | 0.09 ± 0.03 abcd |
| | Spring ('03) | | | | 281 ± 25 abc | 5.6 ± 0.4 ab | 0.04 ± 0.02 a |
| Large flower | Spring ('04) | 45-50 | 100-130 | | 690 ± 27 fg | 13.4 ± 1.0 l | 0.21 ± 0.02 f |
| | Summer 2 | | | | 396 ± 20 cde | 12.7 ± 1.0 hi | 0.19 ± 0.03 ef |
| | Summer 1 | | | | 367 ± 23 bcde | 8.6 ± 0.6 cde | 0.12 ± 0.02 cde |
| | Spring ('03) | | | | 197 ± 13 a | 5.6 ± 0.3 ab | 0.06 ± 0.01 ab |

Bud and flower diameter represents the measurement of 10 buds or flowers of which the minimum and maximum was taken as extremes. The average light intensities measured within the canopy is the average of 50 measurements. Leaf area, photosynthesis rate and stomatal conductance is the average of 10 replicates. Letters next to SE indicates significant differences determined by ANOVA with *post hoc* , LSD tests ($P<0.05$).

### 3.4. Discussion

Flowers, which are large metabolic sinks, had significantly higher $\delta^{13}C$ values than the foliar source tissue that produces carbohydrates for development and maintenance of these large structures (Fig. 3.2). Increase in $\delta^{13}C$ values from the expected values may indicate remobilization of carbon from storage tissue, rather than current photosynthate utilization (Helle and Schleser, 2004; Langley et al., 2002). The seasonal differences in shoot formation failed to show a distinct pattern that can be attributed to water availability or other environmental conditions. All flushes are formed during spring, summer and autumn months when light is not limiting and the formation of flushes could thus be supported by current photosynthate produced in mature flushes. We expected an indication of the use

of stored carbons for the initial regrowth from the bearing shoots and therefore, a more positive $\delta^{13}C$ value. However, it is also possible that resprouting could have been dependent on current photosynthate from surrounding shoots. Shoot to shoot translocation was indicated by a reduction in the $\delta^{13}C$ values of shoots on the same plant when a vegetative shoot was supplied with $CO_2$ enriched in $^{12}C$ (Fig. 3.3). The greatest response was seen in the flowering shoots where a large metabolic sink, the developing flower, was present. The lack of reduction in $\delta^{13}C$ values of the vegetative shoot enriched in fossil fuel $CO_2$ may be explained by the translocation of recently assimilated material to the other components and shoots. The signal was thus 'shared' amongst the other shoots in the form of carbon with a reduced $\delta^{13}C$ value that reduced their $\delta^{13}C$ values slightly. However, the carbon incorporated during the one week of supply represents only a small portion of the total carbon incorporated in the shoots during growth in ambient air and thus the influence on the $\delta^{13}C$ values was expected to be small. The flowering shoot constantly supplies large amounts of carbon to the flower for structural development and nectar production. Thus, the significantly larger amount of carbon imported by this shoot compared to the other surrounding shoots may explain why a significant effect was seen in this shoot.

The increase in $\delta^{13}C$ values as the shoot formed more flushes (Fig. 3.2) was not evident in the vegetative shoot with only two flushes. Thus the older shoots might lose $^{12}C$ during their development as a result of heterotrophic support of floral development and young developing shoots. Helle and Schleser, 2004, observed a similar pattern in four deciduous tree species, with a $^{13}C$ enrichment in the leaves formed during Spring regrowth. The remobilisation of $^{13}C$-enriched starch stored during the day in the form of $^{13}C$-enriched sugars and incorporation in developing flushes might also contribute to the pattern observed. This has been suggested by other authors to be the reason for a difference in the $\delta^{13}C$ values of wood versus foliage (Helle and Schleser, 2004). Previous experiments have suggested that the refixing of respired $CO_2$ (Gillon and Griffiths, 1997; O'Leary, 1981) may lead to a more negative $\delta^{13}C$ value being incorporated. Leaves near the ground in dense forests have significantly more negative $\delta^{13}C$ values than leaves from higher elevations (O'Leary, 1981). This may also explain the more negative $\delta^{13}C$ values of the bottom flushes (Fig. 3.2) that are surrounded by a number of respiring leaves on other shoots within the canopy (Fig. 3.2), as well as the pattern between young and old leaves in the labeling experiment (Fig. 3.3). The very positive $\delta^{13}C$ values of flowers could thus be attributed to the large contribution of photosynthate from the top flush that has a more positive signal $\delta^{13}C$ (Fig. 3.3, Fig. 3.7). However, the Protea leaf canopy is not very dense and high average wind speeds (annual

average of 5.2 m $s^{-1}$, data from South African Weather Bureau) would ensure high turnover of canopy $CO_2$. Thus remobilisation of stored reserves, from underground storage system and other shoots may well contribute to the increase in $\delta^{13}C$ values in younger tissue and in the floral structures (Fig. 3.8).

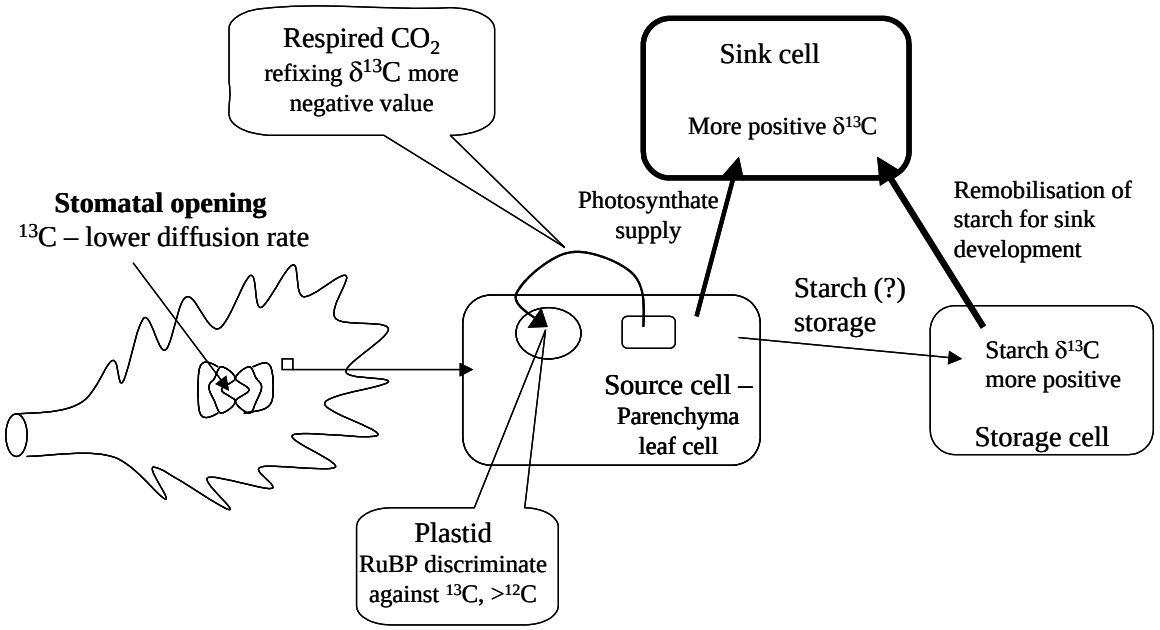


**Figure 3.8**. Diagrammatic representation of $\delta^{13}C$ abundance in different compartments of the plant. The first level of discrimination is the diffusion rate of the $CO_2$ isotopes into the leaf through the stomatal opening. Stomatal conductance controlled by environmental conditions may also influence the fractionation. Secondly, rubisco bisphosphate carboxylase discriminates against $^{13}CO_2$, preferentially fixing $^{12}CO_2$. Refixation of respired $CO_2$ has been shown to decrease $\delta^{13}C$ values (Gillon and Griffiths, 1997; O'Leary, 1981). The starch and lipid fractions in plants are enriched in $^{13}C$ compared to the other metabolite fractions (O'Leary, 1981). The remobilization of starch from storage tissue as well as the supply of current photosynthates, usually from the young mature leaves (more positive $\delta^{13}C$ values), could lead to a more positive $\delta^{13}C$ signal in strong sink tissue.

$^{14}CO_2$ labeling of different seasonal flushes at two time points during the development of the top flush (Summer 2), firstly young and soft and at the second matured, showed the translocation of carbon from each flush to the surrounding flushes and the apical meristem. Flushes kept most of the incorporated $^{14}C$, present after 24 h, for sustaining their own development, with the percentage of $^{14}C$ translocated to other flushes and the apical meristem ranging from between 0.3 and 5%. Although the amount of carbohydrates transported from these flushes are only a small percentage of the total, $^{14}C$ feeding was only conducted for 2 h and shoots were harvested 24 h later. In the fossil fuel $CO_2$ feeding experiment, feeding was done for one week and that may be why most of the carbohydrates with the altered $\delta^{13}C$

values were dispersed between the different shoots. The oldest flush, Spring (2003), retained 99.7% (Fig. 3.4A) of the incorporated $^{14}C$ and only contributed 10% to the total photosynthesis of a shoot with a developing flower (Fig. 3.7). Summer 1 and Summer 2 flushes translocated the largest amount of label to the dormant apical meristem (Fig. 3.4). Although the shift in carbon translocation from the different flushes at the two developmental stages was not large, differences can be seen between the contributions of the Summer 1 flush at the two time points (Fig. 3.4B). However, at both stages the Summer 2 flush contributed the largest amount of $^{14}C$ to the apical meristem (Fig. 3.4C). It thus seems that the dormant bud needed a fixed amount of carbon to stay metabolically active and the source of this carbon only shifted slightly between the Summer 1 and Summer 2 flushes as the Summer 2 flush developed.

BA application had no significant effect on the carbon allocation from source tissue to the treated apical meristem (Fig. 3.5). BA is used in the fruit industry to increase fruit size in apple and pear (Stern and Flaishman, 2003; Stern and Flaishman, 2004). BA increases cell division and therefore increases the sink strength of fruit, leading to increased carbon import (Machackova et al., 1993; Wismer et al, 1995). This assay was however a rather weak way to determine the effect of BA on source/sink relationships because of the relatively high turnover of carbon as a result of respiration. Thus arriving $^{14}CO_2$ may have been lost through respiration. The increase in sink strength might also have been met by the remobilisation of stored carbohydrates, as was shown by the $\delta^{13}C$ natural abundance studies to be the case for the very positive $\delta^{13}C$ value of the flowers (Fig. 3.2). Labeling was conducted 48 h after BA application, a greater response might have been seen if the shoot was allowed a longer period to respond to BA application.

The percentage photosynthetic contribution of each flush of a shoot carrying a developing flower differed significantly, with the older flushes contributing less than the younger ones (Fig. 3.7). Flushes deeper in the canopy are exposed to lower light intensities (Table 3.1), with the Spring 2003 flush only receiving 16% of the light that the Spring 2004 flush was exposed to. This may explain why the Spring 2003 flush only contributes 10% to total photosynthesis of the shoot. The respiration rate provides an indication of the sink strength of developing tissue. Big flowers showed lower respiration than the developing flowers. This reduction in respiration rate might be due to the fact that the structural development in this organ has been completed. The fact that flowers represent a significant carbon drain on the shoot was demonstrated by the fact that the respiration rates of the flowers were estimated

to consume a large proportion (up to 35%) of the daily photosynthate. Development of floral structures in *Protea* spp. can take up to three months from the first morphological observations of a floral bud to the formation of a mature flower. This makes flowering a very expensive endeavour in *Protea* spp. As the flower develops and increases in weight the respiration rate decreases. Although the respiration rates of the small structures were high compared to the larger flowers, the weight is also so small that these developing sinks need a smaller proportion of carbon.

## Conclusions

The significant differences between the $\delta^{13}C$ abundance of leaves and flowers may indicate that sink development incorporates carbon from both storage and current photosynthesis in *Protea* spp. Photosynthetic capacity of flushes differed significantly, but in all cases the oldest, bottom flush contributed the least to flower development, while the subtending flush contributed the most. This was true even for a young, developing flush carrying a dormant apical meristem. Flowers form significant carbon sinks to meet structural costs, maintenance and nectar production. The fact that carbohydrates are essential for floral development and support makes it likely that they play an important role in floral initiation and control, perhaps directly controlling the key genes in floral initiation.

## Acknowledgments

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# CHAPTER 4

## Identification of a homologue of *LEAFY* in Proteaceae and profiling of expression during the transition to flowering


### Abstract

A partial sequence of a *LEAFY* (*LFY*) homologue, dubbed *PROFL*, was amplified from the *Protea* hybrid 'Carnival'. *PROFL* shows 77% sequence homology with the meristem identity gene *LFY* from *Arabidopsis* and 84% homology with *AFL1* and 85% homology with *AFL2* from apple. *PROFL* was found to be expressed in other Proteaceae such as naturally occurring *Protea repens*, the commercial *Protea* hybrid 'Sylvia', the *Leucospermum* hybrid 'Succession' and the *Leucodendron* hybrid 'Safari sunset'. Therefore, it appears that *PROFL* is conserved throughout the Proteaceae. *PROFL* was expressed in vegetative meristems as well as reproductive meristems and in leaves. Expression was high in dormant buds and decreased at the onset of bud break (release of dormancy). Before complete elongation of the developing flush, *PROFL* expression increased gradually until floral bud formation possibly indicating that floral initiation occurred at the time the expression started to increase. mRNA levels in leaves stayed low, except when the expression started to increase in the apical meristems. At this time a sharp increase in leaf *PROFL* expression was observed. High levels of *PROFL* expression in leaves may inhibit the formation of new leaves and in such a way inhibit vegetative growth. Although BA (Benzyl adenine, 6-benzylaminopurine) promoted the release of apical meristem dormancy and led to early flower initiation, there was no evidence of a direct interaction of BA with the flowering gene *PROFL*. Morphological characteristics of the BA treated buds showed developmental steps associated with early bud break compared to untreated shoots to be advanced by approximately one month. *PROFL* expression in BA treated meristems started to increase about one month before the untreated plants, but showed the same *PROFL* expression patterns in the apical meristems and leaves as the untreated plants. Thus *PROFL* expression is likely to provide a convenient marker for the induction of flowering, and a tool for investigation of control of flowering.


### 4.1. Introduction

*Protea* spp. and commercially cultivated hybrids exhibit a seasonal flush growth habit with the number of flushes ranging from one to two per season. The production of vegetative flushes and transition to reproductive growth are influenced by the environmental conditions and the genotype. Flowers (the

term ‘flower’ is used colloquially to describe the inflorescence) are borne terminally on a shoot consisting of two or more flushes (Coetzee and Littlejohn, 2001). Naturally occurring species flower during the autumn and winter months, but the flowering times of commercially developed hybrids differ significantly from parent species and each other. Therefore a single environmental signal responsible for the initiation of flowering in this diverse genus is unlikely. For instance, some members of the Proteaceae may be day-length sensitive (Hettasch, personal communication), whereas this does not appear to be the case in the genus *Protea*.

Expression of the floral meristem identity gene *LEAFY* (*LFY*) in *Arabidopsis thaliana* (Arabidopsis) and *FLORICAULA* (*FLO*) in *Antirrhinum* (Snapdragon) is responsible for the transition of vegetative meristems to a floral fate (Coen et al., 1990; Weigel and Nilsson, 1995). LFY in Arabidopsis has been shown to code for a transcription factor that controls developmental transition by inducing the expression of a second transcription activator *APETALA 1 (AP1)* as well as the expression of at least five other genes involved in the switch to reproductive growth (William et al., 2004). The importance of *LFY* in the floral transition of annuals has resulted in a search for *LFY/FLO* homologues in a range of woody perennial plants (Carmona et al., 2002; Rottmann et al., 2000; Mouradov et al., 1997; Mellerowicz et al., 1998; Frohlich and Parker, 2000; Dornelas et al., 2004; Southerton et al., 1998; Wada et al., 2002). Many of these studies have led to the genetic transformation of these agriculturally important plants to ultimately reduce the duration of their juvenile phase (Peña et al, 2001; Rottmann et al., 2000). *LFY* expression in vegetative structures such as leaf primordia has been found in Arabidopsis (Blázquez et al., 1997), suggesting that *LFY* has a more diverse role in flowering other than simply meristem determination. The expression of *LFY* homologues in a range of angiosperms has also been shown not to be specific to the reproductive phase, but is expressed to varying degrees during vegetative growth (Mouradov et al., 1998), although its function during this time is unclear. Genetic transformation of *Arabidopsis* with homologues of *LFY* and *FLO* from woody perennial has shown flower-promoting effects comparable to constitutive expression of the endogenous genes (Mouradov et al., 1998; Rottmann et al., 2000; Southerton et al., 1998).

Synthetic cytokinin (BA; Benzyl adenine, 6-benzylaminopurine) applications have been shown to shift flowering time in the Protea hybrid ‘Carnival’ (*P. compacta x P. magnifica)* (Hoffman, personal communication). Cytokinin application to ‘over-wintering’ dormant meristems of ‘Carnival’ leads to an earlier release of dormancy in the treated buds, and production of flowers nearly two months earlier than normal. The plant hormone gibberellin ($GA_3$) has been shown to promote flowering in

*Arabidopsis* by acting on the *LFY* promotor (Blázquez et al., 1998). Cytokinins may also be involved in the control of gene expression, especially in the shoot apical meristem. Cytokinins can initiate shoots from callus in tissue culture and the initiation of ectopic meristems in cytokinin overproducing plants has also been previously described (D'Agostino and Kieber, 1999).

The genetic control of flowering in annuals has been studied extensively, especially in *Arabidopsis*. Control of flowering in woody perennials where some meristems are marked for a floral state and others have to continue vegetative growth is a very complex system and still largely unknown. Large size and long juvenile periods make studying flowering in woody perennials difficult, therefore the compatibility of the annual flowering model with perennials such as *Protea* needs to be determined. The aim of this work was to identify a *LFY* homolog in *Protea* spp, *PROFL* (***PR****OTEA* ***F****LORICAULA* ***L****EAFY*), and determine if its expression pattern is comparable to that of *LFY* from Arabidopsis. We studied *PROFL* expression patterns during the vegetative and reproductive development of the *Protea* hybrid 'Carnival'. *PROFL* expression was also investigated after BA application.

## 4.2. Materials and Methods

### *4.2.1. Plant material*

Plant tissue was collected from a farm, 'Protea Heights', in the Stellenbosch district (latitude 33°54'S; longitude 18°40'E), South Africa. All plants are grown without fertilization and irrigation. Weed management is practiced on all cultivars except *P. repens* that grows unmanaged on the farm.

### *4.2.2. Extraction of RNA for RT-PCR*

RNA was extracted from tissue frozen in liquid nitrogen and stored at –80°C. Tissue was ground in an IKA A11 mill (IKA-Werke, Germany). Tissue (3 g FW) was added to 15 ml pre-warmed (65°C) CTAB-buffer (2% CTAB (w/v), 2% PVP-40 (w/v), 100 mM Tris (pH 8.0), 25 mM EDTA, 2.0 M NaCl) containing 2% (v/v) β-mercaptoethanol and 3.4 mM spermidine. Tissue was extracted at 65°C for at least 30 min, mixing frequently, and subsequently centrifuged at 10 000 g at 25°C for 10 min. The supernatant was extracted twice with an equal volume of chloroform, centrifuging at 10 000 g, 25°C for 10 min to separate the two phases, removing the top phase after each extraction. RNA was precipitated by addition of 0.5 volumes of 10 M LiCl and incubation at -20°C overnight. Precipitated RNA was recovered by centrifugation at 10 000 g at 4°C for 20 min. The pellet was subsequently washed with 1 ml 70°C ethanol and transferred to an eppendorf tube. RNA was recovered by micro

centrifugation at 13 000 g for 10 min. The pellet was dried under a laminar flow to allow evaporation of ethanol, after which it was dissolved in 100 µl autoclaved milli-Q (Millipore,USA) water.

*4.2.3. RT-PCR*

RNA (4 µg) were treated with RNnase-free Deoxyribonuclease I, as described by the manufacturer (Fermentas Life Sciences, Madison, USA). Treated RNA was used for first strand synthesis using Supercript III (Invitrogen life technologies, California, USA), and subsequently treated with RNnase H (Promega, Madison, USA) to remove any remaining RNA. 2 µl of the reaction was used as template for PCR with Super-Therm Polymerase (Southern Cross Biotechnology, Cape Town, South Africa), using 1.5 mM $MgCl_2$. The linearity of the amplification was determined by removing 5 µl of the reaction after 20, 25 and 30 PCR-cycles. Primers used to amplify the *LFY* homologue, *PROFL*, were designed for apple by Kotoda et al. (2000) and amplify a 440 bp fragment within exon 2 that is highly conserved between species (Fig. 4.1). This fragment was cloned into pGEM®-T easy vector system (Promega, Madison, USA) and sequenced using Basecaller-3100 version 3.7 (Department of Genetics, University of Stellenbosch, South Africa). Quantitative RT-PCR's were normalised by simultaneous amplification of actin by using primers designed for grapevine: 5' -TCACACTTTCTACAATGAGCT-3' and 5'-GATATCCACATCACACTTCAT-3'.

*4.2.4. Effect of cytokinin application on PROFL expression*

'Carnival' shoots of similar size and developmental state was selected and the apical meristem painted with 500 ppm BA (Maxcel$^{TM}$, Valent biosciences corporation, USA), by thorough wetting of the apical meristem, at the start of the experiment. Ten shoots were harvested 48 h later and then every two weeks thereafter. Untreated shoots were also harvested. Six sample collectings, each two weeks apart, were done in total of the treated shoots and eleven of the control shoots. At the end of the experimental period, flowers were collected and the tissue divided into involucral bracts and individual flowers from the inflorescence. Buds were excised and vegetative leaves from under the bud were removed, frozen in liquid nitrogen and stored at –80°C for RNA extraction. RNA was extracted as described previously.

## 4.3. Results

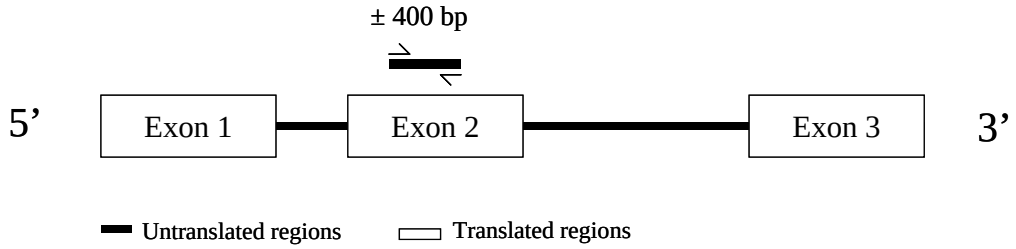


**Figure 4.1**. Diagrammatic representation of the *LFY* gene from *Arabidopsis* (www.ncbi.nlm.nih.gov/entrez). The whole sequence is approximately 2638 bp. 400 bp were amplified from a highly conserved region in exon 2.

```
                    10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|
PROFL               GTGGCCCGTGGCAAAAAGAACGGTATTGATTATCTCTTCCACCTCTACGAGCAGTGCCGTGATTTCTTGA
APPLE AFL1          .....A..................C.......C......T.T.........T..............
APPLE AFL2          .....G..................CC.C.....C........T.....................C.........
ARABIDOPSIS LFY     .....A..................CT.A........G......T.G.....A..A........G...C.TC

                    80        90        100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
PROFL               TTCATGTTCAGAACATCGCCAGGGAGCGTGGTGAAAAATGCCCCACCAAGGTGACGAACCAAGTGTTCAG
APPLE AFL1          .C..G..C........T....A......C..........T..A..........A.....G.....T..
APPLE AFL2          .C..G..C........T....A......C..........T.............A..........T..
ARABIDOPSIS LFY     ....G..C....CA..T..T.AA..C.....C.....................................A.....

                    150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
PROFL               ATATGCAAAGAAGGTAGGAGCAAGCTACATAAACAAGCCGAAAATGAGGCATTACGTGCACTGCTACGCA
APPLE AFL1          G.....C..A...TC...C..........C..........G...C....C..T..............C
APPLE AFL2          G.....C..A....C...G..C........C........A..G...C....C..............C
ARABIDOPSIS LFY     G..C..G.....ATC......G..T.............T......C.A..C.....T.....T.....T

                    220       230       240       250       260       270       280
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
PROFL               TTGCATTGCCTGAACGAAGAGGCATCGAATACCCTGCAGGAGAGCTTTCAAGGAGAAAGGAGAGAACGTG
APPLE AFL1          C..........G...TG.....G......GTG...-........G..........GG..C..A..T..C
APPLE AFL2          C........T..G.T..G............G.G...-........G..........GG..C..A..T..C
ARABIDOPSIS LFY     C.C..C.....AG.......A..T..A...G.T..C-..A.....G..T..A..ACGC..T........T

                    290       300       310       320       330       340       350
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
PROFL               GGAGCTTGGAGGCAGGCCTGCTACCAGCCCCTTGTTGCCATGGCAGCCGGCCAAGGTTGGGACATTGACT
APPLE AFL1          ..G..A...C.......G..T...A....T.....G.T...T.....A.C......C........C..TG
APPLE AFL2          ..G......C.......A..T...A....T.....G.....T.....A........C..........TG
ARABIDOPSIS LFY     ..CT.A...C.T.....T..T...A....A.....GAA...C..TTGTC.T..T..C.....T..A...G

                    360       370       380       390       400       410
                    ....|....|....|....|....|....|....|....|....|....|....|.
PROFL               CCATCTTCAACTCCCACCCTCGTCTCTCCATCTGGTATGTCCCNACCAAAGCTTCGTCAC-
APPLE AFL1          ....T.....T..T..T..C..............C..T.....-G.....C.....G
APPLE AFL2          ..........T.....T..C........T..T.....T.....-G..........G
ARABIDOPSIS LFY     ..G....T...G.T..T..........T..T........T.....-......G.....G
```

**Figure 4.2**. Comparison of the *PROFL* sequence with homologues from apple, *AFL1* and *AFL2* (Wada et al., 2002) and *Arabidopsis* (Weigel et al.,1992). Identical bases are indicated by ‘.’ and where discrepancy exists the base in question is indicated. *PROFL* shows 83% and 85% homology to *AFL1* and *AFL2* respectively, and 77% homology to *Arabidopsis LFY*.

A fragment of approximately 400 bp was successfully amplified from the Protea hybrid 'Carnival' (Fig. 4.1). This fragment showed 84% and 85% homology to two *LFY* homologues, *AFL1* and *AFL2*, which are orthologs of each other identified in apple. *PROFL* also showed 77% homology to the *Arabidopsis LFY* gene (Fig. 4.2). *PROFL* is also expressed in other Proteaceae. Strong expression was observed in the florally determined buds of the different family members and low expression was observed in the leaves (Fig. 4.3). In the case of the *Leucodendron* hybrid 'Safari Sunset', the cone had very low expression while the coloured involucral bracts surrounding the cone showed strong expression. The Protea hybrids 'Sylvia' and 'Carnival' showed similar expression patterns to the naturally occuring *P. repens* and the *Leucospermum* hybrid 'Succession'.

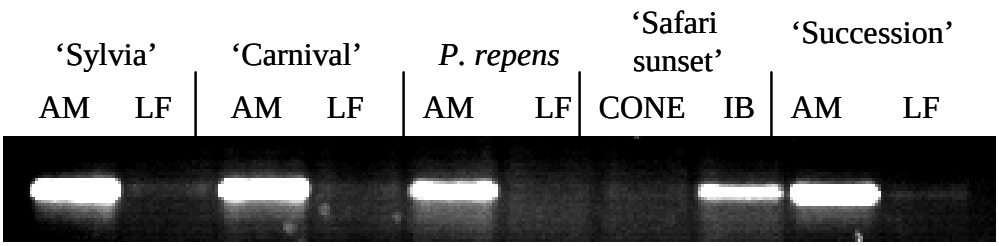


**Figure 4.3**. *PROFL* expression in Proteaceae measured by RT-PCR. 'Sylvia' and 'Carnival' are commercial *Protea* hybrids, while *P. repens* is a naturally occurring species. 'Safari sunset' is a commercial *Leucadendron* hybrid and 'Succession' a *Leucospermum* hybrid. AM=Apical Meristem (floral), LF=Leaf, IB=Involucral bract.

*PROFL* expression was followed by RT-PCR starting at the end of June when apical meristems on the Summer 2, which is the second summer flush, were still dormant. Expression patterns changed in both the developing meristem as well as the vegetative leaves (Fig. 4.4). mRNA levels were very high in the dormant meristem. Preliminary experiments showed high levels of *PROFL* expression in meristems of shoots consisting of only one seasonal flush and two seasonal flushes (data not shown). Expression declined in the apical meristem as dormancy was lost and extension of the Spring 2004 flush commenced. This flush carried the flower on the 'Carnival' shoots that consisted of four flushes. Expression started to increase around the third of September, until a floral bud could be seen (Fig. 4.6). *PROFL* expression in leaves stayed more or less constant except for a sharp increase at the same time as the expression levels in the apical meristem was at its lowest. The expression in both the apical meristem and leaves of the BA treated shoots followed a similar pattern to that of the natural expression (Fig. 4.5). However, expression in the BA treated apical meristems had already started to increase somewhere between the 6$^{th}$ and 20$^{th}$ of August. This was nearly a month before expression in

the un-manipulated plants started to increase. As with the BA untreated plants, high levels of expression were detected in the leaves at the time *PROFL* started to increase in the apical meristem.

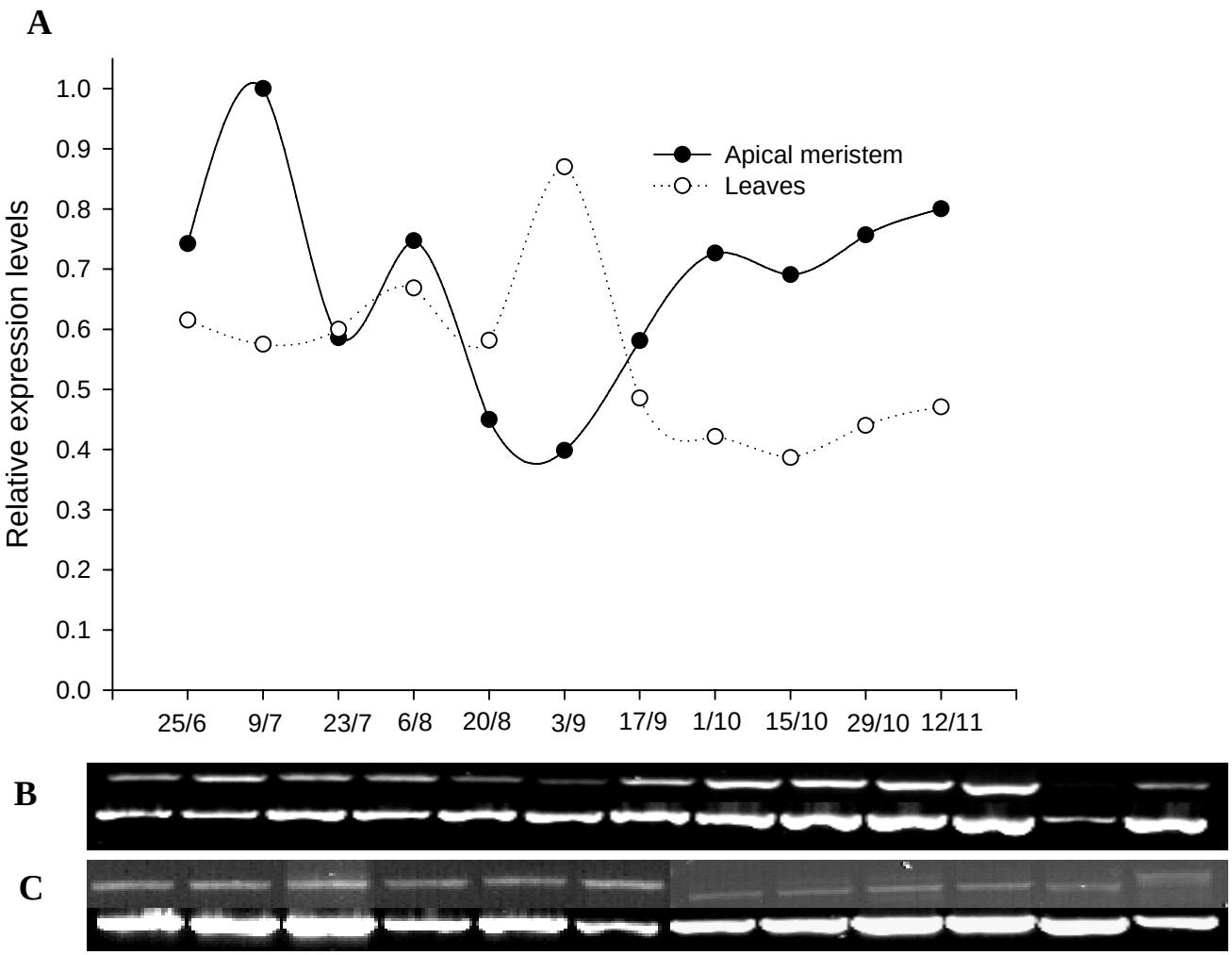


**Figure 4.4**. Expression patterns of *PROFL* during flush and flower development measured by RT-PCR. **A**. Relative expression levels normalized to actin expression. Numbers indicates the dates of harvest (day/month). **B**. *PROFL* expression in apical meristem and (**C**) in leaves. The top bands are *PROFL* expression and the bottom actin. Lanes correspond to the dates indicated by the graph. The final two lanes in B are the floral parts and involucral bracts harvested from a mature flower, and the final lane in C is the leaves of the shoot carrying a mature flower.

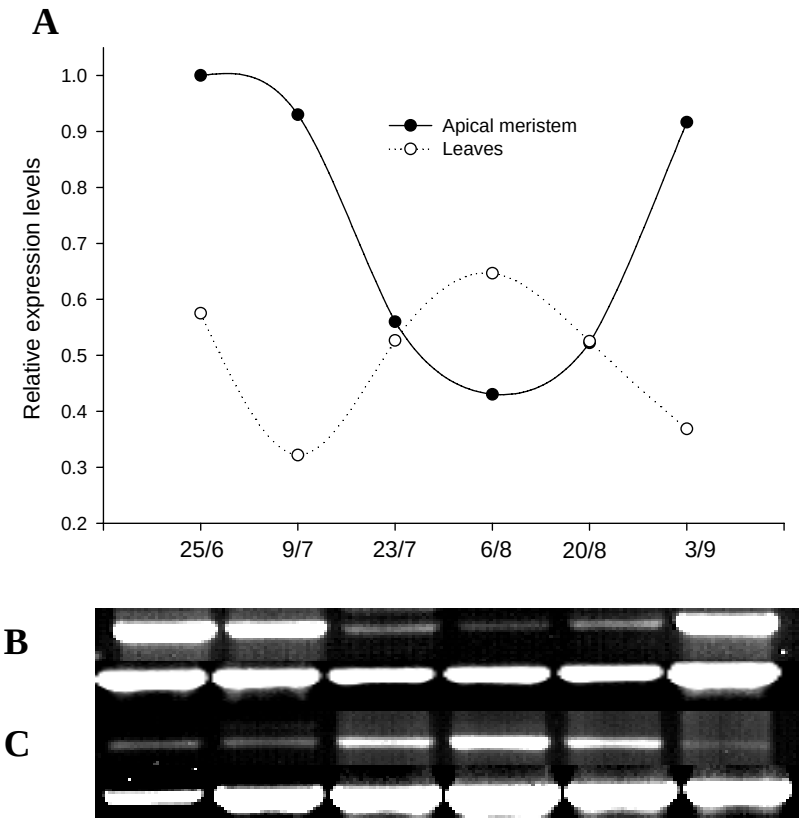


**Figure 4.5**. *PROFL* expression after BA application measured by RT-PCR. **A**. Relative expression patterns normalized to actin expression. The numbers indicates the dates of harvest. *PROFL* expression in apical meristems (**B**) and leaves (**C**). The top bands indicate *PROFL* expression and the bottom actin. Lanes correspond to the dates indicated on the graph.

BA application to dormant buds induced bud break more than one month earlier than in the untreated plants (Fig 4.6). Six stages were noted for the BA treated apical meristems and 11 for the untreated plants. At the final stage (C11) the apical meristem had the morphological characteristics of a floral bud.

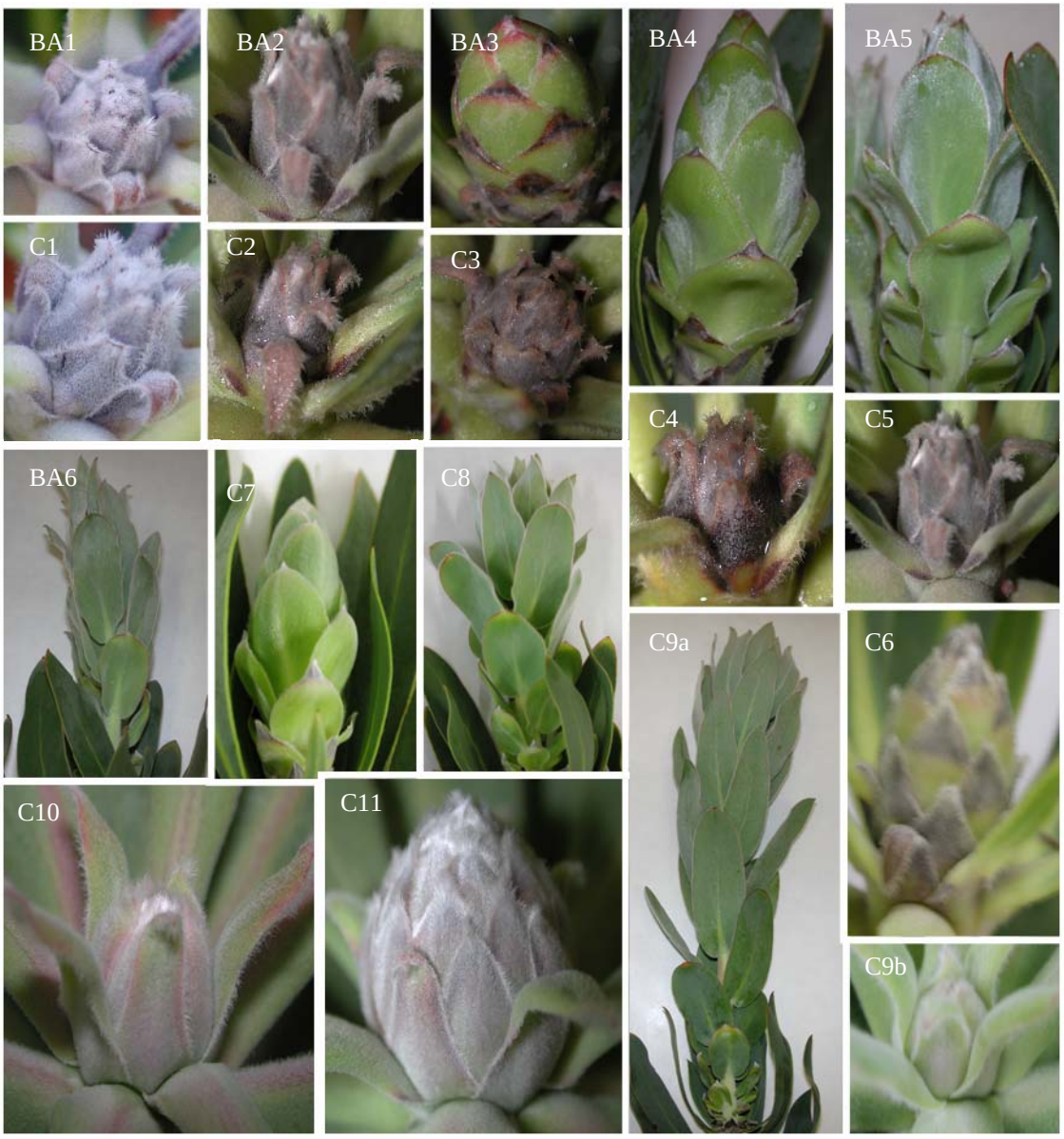


**Figure 4.6**. Morphological characteristics of the apical meristem with (BA) and without (C) BA treatment. The numbers indicates the dates of harvest: 1=25/6, 2=9/7, 3=23/7, 4=6/8, 5=20/8, 6=3/9, 7=17/9, 8=1/10, 9=15/10, 10=29/10, 11=12/11. C9a is the extended shoot on the 15/10 and C9b the apical meristem of the same shoot.

## 4.4. Discussion

A partial sequence of a *LFY* homologue comprising ± 400 bp was identified in Proteaceae by RT-PCR amplification (Fig. 4.2), and dubbed *PROFL*. This fragment was amplified from a highly conserved region of exon 2 (Fig. 4.1). It showed 77% homology to *LFY* from *Arabidopsis* (Weigel et al., 1992) as well as 84% and 85% to two orthologues from apple, *AFL1* and *AFL2*, respectively (Kotoda et al., 2000). These apple orthologues were amplified with the same primer set as the *Protea* homolog. *LFY* homologues have been identified in a great number of woody perennial species. Apple cv. Jonathan has shown to have at least three genes homologous to *LFY*, two of which have already been characterized (*AFL1* and *AFL2*; Wada et al, 2002). *AFL1* seems to have a similar function to the floral meristem identity gene *LFY* in *Arabidopsis*, and is only expressed in floral buds. *AFL2* was expressed in floral buds but also floral organs, vegetative apexes and roots. Homologs of *LFY* have also been identified in *Eucalyptus globulus* and *Eucalyptus grandis.* Southern analysis of these *LFY* homologues showed at least two copies of the gene to be present, one of which is a functional equivalent of *LFY* and the other a pseudogene (Dornelas et al., 2004; Southerton et al., 1998). A search for *LFY* homologues in gymnosperms has lead to the identification of *NEEDLY* (Mouradov et al., 1998) and *PRFLL* (Mellerowicz et al., 1998) in *Pinus radiata*; both are expressed in vegetative and reproductive buds. *VFL*, the *Vitis vinifera LFY* homolog is also found in both vegetative and reproductive meristematic regions, as well as leaves (Carmona et al., 2002). In most of these complex woody perennial systems, it is possible that at least two copies, or orthologues, of *LFY* may be present. Phylogenetic studies by Frohlich and Parker (2000) suggested that there was a loss of *LFY* copies during the divergence of angiosperms. It is possible that some of the woody perennial species maintained at least two functional copies of *LFY*, one with a function equivalent to the meristem identity function in *Arabidopsis* and the other a 'housekeeping' gene. Southern analysis of DNA from the Protea hybrid 'Carnival' did not produce any significant results (data not shown). As most commercially cultivated *Proteas* are hybrids, such as 'Carnival' used for this study, and possibly have complex polyploid genomes, it is very possible that this meristem identity gene might be present in more than one copy. The fragment amplified in this study most probably represents the expression of all the copies present in 'Carnival', as the region is highly conserved. Therefore the results obtained by RT-PCR may be misleading.

*PROFL* expression was found in the floral buds and vegetative leaves of a number of Proteaceae (Fig. 4.3). Therefore, it can be assumed that *PROFL* is conserved over the Proteaceae family. Expression patterns leading up to floral determination in family members such as *Leucodendron* and *Leucospermum*, may differ significantly from that of *Protea* spp., because the former have been shown

to be inducible by short days (Jacobs and Minnaar, 1980). The diverse timing of flowering in *Protea* spp. and hybrids indicates that flowering in this genus in not inducible by a single environmental condition across all species. *PROFL* expression patterns in dormant meristems are inconsistent with the expression patterns found for *LFY* (Weigel et al., 1992), with extremely high expression during this time. *PROFL* expression in dormant buds was higher or at least similar to the expression levels of *PROFL* in florally determined buds (Fig. 4.4A). The lack of response to *PROFL* expression, i.e. the transition to flowering, at this stage might be due to the presence of floral inhibitor such as those previously identified in Arabidopsis. *TERMINAL FLOWER 1 (TFL1)* may influence the interaction of *LFY* with *AP1 (APETALA1)* negatively. *AP1* is the floral identity gene responsible for the formation of sepals and has been identified as a direct target of *LFY* (Yanofsky, 1995). If a floral inhibitor such as *TFL1* is present in Protea and works in a similar way as has been suggested for *TFL1* in Arabidopsis, the *PROFL* protein will still be expressed but will fail to continue the inductive flowering pathway. As the Spring 2004 flush extended the expression pattern became consistent with the expression pattern expected for floral determination. *LFY* expression has also been reported in vegetative primordia of Arabidopsis; Blázquez et al. (1997) proposed that *LFY* is expressed in these primordia because they have the potential to commit to a floral fate, and that a critical or threshold level of *LFY* expression is necessary for the ultimate switch to flowering. This threshold may change with the age of the plant. A very important factor that determines the response of a meristem to a floral stimulus is floral competence. Competence is demonstrated if a cell or group of cells exposed to a developmental signal responds in the expected manner (McDaniel, 1992). It may be that these meristems are potentially marked for a floral fate, but that floral inhibitory signals override floral development at this stage.

The expression pattern in leaves was, however, puzzling. The expression in the leaves are consistent with that of an orthologue with a vegetative function as we see high expression during strong vegetative growth and then inhibition when the switch to reproductive development, marked by an increase in the expression of *PROFL* in the meristem, occurred. Floral commitment is thought to occur late in September coinciding with the time when the expression of *PROFL* in the apical meristem started to increase. At this time the expression in the leaves was starting to decrease. It has been suggested that strong *LFY* expression may have an inhibitory effect on new leaf formation (Blázquez et al., 1997). Arabidopsis plants with mutant alleles of *LFY* continue to produce leaves and lateral shoots after wild-type plants have already produced flowers (Okamuro et al., 1996; Weigel et al., 1992). Phenotypical differences in these late flowering mutants include structures that combine characteristics of both flowers and shoots. The most obvious of these are the leaf-like organs, the bracts which

subtend the flowers. Constititutive expression of *LFY* from the cauliflower-mosiac-virus 35S promoter, in *Arabidopsis*, causes an early flowering phenotype with a decreased number of leaves and no 'bracts' subtending the flowers (Steynen et al., 2001). *LFY* expression has been identified in emerging leaf primordia of Arabidopsis and a gradual increase was observed during flower inducive conditions (Blázquez et al., 1997). These results may suggest an inhibitory function of *LFY* expression in leaves. This may explain the expression pattern observed in 'Carnival' leaves during flush development and floral initiation. Thus increased levels of *PROFL* prior to floral determination would lead to a reduction in new leaf formation and a subsequent switch to reproductive growth.

BA application accelerates the morphological development of the Protea hybrid 'Carnival'. Accelerating the development of apical meristems from bud break, to the extension of the Spring 2004 flush and then ultimately flowering more than two months earlier compared to the natural system (Fig. 4.6). BA also accelerates the *PROFL* expression pattern as seen in the natural season. The same pattern as for the natural system can be seen in the leaf expression pattern with an increase in *PROFL* expression when flowering is initiated. BA application is unlikely involved in the direct control of *PROFL*, because of the initial low expression levels after BA application. BA is most probably involved in the release of dormancy of the apical meristem (Li and Bangerth, 2003; Ohkawa, 1984). This release of dormancy may be linked to an increase in sink strength after BA application and therefore a mobilization of resources to the apical meristem. The increase in carbohydrates at the apical meristem is thought to effect the expression of meristem identity genes such as *PROFL*, possibly through interaction with the promoter region.

**Conclusions**

*PROFL* expression is consistent with that of other homologues identified in woody perennials, especially in the vegetative and floral meristems. Very few studies have, however, investigated the expression patterns in leaves and the effect that expression has on growth and development of these vegetative structures. Conclusions from this work indicate an inhibitory function of *PROFL* in the leaves of *Protea* spp, because of the increase in expression during the time of floral initiation. The increase in *PROFL* expression in the apical meristem at the time of floral evocation strengthens its use as a marker to identify the time of floral transition in *Protea* spp.

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# CHAPTER 5

# GENERAL DISCUSSION

A variety of environmental conditions control floral transition in flowering plants. The control of flowering is crucial to coincide flower formation with the time of the highest pollination rate and maximum reproductive success. The control may be seasonal or triggered by a developmental cue. Different plants respond to a specific combination of environmental and endogenous signals to control flowering. Not all of the apical meristems of the plant will switch to a reproductive fate at the same time; therefore, floral development requires the co-ordination of a complex set of events, particularly in perennial plants. Considerable progress has been made towards understanding the mechanisms by which a plant determines at what time and in which meristems the floral "program" is activated (Pidkowich et al., 1999). This progress has been marked by the identification and cloning of the genes required for determination of the floral fate, firstly in the model species Arabidopsis and Antirrhinum and more recently in a range of woody perennials. The use of expression studies and genetic manipulation together with physiological studies has proven to be very useful in the elucidation of floral signals in these annual species. However, no single molecule has as yet been found to induce flowering in plants.

## 5.1. Carbohydrates and flowering in Protea spp

The complexity of flowering in *Protea* spp has made investigating flowering very challenging. Natural species flower in the autumn and winter months, while the hybrids have a very dissimilar flowering pattern. Some hybrids have very specific flowering times while others flower throughout the year with a peak flowering time mostly during the summer months and is inconsistent with the flowering pattern in parent species. Woody perennials, such as *Protea* spp., are dynamic systems with constantly changing source/sink relationships. The large size of Protea flowers (Chapter 3) and the complex nature of the arrangement of floral parts in the flowerhead, makes carbon input into floral development a very carbon expensive endeavour. Apart from the respiratory costs, other factors that require carbon input, but were not investigated in this study, include the large amount of nectar produced by some of the *Protea* spp. *P. repens* produces copious quantities of nectar to such an extent that the nectar was boiled to make a syrup (locally known as 'bossiestroop') which was an essential component of the 19$^{th}$ century medicine chests in the Cape (Rebelo, 1995). Floral respiration was estimated to consume up to

39% of the daily $CO_2$ fixed by photosynthesis (Chapter 3); this excludes the carbon needed for structural development as well as the large amounts of sugar used for nectar production. Large amounts of sugar are transported via the phloem to the nectaries and used for nectar production. Nicolson (2002) reported that nectar of *P. repens* contained up to 20% (w/w) sugar. This copious and concentrated nectar that serves as a food source for bird, and sometimes rodent, pollinators contributes to the large sink size of the floral structure. As *Protea* spp. are perennial, a great number of developing shoots and meristems are maintained on the plant as the flower develops. The carbohydrates needed for the growth and maintenance of these developing structures, as well as the underground root system, are supported by current photosynthate during the spring, summer and autumn months. During months of high photosynthate production most tree systems additionally store carbohydrates in underground storage systems (Langley et al, 2002; McQueen et al., 2004; Wong et al., 2003). These storage carbohydrates may be utilized for growth and development during months of low photosynthesis and have been found to play a significant role in the development of reproductive structures. *Protea* spp. naturally grow in a fynbos habitat that is prone to regular fires and underground storage systems have been identified in *P. cynaroides.* These underground storage structures are called lignotubers and the carbon stored in these is used for regrowth after fire. Results from $\delta^{13}C$ abundance studies (Chapter 3) may have provided evidence for the use of storage carbohydrates for the development and maintenance of carbon-expensive floral structures. Protea flowers can take up to two months to develop from a floral bud until a mature flower. This is an extremely long time to support these large, nectar-producing flowers.

A post-harvest problem for many Protea exporters, termed leaf blackening, is encountered in several important Protea cultivars. Data suggest that the rapid and substantial loss of starch and sucrose soon after harvest initiate the physiological events that lead to leaf blackening (Jones et al., 1995). A part of these carbohydrates are used for respiration of both the leaves and flowers as well as the continuation of nectar production even after harvest. After harvesting of the flower all carbohydrates are imported from the leaves on the flowering shoot and these are not sufficient sources of photosynthate. Harvested shoots stored in light at optimum temperatures for photosynthesis have shown a delay in leaf blackening and this is though to be because of $CO_2$ fixation by photosynthesis. Glucose pulsing has been found to increase the vase life and reduce leaf blackening in a number of commercially grown and exported Protea hybrids (Stephens et al., 2003). The inclusion of sugars in the vase water of these large flowers is thought to reduce the amount of photosynthate exported from the leaves and therefore delays leaf blackening. This post-harvest blackening, sometimes occurring within hours of harvesting,

demonstrates the intensive requirements for maintenance of the fully developed flower. This large sink size of the flower and the carbohydrates needed for development, maintenance and nectar production of un-harvested shoots are supplied by the source leaves on the subtending shoot (Fig. 3.7), although, translocation from the surrounding shoots (Fig. 3.3) and utilization of stored reserves (Fig. 3.2) may also contribute.

Sugars have been implicated as key regulatory molecules in a number of important physiological processes. The supply of dilute sugars to Arabidopsis *in vitro* has been shown to promote flowering in this annual. Treatments that are thought to increase carbohydrate levels in the shoots of fruit trees have also been shown to induce flowering (Jackson and Sweet, 1972), these include fruit thinning and BA treatment (Stern and Flaishman, 2003; Stern and Flaishman, 2004). The physiological effects of BA application have not been investigated, but an increase in sink strength and cell division has been implicated in the response of the apical meristem to BA application. A signaling role of sugars has been implicated in a range of plants, with sugar levels having a direct effect on flowering (Gibson, 2005). Carbohydrate status may be a major factor controlling flowering in *Protea* spp. The availability of carbon from photosynthate and storage for the development of these expensive structures may be established through sugar signaling, prior to the initiation of flowering. The effect of BA on the growth of *Protea*, may be a response to the import of sugars to the apical meristem after BA application, although no direct evidence of this can be seen from these studies.

**5.2. Molecular control of flowering in Protea spp.**

This study included a search for the meristem identity gene responsible for the switch to flowering in *Arabidopsis thaliana* (Arabidopsis), *LEAFY* (*LFY*). *LFY* homologues have already been identified in a wide range of woody perennial species. Models of the function of *LFY* suggest that it is a transcription factor that is activated by exogenous factors, such as environmental conditions, and endogenous factors such as the meristem competence and carbohydrate status of the plant (Pidkowich et al., 1999). Many of the woody perennial species in which *LFY* homologues have been identified have more than one orthologue of the gene (Dornelas et al., 2004; Southerton et al., 1998; Wada et al., 2002). It has been speculated that one of these genes is a functional equivalent of *LFY* and the others may be pseudogenes. We have identified *PROFL* (***PR****OTEA* ***F****LORICAULA* ***L****EAFY*) a homologue of *LFY* in Proteaceae. The expression of *PROFL* is not always consistent with the expression patterns seen for *LFY* in Arabidopsis (Blázquez et al., 1997) with the most obvious difference being the high expression levels present in vegetative meristems. These expression levels were higher than expression levels

during the reproductive development of the apical meristem, i.e. after floral transition. *Protea* spp. exhibit a flush growth habit and it is during the dormant stage of the meristem, before the formation of the next flush, that *PROFL* expression is extraordinary high. Floral signals received at this time may be overridden by meristem competence or the lack of sufficient carbohydrates for the support of a developing flower. Although high levels of sucrose (5% [w/v]) have been found to inhibit floral transition in Arabidopsis, growth on low concentrations (1% [w/v]) *in vitro*, have been found to promote flowering (Ohto et al., 2001). This may be one of the differences between annuals and perennials, when it comes to floral transition. Annuals will continue and even accelerate vegetative growth when an abundance of carbohydrates is available. Perennials that continue vegetative growth in most meristems will most probably initiate flowering in physiologically competent shoots. Evidence for this is lacking, but carbohydrates have been shown to influence *LFY* expression through the interaction with the promoter region (Ohto et al., 2001). *LFY* expression has been found in the vegetative meristems as well as leaf primordia of Arabidopsis (Blázquez et al., 1997) and this is consistent with *PROFL* expression. It has been suggested that *LFY* needs to reach a threshold level of expression before flowering is induced. During strong vegetative growth such as the extending of the next seasonal flush, *PROFL* expression was low compared to the pattern during dormancy and floral evocation (Fig. 4.4 and Fig. 4.5).

We propose that *PROFL* is a transcription factor comparable to *LFY* from Arabidopsis. *PROFL* is thought to be controlled by both exogenous and endogenous factors that induce flowering in Protea, i.e. most probably carbohydrate levels and to a lesser effect environmental cues. Although the environmental factors that control flowering in the many species and hybrids of Protea may be diverse, which is emphasized by the varying flowering times, the main factor of control is believed to be the carbohydrate status of the plant. Carbohydrates may directly influence the expression of *PROFL* by interaction with the promoter region of the gene. The *PROFL* protein will in turn promote the expression of the floral organ genes responsible for the formation of the flowers.

### 5.3. Future prospects for the elucidation of flowering in Protea spp.

No response to day length has been found in *Protea* spp., although, in *Leucospermum* and *Leucodendron*, both Proteaceae members, floral response to light treatments have been identified (Malan and Jacobs, 1986; Hettash, personal communication). Future research will include searching for a *CONSTANS* homologue in *Protea*. *CONSTANS* is the final gene in the photoperiodic response pathway and in turn induces the expression of LFY, previously identified as the gene responsible for

floral transition. The effect of growth regulators on plants can be readily observed, but the best scenario will be if the morphology, physiology and genetic aspects of flowering in *Protea* spp are well understood. *Protea* flowers are arranged in a complex flowerhead (inflorescence) and therefore it is unlikely that the genetic make-up will be comparable to the simple flowers from the well-studied model plants. The need exists for a complete morphological assessment of *Protea* spp., beyond the flower, that will include developmental stages that can be used as landmarks to describe crucial events in plant development. These landmarks can then be linked to the genetic profile at that time and help with the elucidation of floral evocation in *Protea* spp.

Commercially grown *Protea* hybrids are the result of successful hybridization events using naturally occurring species. These hybrids possibly have complex polyploid genomes, although this has never been established. As mentioned previously, most of the woody perennial species in which homologues of *LFY* have been identified, more than one functional copy of the gene may be present. The first aim of future work will be to determine the copy number of *PROFL* as well as to clone the complete sequence. This pilot study might have identified the presence of a *LFY* homologue in Protea but it is possible that there is more than one functional gene, because of the highly conserved region that was amplified by RT-PCR. These genes can then be characterized and the function of each in Protea can be determined. The promoter region of the gene ultimately controls expression and levels of expression. The effect of various treatments on the expression of a reporter gene placed in control of the *PROFL* promoter expressed in a model system will also be investigated. These treatments will include environmental conditions as well as the effect of sugars on the control of the promoter.

A large amount of work will be required to completely understand flowering in this complex family of plants. These studies have laid a groundwork that is invaluable for future work. Most of the basic methodologies needed for genetic studies have been optimized and from here the large amount of gaps can be filled in.

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