Chapter 1 General Introduction

1.1 Introduction

Australia produces an extensive range of fresh cut flowers and foliage, which includes unique native flora as well as traditional varieties. With the development of new varieties, extended seasonality, improved quality control and shipment arrangements, Australia is a competitive supplier of unique cut flowers to global markets. *Swainsona formosa* (Sturt's Desert Pea) has been amongst the many native export floricultural plants with commercial value, as a cut flower, as a hanging basket specimen, or container grown plant, both within and outside Australia (Kirby, 1996b; Austrade, 2007). Japan is one of the largest markets in the world for *S. formosa*, because the plant is suitable for use in floral arrangements or ikebana (Barth, 1989; Barth and Bennell, 1989).

As a result of the ornamental attractiveness of *S. formosa*, research to increase yield, improve quality and shelf-life (Jones *et al.*, 1998) and reduce the production costs has been carried out (Kirby, 1996a). Time of flowering is often the decisive factor determining production and profit in cut flowers and potted plants to meet peak market demand periods. To date the commercial use of *S. formosa* has been marred by difficulties; this is partly due to our lack of understanding of the specific mechanisms involved in the regulation of flowering in *S. formosa*.

Flowering is a developmental process which depends on the ontogeny of the plant (Jack, 2004a, b). Meristems function as genetic sources of cells for differentiation into organs, with each type of meristem programmed to produce only certain structures (Silva *et al.*, 2005). The initiation of a flower by a shoot apical meristem results from a sequence of events that occurs in the various tissues and organs of the plant, including the shoot apical meristem (Hay and Tsiantis, 2005). The problem is to determine the underlying causes for the relatively sudden change from the vegetative to the floral stage.

The transition to flowering is often determined by the interaction of environmental cues with endogenous developmental signals (Bernier and Perilleux, 2005). Internal factors

involved in the induction to flowering include plant maturity, nutritional status, hormones and other compounds, while the external (environmental) factors include day length, light spectrum, light intensity and ambient temperature. Some of these factors may act in sequence, since some of the cellular changes seem to occur before the floral stimulus arrives at the apex (Corbesier and Coupland, 2005).

Environmental responses differ among species and the mechanisms of transport and response to the signal at target tissues could be diverse as well (Bouveret *et al.*, 2006). Many plant growth regulators and other compounds that can induce or inhibit flowering in one species or another when applied at appropriate concentrations are now known (for example see Chapter 2 section 2.4.1.4). Therefore, anything that can be demonstrated for a particular species will have been tested in many others before making any general conclusion. However, plants with different responses can have various experimental advantages and can provide complementary information.

The switch from vegetative to floral development is associated with biochemical changes in the shoot apex (Poethig, 2003). Quantitative changes in any of several major compounds, e.g. carbohydrate (soluble sugars), carbon and nitrogen pools, protein or other components, or the combinations of them, have been found to be involved in the regulation of flowering. Progress towards understanding the mechanisms of floral induction requires an integrated approach of these multiple external and internal factors. These biochemical processes for the generation and transport of compounds, which control plant metabolism, including flowering, are mediated by specific genes (Suarez-Lopez, 2005).

An ideal flowering pot plant is one that is compact and can flower for an extended period under the typical low light conditions of the indoor environment, which is far removed from the high light intensity of its natural environment. Earlier work by Yusuf (2000) revealed that plants of *S. formosa* produced no flowers if grown under the low light intensity of 140 μmol $m^{-2}s^{-1}$. The objective of the work presented here is to investigate the changes in sugar levels, C:N ratio, and protein expression in the shoot apices of *S. formosa* plants during the transition period from vegetative to floral to better understand the mechanisms of flowering in this plant. Such an understanding may

provide commercial growers with practical strategies in aiding control of flowering in this uniquely spectacular plant.

1.2 Research aims

The broad aim of this research is to improve on the understanding of the flowering mechanism in *S. formosa* through :

- establishing the morphological marker for changes in the shoot apex of *S. formosa* during the transition from vegetative to floral;
- determining changes in sugar production and the C:N ratio as possible biochemical markers of flowering in *S. formosa*; and
- examining the protein expression during developmental stages in shoot apices of *S. formosa* as possible molecular markers of flowering.

1.3 Thesis outline

An overview of the thesis and the research aims are provided in Chapter 1. The literature review (Chapter 2) covers an introduction to *S. formosa*, its description, cultivation and factors affecting its growth and development, and a review of various aspects of plant flowering, including flowering processes and the factors that can affect flowering. Chapter 3 presents the general materials and methods used throughout the experiments in this thesis. A morphological feature of flower development of *S. formosa* is examined in Chapter 4, and details of the changes in sugar levels accompanying flower development are reported in Chapter 5. Chapter 6 presents the role of the C:N ratio on *S formosa* flowering. The five methods dealing with the exogenous application of sugar are reported in Chapter 7. In Chapter 8, protein expression in the shoot apex at the vegetative and flowering stages in *S. formosa* is examined, and finally, Chapter 9 integrates the whole research work and proposes directions for future work.

Chapter 2 Literature Review

2.1 Introduction

Flowering is a unique biological phenomenon by which most plant species perpetuate themselves from one generation to another. The transition from vegetative to reproductive involves major changes in the pattern of morphogenesis and cell differentiation at the shoot apical meristem, ultimately leading to the production of the floral organs. The developmental signals that bring about floral evocation include endogenous factors, such as circadian rhythms, phase change, hormones, and external factors, such as photoperiod, light quality and temperature. The interactions of these endogenous and external factors enable plants to synchronise their reproductive development with the environment.

Much of the research on phase change has focused on a single event – floral induction. How this process is related to earlier developmental transitions remains to be determined, as does the mechanism of these transitions. Model organisms, such as *Arabidopsis* and pea (*Pisum sativum*), have produced rapid progress in the understanding of plant development in recent years and will continue to be an important source of information about the central problem of floral induction in shoot morphogenesis.

This literature review will:

- outline the biology and physiology of *S. formosa*,
- provide a general frame work for understanding the process of flowering,
- review published and unpublished work on flowering in *S. formosa* and related species, and
- identify areas of potential research interest and develop hypotheses that can be tested empirically.

2.2 *Swainsona formosa*

Swainsona formosa (G.Don) J. Thompson (syn. *Clianthus formosus* G.Don) is commonly known as Sturt's Desert Pea or Glory Pea. The genus name *Swainsona*

honours Isaac Swainson who maintained a private botanic garden at Twickenham near London in about the year 1789. The specific name *formosa* is Latin for beautiful. The original author of the species was the Scottish botanist, George Don (1798 – 1856). The plant, formerly known as *Clianthus dampieri*, was first collected in the seventeenth century by William Dampier when he visited the north-western coast of New Holland. In 1961, *S. formosa* was adopted as the Floral Emblem of the State of South Australia, and the species is protected in that State. The flowers and plants must not be collected on private land without permission (Boden, 1985).

Swainsona formosa belongs to the family Fabaceae (also known as Leguminosae), and is in the subfamily Faboideae. The genus *Swainsona* is placed in the tribe Galegeae sub-tribe Coluteinea (Zulkarnain *et al.*, 2002). *Swainsona formosa* is found in the arid areas of southern, western and central Australia (Figure 2.1), growing mainly on deep sands or on stony ground in hot, dry and exposed locations (Williams, 1996).

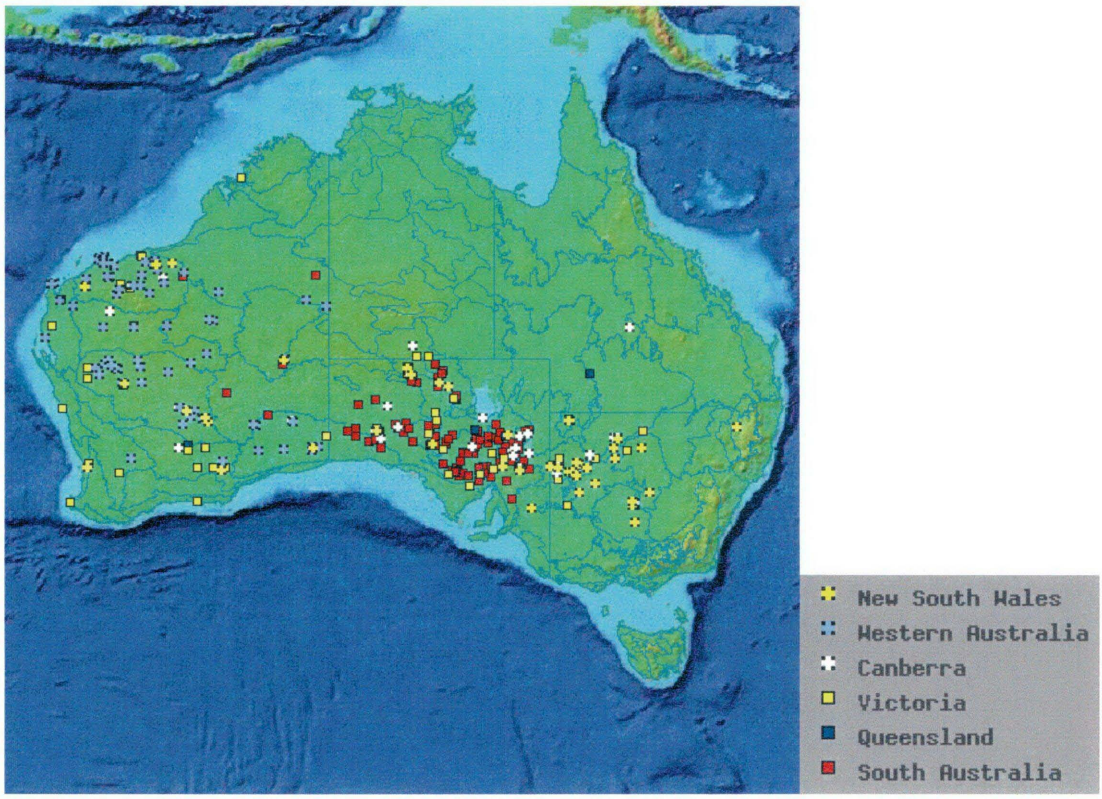

Figure 2.1 Distribution of *Swainsona formosa* in Australia from 439 recorded mapped sites (Australia's Virtual Herbarium, 2003).

Swainsona formosa is an annual or biennial herb with thick upright flowering stems up to two metres long. Leaves are pinnately divided into 11 – 17 narrow to broad elliptic or

oblong leaflets, 1 – 3 cm long and 6 – 15 mm wide, grey-green and covered with long, soft hairs. The flower is a typical legume flower (Figure 2.2*A*). The fruit is a legume about 5 – 6 cm long, which splits at maturity releasing several flat kidney-shaped seeds (Figure 2.2*B*); the plant flowers in spring (Williams and Taji, 1991).

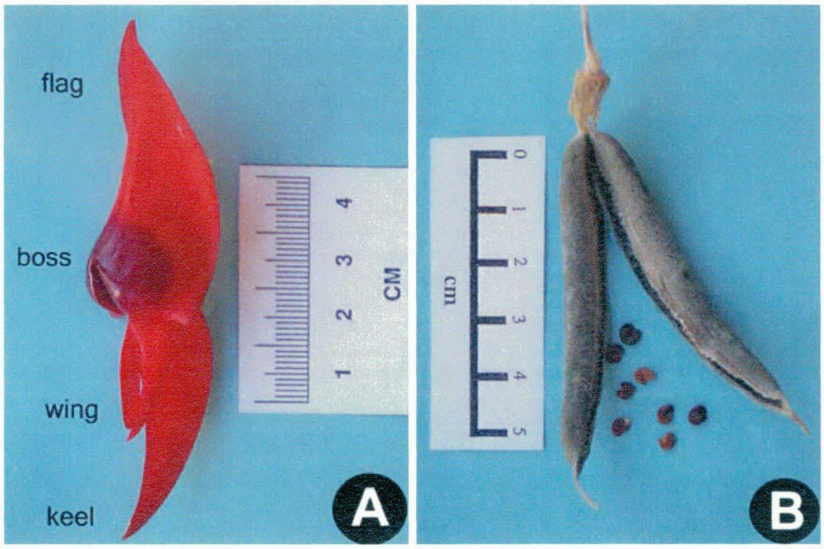

Figure 2.2 (*A*) Flower of *Swainsona formosa*. (*B*) Pods and seeds of *Swainsona formosa*.

2.2.1 The flower

Floral development of *S. formosa* follows a similar pattern to that of most other members of the legume family. Following the period of vegetative growth, which varies depending upon cultivar and environmental conditions, such as daylength and temperature, the plant enters the reproductive stage, during which axillary buds develop into pendulous axillary racemes (Williams, 1996).

Swainsona formosa's growth habit and distinctive long and curiously shaped flowers provide the plant with commercial appeal. Normally, it takes 10 – 12 weeks for *S. formosa* to flower, when grown from seed under favourable conditions, and the flowers are produced continuously over many months (Williams and Taji, 1991). Yusuf (2000) reported that the time taken from germination to the first flower at full bloom was approximately 61 – 87 days. Full bloom was defined as the standard (flag) petal being fully open (stage 8 based on Tade (1992) or stage 6 based on Jusaitis (1994)). However, flowering time is widely variable in the wild, and some plants may take more than 16 weeks to start flowering. Kirby (1996a) also observed that plants growing in hot areas

flower much more quickly than those growing in cold areas. In addition, it was found that plants growing in the autumn took longer to flower than those growing in the spring, possibly due to the temperature and day length.

The flowers are usually 7 – 10 cm long and arranged in clusters of five to eight on short, thick, erect peduncles, although Williams and Taji (1987) counted six to eight flowers per raceme. The flower consists of five sepals (calyx) and a five-part corolla (petals) (Tade, 1992). The petals are usually blood red or scarlet, with a glossy black swelling or 'boss' at the base of the uppermost petal, the standard (Williams and Taji, 1987; Jusaitis, 1994). Although red flowers are typical, the colour can range from white to deep pink, either with or without a boss, and rarely a bicoloured form, in which the standard is scarlet and the other petals, the wings and keel, are white tipped and edged with scarlet. Pedicles (flower stems) are mostly in groups of six arranged in a sub terminal whorl. Sometimes a second whorl forms above the main one (Williams, 1996). The peduncle may be up to 20 cm long (Jusaitis, 1994), and a well-grown plant can produce up to 150 flower racemes (Williams and Taji, 1991).

Swainsona formosa has a typical legume flower which consists of ten stamens, collectively called the androecium. Stamens occur in two groups in which the filaments of nine of the stamens are fused and elevated as a single structure, while the posterior stamen remains separate. The single pistil, which extends beyond the anthers, is unicarpellate, with trichomes covering the outer surface. The style is about half the length of the pistil and curves backwards slightly towards the free posterior stamen, terminating at the stigma (Tade, 1992). The stigma is covered by a stigmatic cuticle that, unless ruptured, prevents self-pollination (Jusaitis, 1994).

2.2.2 Flower development

The growth habit and floral characteristics of *S. formosa* are extremely variable (Jusaitis, 1994). Jusaitis (1994) divided the floral development of *S. formosa* into 7 stages (Figure 2.3) and noted that it took 17 days from bud initiation to anthesis. The seven stages are:

(A) Flowers are tightly clustered in bud, and petals are completely enclosed by calyx.

(B) Petal tips begin to protrude through the calyx, showing first signs of colour.

(C) The flower is oriented horizontally during the process of downward curvature of pedicels.

(D) Pedicels have completed their downward curvature and flowers are vertically oriented.

(E) The standard (flag) petal begins to reflex back from the keel.

(F) The flag is in a half-reflexed position and horizontal.

(G) The flag is fully reflexed and the flower is open.

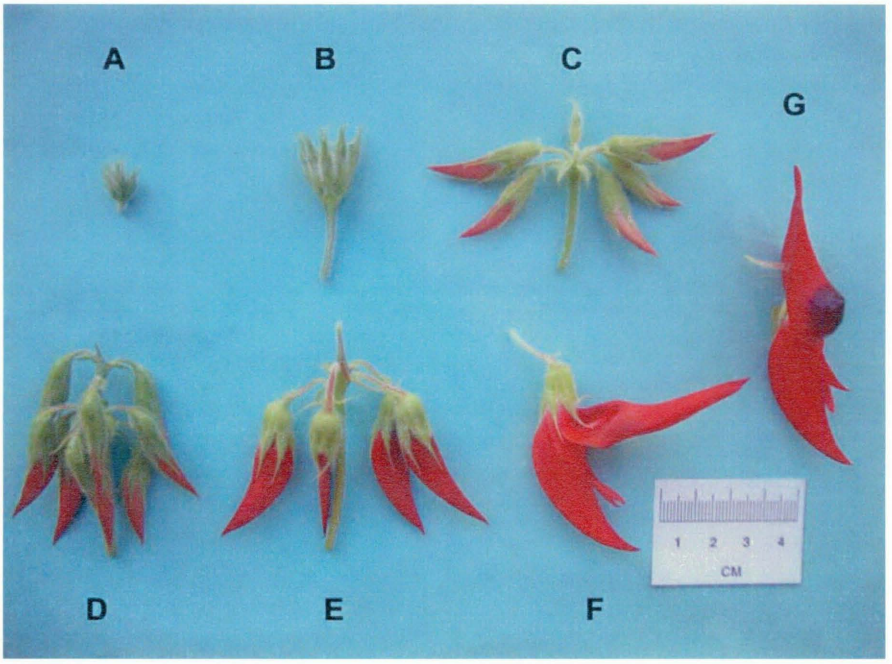

Figure 2.3 The seven stages of flower development of *Swainsona formosa* (adapted from Jusaitis, 1994).

The length of the peduncle follows a sigmoidal growth pattern over the period of floral development, and maximal length is achieved by 19 – 20 days. During the first half of this period, the flowers remained at stage (A), but when the peduncle growth becomes linear, floral development accelerates quickly through stages (B) and (C) to reach stage (D). Floral development slows at stage (D), while the peduncle continues to elongate. Flowers then pass through stages (E) to (G) as the peduncle elongation rate declines. Floral stages (E) and (F) are the most rapid, both occurring on the same day. Floral development is completed 17 days after the emergence of a floral bud, but peduncle growth continues slightly beyond that time (Jusaitis, 1994).

Anther dehiscence commences at stage (E) and is completed by stage (F). This indicates that, for a breeding programme, emasculation should be carried out during stage (E), when the anther is fully developed but has not dehisced. Following anther dehiscence, pollen falls to the bottom of the keel and accumulates immediately posterior to the

stigma. The production of nectar at the base of the filament tube commences during stage (F), and by stage (G) each flower contains 80 – 100 μL of nectar (Barth, 1990b).

2.3 General cultivation

Propagation of *S. formosa* can be carried out from seed, cuttings, or by grafting using the related species *Clianthus puniceus* (G.Don) Lindley (native to New Zealand) as rootstock (Kawaguchi and Taji, 2002). Tissue culture (Williams and Taji, 1987; Williams, 1996) and hydroponic methods are also used to propagate and multiply *S. formosa* (Kirby, 1996a).

In cultivation, *S. formosa* is regarded as difficult to manage (Taji and Williams, 2005). *Swainsona formosa* grown under unfavourable conditions often produces few flowers; therefore, optimal growing conditions are vital for successful commercialisation of this plant. Soil nutrients, light intensity, temperature, and photoperiod are factors that can affect the flowering of *S. formosa* plants (Williams and Taji, 1991). *Swainsona formosa* grows mainly in alkaline soils with high levels of calcium and iron oxide. For successful cultivation, it is advisable to supplement the nutrients in the medium with extra iron chelates and keep the pH over 6.5 to maintain a less acidic medium (Kirby, 1996b). However, Kirby (1996a) found that when using some hydroponic mixes, the pH could drop as low as 3.5 without obvious damage to the plants. In terms of mineral nutrition, slow release fertilisers lasting eight to nine months provide a good base level of nutrients (Williams, 1996). By using a slow release fertilizer, the available nutrients are sufficient for excellent growth until the plant flowers (Kirby, 1996b). Micro elements must also be added, and supplementary N:P:K can be supplied by regular liquid fertiliser applications (Williams, 1996). Bridgeman (1995) recommended the application of sulphate of ammonia at more than 1.5 g m^{-2} or urea at 1.0 g m^{-2} at monthly intervals to maintain adequate N levels. While a variety of potting media have been used for flower production, coarse sandy soils are recommended for garden plantings (Williams, 1996), and *S. formosa* will usually grow well in most potting mixes as long as the media are well drained (Webb, 1972; Williams, 1996).

When *S. formosa* is grown as a pot plant, the trellising and pruning systems are very important. Barth (1989) described the growth of container-grown *S. formosa* plants. Unsupported central stems grew upright (about 30 cm) but supported central stems

continued to climb producing a flower at each node. These stems had a mean diameter of about 1.0 ± 0.3 cm compared with 0.7 ± 0.2 cm for lateral runners. In 105 days, stem lengths of two metres with up to 28 flowers were obtained on a trellised runner. Vegetative buds normally occur at the nodes on the lower two-thirds of the stem with floral development on the upper part of the stems.

2.4 Factors affecting growth and development of *S. formosa*

2.4.1 Environmental conditions

Swainsona formosa prefers a hot and dry climate and has a high light requirement for flower production (Barth, 1990b; Kirby, 1996a, b). Yusuf (2002) found that *S. formosa* plants growing under a light intensity of 600 μmol $m^{-2}s^{-1}$ flowered (73.3 days) earlier than those grown under a higher light intensity of 950 μmol $m^{-2}s^{-1}$ (77.5 days) but the number of flowers was the same. In contrast, plants grown under a low light intensity (140 μmol $m^{-2}s^{-1}$) produced no flowers during the experimental period of 121 days. *Swainsona formosa*, therefore, requires relatively high light intensity for flower production, but potted or pot grown plants should be shaded during the hot summer months to avoid heat stress (Kirby, 1996b).

The flowering of *S. formosa* is not affected by photoperiod and plants will flower all year round (Williams, 1996). However, Yusuf *et al.* (2002) found a longer photoperiod (16 hours per day) was required for faster flower appearance, and a neutral photoperiod (12 hours per day) produced more flowers per plant. According to the research of Zulkarnain (2003), plants grown in growth cabinets under photoperiods of 16, 12 and 8 hours the 12th and 11th nodes were the critical nodes for flower formation in the main and side stems, respectively. Meanwhile, the 10th and 8th nodes were found to be the critical nodes for flower formation for the main and side stems respectively for plants grown in the greenhouse under natural photoperiods ranging from 12 to 16 hours per day. Results from histological examinations indicated that the conversion of shoot apices from vegetative to reproductive occurred at 56 to 65 days after germination for the plants grown in growth cabinets under 12 – 16 hour photoperiods (Zulkarnain, 2003). Yusuf *et al.* (2002) reported that *S. formosa* plants grown under 18/30 °C (night/day) produced more flowers and the time taken to the appearance of the first flower was reduced compared to plants grown under 10/22 °C (night/day) or 25/25 °C

(night/day). It seems that, for *S. formosa*, days to flower depends on the combination of temperature and light, and thus degree-days. Within a certain temperature range, flowering time is linearly related to thermal time under a wide range of environmental conditions; also temperature responses are also often modified by light (Poethig, 2003).

Kirby (1996b) observed that *S. formosa* can tolerate very hot conditions (i.e. temperatures above 40 °C) provided sufficient water is available. The optimum daily temperature is 32.5 ± 2.5 °C, and the plant does not grow well if daily temperature is below 15 °C (Kirby, 1996a). Cold night temperatures (12 – 15 °C) can arrest plant growth without causing any damage (Williams, 1996). High fluctuations in day and night temperatures will produce elongated plants (Barth, 1990b), and warmer night temperatures result in more compact plants (Williams, 1996).

2.4.2 Plant growth regulators

Flowering can be promoted or inhibited by the application of plant growth regulators (PGRs). There have been many studies on the role of PGRs in the control of growth and development in *S. formosa* (Table 2.1).

The effects of PGRs on the growth of *S. formosa* are variable because of the differences in factors, such as the dosage, formulation of the material, method of treatment, time of application, and plant age. The PGRs of most relevance to flowering of *S. formosa* are GA_3, BA and related compounds. The role of PGRs in flowering is discussed further in section 2.7.1.4.

Table 2.1 Effects of some plant growth regulators (PGRs) on *Swainsona formosa*.

Effect	PGR	Dose	References
Increase shoot length	GA_3	1 – 1000 mg L^{-1}	Jusaitis and Schmerl (1993)
	BA	25 – 50 mg L^{-1}	Barth (1990b)
No effect on shoot length	BA, BA + PBZ	100 mg L^{-1}	Jusaitis and Schmerl (1993)
Decrease shoot length	Ancymidol	10 – 100 mg L^{-1}	Jusaitis and Schmerl (1993)
	PBZ	1 – 2 mg L^{-1}, 2 – 4 mg/pot	Barth (1990b)
Increase number of nodes to first flower	GA_3	100 mg L^{-1}	Williams and Taji (1992)
	BA	10 mg L^{-1}	Williams and Taji (1992)
Delay flowering	Cytolin®	1 – 1000 mg L^{-1}	Jusaitis and Schmerl (1993)
	PBZ	10, 20, 50 mg/plant	Hamid and Williams (1997b)
	Daminozide	2.5% per plant	Hamid and Williams (1997a)
	Flurprimidol	1 mg/ plant	Hamid and Williams (1997a)
	Ancymidol	5 mg/plant	Hamid and Williams (1997a)
No effect on flowering	GA_3	1 – 1000 mg L^{-1}	Jusaitis and Schmerl (1993)
	BA, BA + PBZ	100 mg L^{-1}	Jusaitis and Schmerl (1993)
	PBZ	0.1, 1 mg/plant	Hamid and Williams (1997a)
	Ancymidol	5 mg/plant	Jusaitis and Schmerl (1993)
Increase flower size	GA_3, GA_{4+7}	25 – 50 mg L^{-1}	Hamid (1996)

GA_3 = Gibberellic acid; BA = Benzyladenine; PBZ = Paclobutrazol;

Cytolin® = a mixture of GA_{4+7} and BA

2.4.3 Pests and diseases

Although admired commercially, *S. formosa* is notoriously hard to grow outside its natural desert environment as plants are prone to fungal diseases and root rots. Williams and Taji (1991) reported that *S. formosa* is prone to sudden collapse when fully grown and coming to flower, due to root diseases, thought to be mainly *Fusarium* infection (Barth, 1990a). The best approach to disease control is to maintain a vigorous root system, keeping plants separated and off the ground to help prevent the spread of diseases (Williams, 1996).

Swainsona formosa is also susceptible to infection by powdery mildew; this can be controlled with a fungicide specific for powdery mildew. The infection of plants by grey mould (*Botrytis cinerea*) in cold, humid conditions is best prevented by keeping

the foliage dry (Kirby, 1996a, b; Summerell *et al.*, 1997). However, some fungal infections can be controlled using commercially available fungicide sprays.

Several major insect pests affect *S. formosa*, such as two-spotted mite, mealy bugs, aphids (Barth, 1990b) and heliothis caterpillar (Kirby, 1996a, b), all of which can destroy *S. formosa* unless controls are undertaken. Two-spotted mites are a common problem in Australian greenhouses, but can be controlled naturally by using predatory mites (Williams, 1996). However, Kirby (1996a) reported that, while trials have shown that biological controls can be effective for mites, the level of control is probably inadequate for commercial flower production, necessitating the use of additional control methods.

2.5 Developmental stages in the life cycle of flowering plants

The life cycle of flowering plants can generally be divided into three growth phases: a juvenile phase, an adult vegetative phase and an adult reproductive phase (Poethig, 2003). The duration of each phase varies among species, with some taking a few days and others taking several years (Irish, 1999).

The early stages of post-embryonic development in plants involve a juvenile phase characterised by the production of vegetative organs differing in size and shape from adult organs and by the lack of meristematic competence to initiate flowering (Poethig, 2003). The process of floral transition involves the coordinated integration of external or environmental cues with internal regulatory factors, such as the developmental status and age of the plants (Simpson *et al.*, 1999). A second major characteristic of flowering is that, in many species, it occurs only at one particular time of the year and so must be under the control of external environmental conditions (McDaniel *et al.*, 1992).

The flowering process is expressed by changes in the morphology and developmental physiology of the shoot apical meristem (Hay and Tsiantis, 2005). However, the development of the shoot apical meristem can be interrupted or it can be prevented from developing by subjecting the plant to influences that favour vegetative growth or prevent or avoid phase change, such as high nitrogen (N) fertilisation (Levy and Dean, 1998). In some determinate plants under certain environmental conditions, leafy shoots are formed after flowers in a phenomenon known as flowering reversion (Wu *et al.*,

2006), indicating that the genes and processes involved in the transition to flowering are required to both initiate and maintain reproductive development.

2.5.1 The flowering process

Three distinct phases occur in the production of a flower: floral induction, which is the initial biochemical changes that lead to flowering; floral evocation or initiation, during which the flower bud or inflorescence can be first identified under a microscope; and floral development, in which the meristem differentiates into the flower parts until the opening of the flower (anthesis) (Poethig, 2003).

2.5.1.1 Floral induction

The transition of the shoot apical meristem from the vegetative to the reproductive stage is a major developmental switch in the plant life cycle. The timing of floral transition is critical for reproductive success, and many plant species have evolved multiple pathways to regulate flowering time. These pathways are regulated by both endogenous biochemical and exogenous environmental factors (Bernier and Perilleux, 2005; Corbesier and Coupland, 2005).

Grafting experiments on several photoperiodic species clearly demonstrate that the flowering stimulus originates in the leaves in response to an inductive photoperiod, and is translocated via the phloem to the apical meristem (reviewed by Colasanti, 2004). Chailakhyan (1936) proposed a hypothesis that the flowering stimulus 'florigen' is responsible for flower induction; however, this 'florigen hypothesis' was reviewed in Machackova and Krekule (2002) and Milyaeva and Romanov (2002) and, despite extensive studies, a flowering stimulus has not been isolated.

The control of flowering time was believed initially to be essentially regulated by plant nutritional status (Sachs, 1977). The promotion of reproductive development requires a high endogenous ratio of carbohydrates to nitrogen (the so-called C:N ratio) within plant tissues (reviewed by Bernier *et al.*, 2002). Later, floral induction was proposed to be a modification of the source/sink relationships within the plant, so that the shoot apex receives a higher concentration of assimilates (mainly carbohydrates) than under non-inductive conditions (Sachs and Hackett, 1977). This was known as the 'nutrient

diversion theory' (Sachs, 1977; Sachs and Hackett, 1977). The sucrose content of the shoot apical meristem and/or the phloem sap reaching the shoot apical meristem increases in several long-day (LD) or short-day (SD) plant species following floral induction (Bernier *et al.*, 1998).

Following the observation that treating vegetative plants with substances, such as sugars, plant growth regulators and their antagonists, can induce in the shoot apical meristem events specific to floral evocation, Bernier and colleagues (2002) proposed the 'multifactorial theory'. In this theory, floral transition occurs if all factors are present in the shoot apical meristem at appropriate concentrations and times. Gibberellins and cytokinins (and possibly also ethylene and polyamines) have been shown to be involved in flower induction, together with sucrose (Bernier *et al.*, 1993; Bernier *et al.*, 1998). While assimilates and hormones are generally present in most plants, some of these compounds may be absent or not present at optimal levels (or ratios) for floral development. Genetic analysis of flowering time in pea, cereals and *Arabidopsis thaliana* supports the hypothesis that the transition to flowering is under multifactorial control (Koornneef *et al.*, 1998); multiple genes that control flowering time have been identified in all of the above. Moreover, some of these genes act to promote flowering and others to suppress it (e.g. obligative and facultative flowering habits). Nevertheless, in some species floral transition appears to be under the control of a single substance, such as in the LD plants *Samolus parviflorus, Rudbeckia bicolour* and *Lolium temulentum*, which can be induced to flower in SDs by treatment with gibberellic acid (GA_3) (reviewed by Corbesier and Coupland, 2005). Gibberellin treatment appears to increase apex sucrose levels (King *et al.*, 2001).

All these hypotheses propose that floral evocation results from the translocation of signals to the shoot apical meristem (Kinet, 1993). These signals would be transmitted through phloem sap and to a lesser extent, through the xylem. They can potentially be identified by sap collection and comparison of the composition of the sap between vegetative and floral-induced plants (Corbesier *et al.*, 2002; Hoffmann-Benning *et al.*, 2002). However, because of difficulties in obtaining and analysing phloem sap and the lack of a bioassay, the chemical nature of the floral stimulus is one of the major unsolved problems in plant biology.

2.5.1.2 Floral evocation or initiation

The second phase of flowering is flower evocation. Floral evocation is determined by genotype (McDaniel *et al.*, 1996), and involves the transformation of the leaf primordia producing a shoot apex to a flower producing primordium. This transformation involves a radical reorganisation of the apex structure (Roh, 2005). Once the apex has changed to a flower primordium, the process is irreversible. However, the floral apex may be aborted, if subsequent environmental conditions are not favourable for full flower development. In such a case, the axillary buds below the aborted floral apex will usually grow vegetatively until day length or temperature conditions are once again favourable for flower induction (Aloni *et al.*, 2006).

2.5.1.3 Floral development

Once the developmental phase has begun, floral primordia will arise on the flanks of the apical meristem in place of leaves. In many cases, vegetative meristems are not directly converted to floral meristems. Instead, the vegetative meristem is first transformed into an inflorescence meristem. The types of lateral organs produced by an inflorescence meristem are different to those produced by a floral meristem. The inflorescence meristem produces bracts and floral meristems in the axils of the bracts, instead of the sepals, petals, stamens, and ovules produced by floral meristems.

Inflorescence meristems may be determinate or indeterminate, depending on the species. In determinate species, the shoot apical meristem eventually produces floral organs and the production of meristems is terminated with a flower. The shoot apical meristem of indeterminate species is not converted to a floral identity and therefore only produces floral meristems from its periphery, resulting in a continuous growth pattern (Jensen *et al.*, 2001). The number of days from flower initiation to anthesis depends on the species and the cultivar as well as environmental conditions during growth (Day *et al.*, 1995).

Resources/conditions needed or prefered to complete evocation and development are:

- adequate water
- adequate nutrients (soil organic matter and nutrient dynamics)
- adequate light

- no temperature extremes.

Despite intensive research over several decades into the regulation of flowering, plant developmental biologists and physiologists still do not fully understand the process of flower induction, evocation and development. Recent research on the flowering time of mutants of *A. thaliana* has greatly increased the knowledge of the molecular mechanisms of flowering and flower development (Jack, 2004b; Bernier and Perilleux, 2005). Although molecular techniques are helpful, generalisations about flowering are limited and many species act uniquely, based on genotype, and there are a large number of exceptions to each rule. Plants of the same genotype grown under different conditions can appear qualitatively different (for example, see Beveridge *et al.*, 2003).

2.6 The genetic basis of flower development

Extensive genetic and molecular studies of *Arabidopsis* and other plants have identified numerous genes that play key roles during flower development (Kramer and Hall, 2005). Numerous reviews provide a broad overview of the molecular basis of floral development in *Arabidopsis*. Quite a number of excellent comprehensive reviews are available (e.g., Jack, 2004a, b; Bernier and Perilleux, 2005; Simpson, 2005; Smyth, 2005). Important genes in flowering regulation can be classified into four groups, related to the key physiological and developmental phase (Silva *et al.*, 2005):

- flowering-time genes
- meristem-identity genes
- floral-organ-identity genes
- late-acting genes.

Genetic analyses of various plant species have identified many of the genes that regulate flowering time in response to hormones, photoperiod, light quality, temperature, and other environmental factors (Mouradov *et al.*, 2002; Putterill *et al.*, 2004). In *Arabidopsis*, regulation occurs through a complex network of genetic pathways (Figure 2.4), with two main pathways mediating environmental responses, the LD and vernalisation pathways, and two pathways that function independently of environmental cues, the autonomous pathway, which promotes flowering under all conditions, and the GA pathway, which is needed for flowering under non-inductive SD conditions

(Blazquez *et al.*, 2001; Lee *et al.*, 2006). These pathways converge in the induction of floral meristem-identity genes and the floral transition.

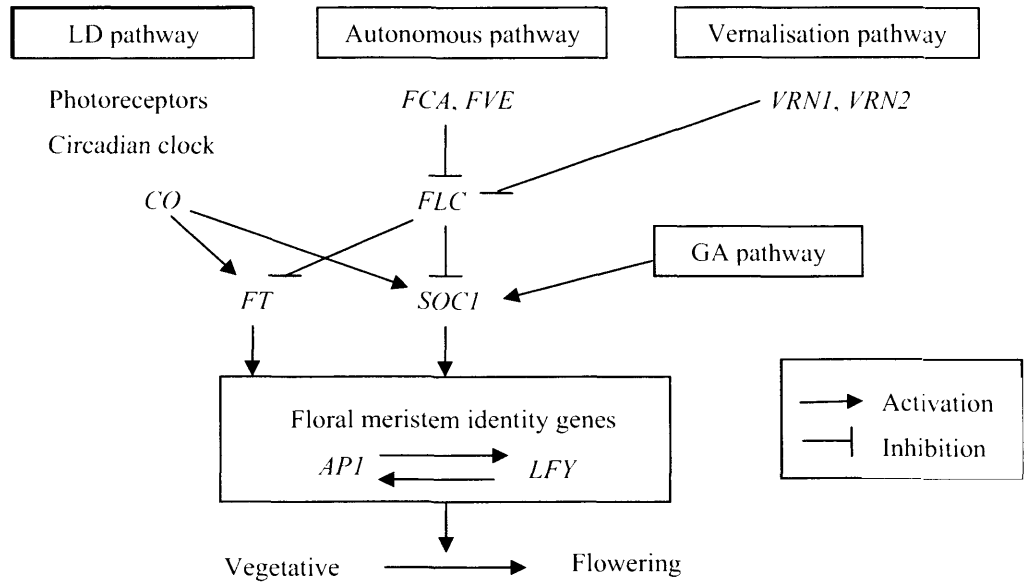

Figure 2.4 The four major genetic pathways regulating flowering time in *Arabidopsis* (Blazquez *et al.*, 2001; Lee *et al.*, 2006). *API* = *APETALA1*, *CO* = *CONSTANTS*, *FCA* = *FLOWERING CA*, *FVE* = *FLOWERING VE*, *FLC* = *FLOWERING LOCUS C*, *FT* = *FLOWERING LOCUS T*, *LFY* = *LEAFY*, *SOC1* = *SUPPRESSOR OF OVEREXPRESSION OF CO1*, *VRN1* = *VERNALISATION1*, *VRN2* = *VERNALISATION2*.

2.6.1 Models of floral development control

The genetic control of flower organ identity was a major discovery in plant developmental biology in the 1990s. The discovery was based on the study of homeotic flower mutants with normal floral organs at ectopic positions replacing the flower organs usually present (Van Lijsebettens and Van Montagu, 2005). Coen and Meyerowitz (1991) proposed that floral organisation in each fold or whorl is determined by a unique combination of three floral-organ-identity genes, A, B and C genes (Figure 2.5). Activity of the Type A gene alone specifies sepals, whereas petal formation requires both A and B gene activity. Stamens are formed by a combination of the activity of both B and C genes, whereas the activity of C alone specifies carpels. The model also proposed that the activity of A and C mutually repress each other. Subsequent studies have modified and refined the ABC model (Figure 2.5) and extended it to many other species (Lohmann and Weigel, 2002; Jack, 2004b). The ABC

model has been verified by double and triple mutants, over-expression constructs and gene expression analyses; it still used today, even though it has been extended with D and E function genes (Pelaz *et al.*, 2000; Honma and Goto, 2001).

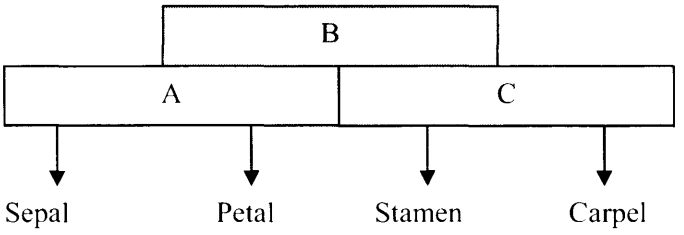

Figure 2.5 Classical ABC model (From Kanno *et al.*, 2003).

Coen and Meyerowitz (1991) developed strategies to understand floral morphogenesis based on the molecular genetic analysis of floral homeotic mutants. Genetic analysis of flower organogenesis in the model systems *Arabidopsis* and *Antirrhinum* generated the proposed ABC model (Figure 2.5) to explain control of floral organ identity. Patterning of the four organ types, sepals, petals, stamens and carpels, along the floral axis is controlled by the coordinated expression of at least three sets of floral-organ-identity genes, whose activities, termed A, B, and C, are arranged in three concentric overlapping rings at floral organ primordia initiation. At a given organ primordium, the expression of A activity specifies sepal identity, A and B together give rise to petals, B and C together control stamen identity, and C alone specifies carpel formation. Most of the floral homeotic genes belong to the MADS-box gene family and encode for transcription factors that show homology to others described in yeast and humans (Schwarz-Sommer *et al.*, 1990; Theissen *et al.*, 2000). Many genes controlling processes such as meristem identity and floral organ identity, genes that regulate the spatial expression of the latter, and genes controlling tissue differentiation within floral organs have been isolated from these models and many other plant systems (Theissen *et al.*, 2000), revealing that the essential mechanisms are widely conserved, although particular differences have to account for the great variety in inflorescence architecture and flower morphology observed in angiosperms.

The identity of the floral organs has been postulated to be specified by tetrameric complexes of floral-organ-identity gene products that bind to promoters of downstream

targets, thereby activating or repressing their activity and resulting in specific floral organ identities (Theiben and Saedler, 2001). ABCDE genes were identified in many species of flowering plants, monocots and dicots, confirming the conservation of the overall molecular mechanism of flower organ formation in evolution (reviewed by Ferrario *et al.*, 2004). Function conservation of key regulators in development, such as the MADS-box transcription factors with a role in flower organ specification, begins to integrate the main aspects of flower morphology in different species, such as the different floral organ types and the floral whorls (Cork and Purugganan, 2004).

2.6.2 Floral development in pea (*Pisum sativum*)

The model plant *Arabidopsis* has provided much information about the genes and genetic pathways that control the flowering process, but little is known about the corresponding pathways in legumes. Hecht *et al.* (2005) found that the majority of *Arabidopsis* flowering genes are represented in pea and in legume sequence databases, although several gene families, including the MADS-box, *CONSTANS*, and *FLOWERING LOCUS T/TERMINAL FLOWER1* families, appear to have undergone differential expansion, and several important *Arabidopsis* genes, including *FRIGIDA* and members of the *FLOWERING LOCUS C* clade, are conspicuously absent from pea. The analysis of *UNIFOLIATA* (*UNI*) and *STAMINA PISTILLOIDA* (*STP*), the pea orthologs of *LEAFY* and *UNUSUAL FLORAL ORGANS*, has revealed a common link in the regulation of flower and leaf development not apparent in *Arabidopsis*. While the *Arabidopsis* genes mainly behave as key regulators of flower development, where they control the expression of B-function genes, *UNI* and *STP* also contribute to the development of the pea compound leaf (Hofer *et al.*, 1997).

The order of floral organ initiation in pea differs from the model species *Arabidopsis* and *Antirrhinum*, where petal and stamen primordia are formed directly from the floral meristem; in pea flower ontogeny, once sepal and carpel primordia have emerged, four structures made up of proliferating cells are formed, called common primordia (Figure 2.6). Proliferation and differentiation processes within each of these common primordia ultimately give rise to second- and third-whorl floral organs in pea flowers (Benlloch *et al.*, 2003).

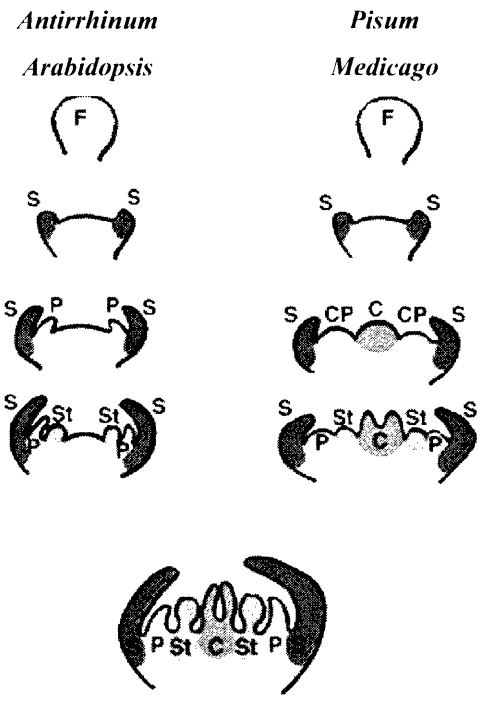

Figure 2.6 Floral ontogeny of *Arabidopsis* and *Antirrhinum* flowers (left) compared to *Pisum* and *Medicago* (right). F = Floral meristem; S = sepal primordium; CP common primordium; P = petal primordium; St = stamen primordium; C = carpel primordium (From Benlloch et al., 2003).

Pisum sativum has been previously proposed as a model system to study the genetic control of flower development in legumes (Ferrandiz *et al.*, 1999; Berbel *et al.*, 2001). Several studies have been done to understand the genetic control of flowering in pea (Murfet and Reid, 1993; Berbel *et al.*, 2001; Beveridge *et al.*, 2001). In *P. sativum*, flowering time is mainly determined by genotype-specific responses to photoperiod and temperature (Alcalde and Larrain, 2006). At least 12 genes controlling various aspects of the flowering process have been identified in pea (Table 2.2) (reviewed by Beveridge and Murfet, 1996). However, the number and molecular identity of the genes responsible for the organ identity functions have not yet been established. Molecular genetic studies on most legume crops are difficult owing to their large genome size, polyploidy and inefficient *in vitro* regeneration and genetic transformation (Benlloch *et al.*, 2003). Knowing the relationship between flowering genes in model species would enable researchers to combine information and capitalise on the advantages of each species.

The genes involved in pea flowering were extensively characterised in several reviews (Weller *et al.*, 1997; Hecht *et al.*, 2005; Domoney *et al.*, 2006) and more recent analysis

of genomic data from model legume sequencing projects has identified homologues in pea for many of the key regulators of *Arabidopsis* flowering time (Domoney *et al.*, 2006).

Table 2.2 Critical genes in flower development in pea (*Pisum sativum*) (Beveridge and Murfet, 1996).

Gene	Phenotypic effect
Synthesis pathway:	
GI (*gigas*)	Synthesis of the floral stimulus
Photoperiodic induction pathway:	
SN (sterile nodes), *DNE* (day-neutral) and *PPD* (photoperiod)	Delays flowering; Responsiveness to photoperiod
E (early) and *HR* (high response)	Enhances effect of *SN*, *DNE* and *PPD*
LF (late flowering)	Specifies minimum flowering node
DM (diminutive)	Delays flowering; Reduce internode length, leaf size and leaflet number
Meristem identity:	
VEG1, *VEG2* (vegetative), *DET* (determinate) and *PIM* (proliferating inflorescence meristem)	Required for flower initiation

Pea and *Arabidopsis* have developed different strategies to control flowering time; these may represent different strategies to respond to environmental conditions. In nature, flowering time variation in *Arabidopsis* populations is explained mainly by response to vernalisation, whereas in pea it seems to be explained mostly by response to light (Foucher *et al.*, 2003). In an ephemeral desert plant, such as *S. formosa*, seasonal flowering might be evolved to respond to both temperature signals and light cues.

The relationships between flowering time and floral-meristem-identity genes are complex and genetic interpretations are complicated by functional (phenotypic) redundancy. Gene control of flowering initiation is becoming a 'hot topic' of plant developmental biology. As more and more genes are isolated, it becomes more and more important to understand the internal pathways and the relationship between different pathways.

2.7 The control of flowering

Flowering is controlled via several interacting pathways influenced by both internal and external (environmental) factors. Internal factors include plant maturity, nutrition status, hormones and other compounds, while the external factors include light, temperature, and stress conditions. These factors are outlined and discussed in the following section.

2.7.1 Internal factors

2.7.1.1 Plant maturity

Because plants must reach a certain age or size before they can flower, the vegetative meristem is thought to first pass through a juvenile phase in which it is unable to respond to internal or external signals that would trigger flowering in an adult meristem. In some species, the juvenile phase is clearly distinguished from the adult phase on the basis of their phyllotaxis, leaf retention, thorniness, and many other traits (Mouradov *et al.*, 2002; Poethig, 2003). It is possible that some of the genes identified as important in controlling the transition from vegetative to reproductive development are also involved in vegetative phase change (see section 2.6) (Levy and Dean, 1998).

Herbaceous annual plants usually have a very short juvenile period, and are considered competent for flowering during their growth period (Ziv and Naor, 2006). In contrast, the juvenile phase of woody plants can last as long as decades. For example, juvenile phases in bamboo (*Bambusa* sp.) range between 2 to 120 years (Ziv and Naor, 2006). In strawberry (*Fragaria*), a perennial herbaceous plant, Verheul *et al.* (2006) found that the start of flowering was delayed with increasing plant age, but the number of inflorescences and the number of flowers per inflorescence increased with plant age. Strawberry appears to have a minimum plant size that has to be exceeded before flower induction can occur (Jahn and Dana, 1970).

Flowering in some plants occurs after the apex has produced a particular number of leaves, while some plants must achieve a certain number of nodes (Irish and Nelson, 1991). Adkins and Dirr (2003) reported that the stage of meristem development was highly correlated with number of nodes, but not with shoot length in the floral development of *Hydrangea macrophylla*. In *Swainsona formosa*, the number of nodes

produced before the terminal inflorescence is tightly controlled (Taji, A., 2004 pers comm., 15/03/2004). In *Hibiscus rosa-sinensis*, flowers are first initiated after six or seven leaves have appeared, depending on cultivar (Karlsson *et al.*, 1991). In *Plantago coronopus*, after the formation of about 14 leaves, plants started to form flowering buds, independent of weight or leaf size. The shoot : root ratio stayed the same during both the vegetative and generative stages (Koelewijn, 2004).

Environmental and agronomic factors, such as photoperiod, varying nutritional levels or supplying carbon dioxide (CO_2) enriched atmosphere, may increase vegetative growth and, if properly controlled, can shorten the time to maturity (Thomas and Vince-Prue, 1997). The effect that environmental (discussed in section 2.7.2) and hormonal factors have on the length of the juvenile phase will ultimately depend on genetic control (see section 2.6). A plant is considered mature when it becomes potentially capable of reproducing. Although a plant may be mature, flowering may not occur until environmental conditions are favourable for floral initiation and/or development.

2.7.1.2 Nutrition

Carbohydrates

It is known that nutrient status directly enhances vegetative growth, flowering, and fruiting in plants (Munoz *et al.*, 2005), and changes associated with evocation of flowering have been an area of long-standing interest (Bernier and Perilleux, 2005). One of the internal stimuli considered by Schwabe (1987) considered as a possible activator of the transition of the shoot apex from the vegetative to the reproductive phase is a quantitative change in carbohydrates.

Carbohydrates are polyhydroxy aldehydes or ketones, or substances that yield such compounds on hydrolysis. Many, but not all, carbohydrates have the empirical formula $(CH_2O)_{ni}$ and some also contain nitrogen, phosphorus, or sulphur (Nelson and Cox, 2005). There are three major size-classes of carbohydrates: monosaccharides, oligosaccharides, and polysaccharides. Glucose and fructose are monosaccharides, which consist of a single polyhydroxy aldehyde or ketone unit. Sucrose is a disaccharide (oligosaccharide) of glucose and fructose, joined by characteristic linkages called glycosidic bonds (Figure 2.7).

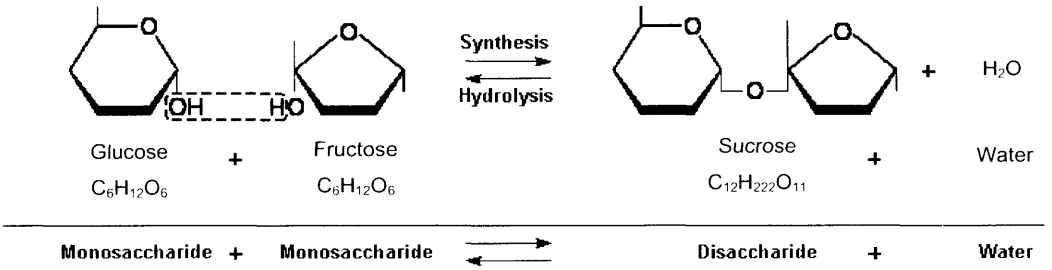

Figure 2.7 The synthesis and hydrolysis of sucrose (disaccharide) (From Uno *et al.*, 2001).

The carbohydrate translocated to a sink floral organ originates for two sources: carbohydrate accumulated during the vegetative stage and photosynthate produced during the reproductive stage (Krapp *et al.*, 2005). Sucrose is the main form in which carbon (C) is translocated through the phloem of the vascular system from the leaf to other parts of the plant, but sucrose and other sugars can also be isolated and stored in vacuoles in the mesophyll cells (Wardlaw, 1990). There are two distinct pathways for cleaving sucrose and yielding glucose and fructose: via invertase (acid and neutral forms) and via sucrose synthase (SS) (Sergeeva *et al.*, 2006). The activity of these sucrose-splitting enzymes varies with plant species, organ type and stage of development (Krapp *et al.*, 2005). The synthesis of sucrose occurs in different cellular compartments, and these processes are coordinated by a variety of regulatory mechanisms that respond to changes in light level and photosynthetic rate (Krapp *et al.*, 2005).

Studies with several plant species indicate that the levels of sucrose in the phloem sap and/or soluble sugars in the apical bud increase early upon photoperiodic floral induction in both LD plants and SD plants. For example, plants in which increased carbohydrate level at the apex is correlated with floral initiation include the SD plant *Xanthium strumarium* (Ireland and Schwabe, 1982), the LD plant *Sinapis alba* (Bodson and Outlaw, 1985; Lejeune *et al.*, 1991; Corbesier *et al.*, 2002) and *Arabidopsis thaliana* (Corbesier *et al.*, 2002). Also, strong relationships between flower development and carbohydrates in woody plants, such as grape (*Vitis Vinifera*) (Lebon *et al.*, 2005), and in cold-requiring plants, such as *Cheiranthus cheiri* (Diomaiuto, 1988), have been demonstrated. Roh *et al.* (1992) also found increased quantities of

sucrose in the apex of the flowering bulb *Eucrosia bicolor* during its transition from the vegetative to the reproductive stage. According to defoliation experiments (Corbesier *et al.*, 1996), this increase in carbohydrate levels coincides with the start of mobile signal transport and occurs before the activation of cell division in the meristem (Corbesier *et al.*, 1998), suggesting that sugar could play a signalling role. However, in *Dryobalanops aromatica*, total non-structural carbohydrate (TNC) in the branch decreased during the flowering period, but TNC was still present in all organs even after flowering (Ichie *et al.*, 2005). It suggests that *D. aromatica* supplied the carbohydrate resources needed for flowering over a short period mainly from storage assimilates, whereas the resources for fruiting came from current photosynthesis on reproductive shoots.

Carbon:Nitrogen (C:N) ratio

Nutrient content at the shoot apical meristem changes at the time of floral transition, induced by changes in the transport of sucrose and other sugars to the apex (Bodson, 1977). In the nutrient diversion hypothesis of floral development, Sachs (1977) proposed that the flowering process is promoted if carbohydrate, or energy, availability to the apical meristem is increased and it is inhibited if this availability is decreased. However, other studies have indicated that carbohydrate concentration is not the only controlling factor (King and Evans, 1991) and that N concentration also may be involved in the control of floral onset (Bernier *et al.*, 1981a, b). Raper *et al.* (1988) proposed that floral transition is stimulated by an imbalance in the relative concentrations of carbohydrates and N in the apical meristem rather than by the absolute concentration of either within the meristem. However, more recent work has shown that increases in sucrose levels at the apex change at the time of flowering in *Fuchsia hybrida*, regardless of whether or not an inductive photoperiod occurred (King and Ben-Tal, 2001). It is possible that changes in sucrose concentrations, or other nutrients, are a result of floral transition and not the signal that induces flowering. Indeed, when LD inductive signals at low light levels were applied, the amount of sucrose at the apex did not increase, while plants still flowered in response to the day length changes (King and Ben-Tal, 2001). While these results indicated that nutrient changes at the apex are not required for floral evocation, it is still not known if these changes can induce flowering.

Generally, high concentrations of N inhibit flowering and promote vegetative growth (Dielen *et al.*, 2001). It has been shown that a high C:N ratio promoted flower initiation

in *P. sativum* (Wareing and Phillips, 1978). In rice (*Oryza* sp.), the C:N ratio increases exponentially with growth (Osaki *et al.*, 1992), with starch and soluble sugar concentrations in rice leaves decreasing with increasing N rates (Wang *et al.*, 2006). In studies on the marine phanerogam *Posidonia oceanica*, Gobert *et al.* (2005) reported that flower formation caused a significant increase in carbohydrate and a significant decrease in N concentrations in intermediate and adult leaves. The importance of the C:N ratio for flowering was also observed *in vitro*, for example in *Pharbitis nil* (Ishioka *et al.*, 1991) and tomato (*Lycopersicon esculentum* (now known as *Solanum lycopersicon*) (Dielen *et al.*, 2001). In *Sinapis alba* and *Arabidopsis thaliana* leaves, the C:N ratio of the phloem sap, mainly the sucrose/glutamine ratio, increased markedly and early during a single LD inductive treatment, suggesting that a flux in organic C and/or decrease in N supply to the apical meristem may be important at floral transition (Corbesier *et al.*, 2002).

The induction of flowering in some species can be achieved by mild plant water stress or low N fertility (Davenport, 2003). Vaz and Kerbauy (2000) also reported that a low concentration of N stimulated flower formation in *Psygmorchis pusilla* (Orchidaceae) *in vitro*. Flower formation was also enhanced by reducing phosphorus (P) and potassium (K) to half strength, whereas the highest calcium (Ca) concentration showed some floral stimulation (Vaz and Kerbauy, 2000). In sweet pepper (*Capsicum annum*), a low concentration nutrient solution containing 3:0.5:1.25 mM of N:P:K, when combined with the plant being grown in a small container, induced an early flowering of short duration (Xu *et al.*, 2001). Overcrowding of roots in the small container may have caused water and/or nutrient stress, which promoted the flowering.

In day-neutral tobacco (*Nicotiana tabacum*), flowering time may be accelerated by different stresses (e.g. low temperature, N withdrawal, restriction of N uptake) and these treatments result in an imbalance in the relative availability of carbohydrate and N in the shoot apical meristems (Raper *et al.*, 1988; Rideout *et al.*, 1992). In pea, Truong and Duthion (1993) found that water stress, which was closely related to deficient N nutrition, also promoted floral development. Deficiencies in essential macronutrients (N, P, K and magnesium) result in the accumulation of carbohydrates in leaves and roots (Hermans *et al.*, 2006).

In contrast, Stutte and Martin (1986) showed that carbohydrate levels did not limit floral induction of olive (*Olea europaea*), similar to the observations of Ulger *et al.* (2004). Ulger *et al.* (2004) found that N levels during the differentiation period may play a role in bud differentiation in a heavy fruiting year, and the C:N ratio seems to have no effect on induction in olive. Clearly, flowering in different plant species is affected by different mineral nutrition. Such complex responses make it difficult to argue that transition to flowering requires low N status coupled with enhanced C supply.

Carbon and Nitrogen metabolisms

Many interactions exist between C and N metabolisms. Carbon skeletons are essential for the incorporation of inorganic N into biologically relevant molecules (Figure 2.8), such as amino acids, proteins, nucleic acids and a large number of secondary metabolites (Krapp *et al.*, 2005). Osaki *et al.* (1988) demonstrated that when N accumulation had almost ceased in rice, and the major part of N in the reproductive organs was derived from N absorbed and incorporated into proteins in leaves.

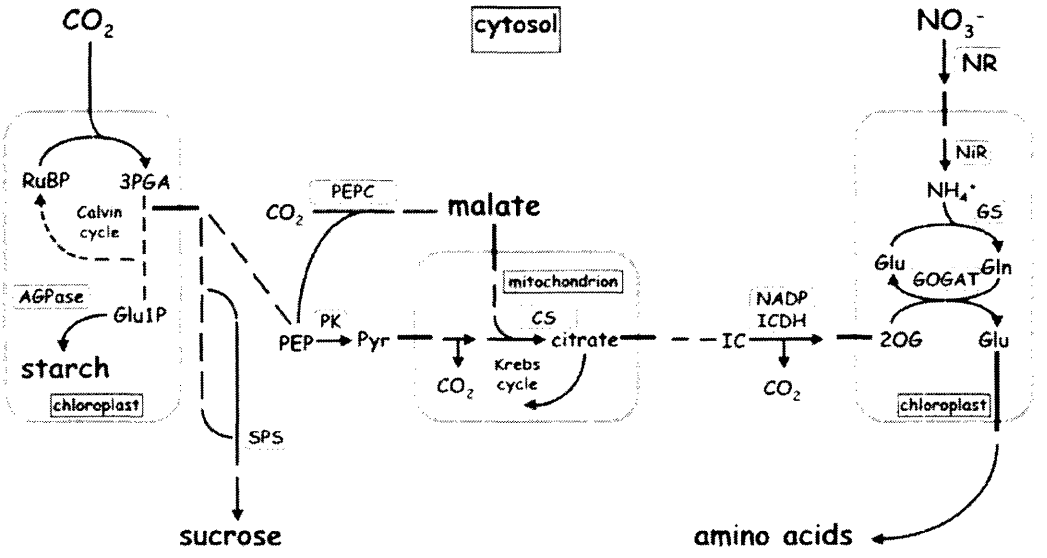

Figure 2.8 Simplified overview of primary C and N metabolism in photosynthetic cells of higher plants (Krapp *et al.*, 2005). Enzyme name are given in boxes, PK = pyruvate kinase, AGPase = ADP glucose phosphorylase, CS = citrate synthase, ICDH = isocitrate dehydrogenase, RuBP = ribulose-1,5-bisphosphate, 3PGA = 3-phosphogycerate, PEP = phosphoenolpyruvate, Pyr = pyruvate, IC = isocitrate, Glu1P = glucose-1-phosphate.

Shinano *et al.* (2006) indicated that leaf position is an important factor controlling photosynthate distribution to the N-pool or C-pool, regardless of the leaf N status. Apart from photosynthesis, C metabolites and plant C:N status both regulate the expression of genes involved in processes as diverse as pathogen attack, environmental stress, storage protein accumulation, and starch, lipid and N metabolism (Pego *et al.*, 2000), and nitrate regulates many genes assigned to sugar metabolism (Harbaugh and Scott, 2001).

In summary, it can be said that when sugar concentrations in the plant increase, there is repression of genes involved in the mobilisation of stored reserves and in photosynthesis. At the same time, genes required for metabolism and storage of C metabolites for future use, e.g. fruit development, are induced. There are many genes whose expression is regulated by this mechanism, and C metabolite-mediated regulation of gene expression seems to be a central and basic process, probably common to all higher plants (Pego *et al.*, 2000).

2.7.1.3 Protein

Changes in the pattern of proteins associated with plant developmental stages have been obtained in *Pisum* vegetative buds (Stafstrom and Sussex, 1988), *Sinapis alba* (Lyndon *et al.*, 1983) and *Silene coeli-rosa* (Taylor *et al.*, 1990) shoot apices. Unique 21 and 103 kDa proteins were evident in one-dimensional electrophoresis of proteins from mature leaves of *Doritis pulcherrima* Lindley, cv. S94-3345 under SD conditions. Two-dimensional gel electrophoresis confirmed that clear polypeptide spots (molecular mass of 21 kDa at an isoelectric point of 5.2 and 103 kDa at an isoelectric point of 5.6) accumulated in the leaves when the flowering shoots had 4 – 5 flower primordia apparent. Possibly, the 21 and 103 kDa proteins play a role during flowering shoot formation in *D. pulcherrima*. Polypeptide sequencing of P21 suggested a possible relationship to the cell division-like protein in *Arabidopsis thaliana* (Wang *et al.*, 2003). It is clear that flowering in *D. pulcherrima* is closely associated with protein synthesis changes in its leaves.

Some studies have focused on the analysis of reproductive-specific polypeptides (Araki and Komeda, 1990; Ono *et al.*, 1991). In longan (*Euphoria longana*), flowering was accompanied with the disappearance of some isozymes, so that there were fewer bands than in vegetative shoots (Zheng *et al.*, 2001). By using the incorporation of ^{35}S-

methionine and two-dimensional polyacrylamide gel electrophoresis in the shoot apex of *S. alba*, a LD plant, before the initiation of floral primordia, a change in the rate of synthesis and appearance of new proteins was noticed (Lyndon *et al.*, 1983). In *Crocus sativus*, Abrishamchi *et al.* (2004) found that, in comparison with the vegetative apex, 89 new polypeptides were identified in the prefloral meristem and 29 polypeptides were missing. In the reproductive meristem, 94 new spots were identified and 44 spots were missing. Substantial quantitative and qualitative changes in the protein composition occurred during the prefloral stage before floral primordia initiation. The qualitative changes indicate that distinct groups of proteins are involved in different steps in the transition to flowering. A significant point of these studies and others is the protein composition of the vegetative meristem, which is qualitatively different from that of the reproductive meristem. In addition, the plant organ development is accompanied by a differential modification of proteins that reflects a pattern of molecular differentiation (Thompson-Coffe *et al.*, 1992).

Some researchers have used other techniques, such as microbore high-performance liquid chromatography (HPLC) (Hoffmann-Benning *et al.*, 2002) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Kehr *et al.*, 1999) and molecular genetic analysis (Marco and Carrasco, 2002) for examining changes in the protein composition associated with plant developmental stages. As an alternative to gel electrophoresis, microbore HPLC separations allowed for the detection of proteins/peptides that were very small and present at very low levels. Recent molecular genetic studies in *A. thaliana* have made progress in identifying genetic pathways and regulatory proteins associated with the control of flowering time (Boss *et al.*, 2004; Putterill *et al.*, 2004). Specifically in the photoperiodic control of flowering, models were proposed in which circadian clock control of gene transcription and post-transcriptional regulation of protein stability by light can combine to confer control of a regulating pathway that mediates the induction of flowering by day length (Corbesier and Coupland, 2005). Hu *et al.* (2003) also suggested that the genes encoding putative regulatory proteins, and at least some proteins with currently unknown functions, might play important roles during flowering.

2.7.1.4 Plant growth regulators and other compounds

Growth hormones are involved in both the genetic and environmental control of flowering. The pattern of distribution of growth hormones in the plant is controlled by interactions between the environmental and genetic factors (Halliday and Fankhauser, 2003). There are five major types of plant hormones: gibberellins (GAs), cytokinins, auxins, abscisic acid (ABA) and ethylene. Table 2.3 shows the effect of plant growth regulators on flowering. Hormones may be either growth inhibitors or promoters or both, depending on the site of action and concentration of the substances (Hay *et al.*, 2004).

In some ornamentals and tree species, GA inhibits flowering, apparently by diverting nutrients from the shoot apex. For example, in the LD plant *Fuchsia hybrida*, sucrose supply regulates flowering because apex sucrose content and flowering increase in parallel with increased light intensity in non-inductive SD. However, applied GA inhibits flowering whether it is induced in LD or by high light intensities (reviewed by King, 2003). Most of the SD and some day-neutral plants did not respond or their flowering was inhibited by GA treatments (Ben-Tal and Erner, 1999).

Rebers *et al.* (1999) indicated that transcript levels of GA biosynthesis genes are all highly regulated during flower bud development. In *A. thaliana*, a separate pathway, including the genes of GA biosynthesis and signalling, was identified among the multiple pathways promoting flowering (Simpson, 2005). In pea, however, dwarfing mutations decreasing the endogenous GAs to very low levels had a minimal effect on the node of first flower initiation or day-length responsiveness (Weller *et al.*, 1997). Moreover, exogenous GA can cause a delay in flowering in pea (Beveridge and Murfet, 1996). In *S. formosa*, the application of GA_3 results in increased plant height, but it does not decrease the time it takes to flower (Jusaitis and Schmerl, 1993). Moreover, the application of paclobutrazol (PBZ) (GA inhibitor) has been reported to delay flowering in *S. formosa* (Hamid and Williams, 1997a). Such complex responses make it difficult to argue whether genes controlling flowering time in pea are either likely or unlikely to act by influencing GA levels and metabolism.

Table 2.3 Effects on flowering in some plant species of plant growth regulators and related compounds that function as hormones.

Effect	Plants	Chemical[1]	References
Promote flowering	*Brassica napus*, *Chenopodium rubrum* and *C. murale*	Cytokinins	(Machackova *et al.*, 1993)
	Iris × *hollandica* cv. 'Sapphire Beauty'	Ethylene	(Botha *et al.*, 1998)
	Lolium temulentum	GA_4	(Bernier *et al.*, 1993)
	Chrysanthemum sp.	GA_5	(Corbesier *et al.*, 2004)
	Philodendron 'Black Cardinal'	GA_3	(Corbesier *et al.*, 2004)
	Olea europaea	GA_4, ABA and Cytokinin	(Chen *et al.*, 2003)
	Curcuma alismatifolia	GA_{4-7}	(Sarmiento and Kuehny, 2003)
	Eucalyptus nitens	GA	(Moncur and Hasan, 1994)
	Arabidopsis thaliana	IAA	(Aloni *et al.*, 2006)
		Steroids	(Janeczko *et al.*, 2003)
		Salicylic acid	(Martinez *et al.*, 2004)
	members of the Bromeliaceae	Ethylene	(Botella *et al.*, 2000)
	Spirodela polyrrhiza	Jasmonic acid	(Krajncic and Nemec, 1995)
	Lemna minor	Jasmonic acid	(Krajncic *et al.*, 2006)
	Sinapis alba	Polyamines	(Havelange *et al.*, 1996)
	Rosa sp.	Polyamines	(Sood and Nagar, 2004)
	Lemna gibba, *Spirodela polyrrhiza* and *Wolffia microscopica*	Salicylic acid	(Crozier *et al.*, 2000)
Delay flowering	*Scaevola aemula*	Ethephon	(Starman and Williams, 2000)
Prevent flowering	*Pisum sativum*	GA_3	(Reid *et al.*, 1977)
	Mangifera indica	GA_3	(Nunez-Elisea and Davenport, 1998)
	Olea europaea	GA_3	(Ulger *et al.*, 2004)
	Pharbitis nil	ABA and IAA	(Wijayanti *et al.*, 1997)
		Jasmonic acid	(Maciejewska *et al.*, 2004)
		Salicylic acid	(Groenewald and van der Westhuizen, 2001)
	Nicotiana tabacum	Jasmonic acid	(Barendse *et al.*, 1985)
	Chenopodium rubrum	Jasmonic acid	(Albrechtova and Ullmann, 1994)

[1] GA_3 = gibberellic acid, GA_4 = gibberellin A_4, GA_5 = gibberellin A_5, GA_{4-7} = gibberellin A_{4-7}, ABA = abscisic acid, IAA = indole-3-acetic acid

A growing number of reports indicate that plant growth regulators are not limited to the five classical plant hormones. Other investigations have identified a variety of compounds that function as hormones in various groups of plants, including plant growth retardants such as paclobutrazol (Gent and McAvoy, 2000), sterols (Janeczko *et al.*, 2003), jasmonate (Creelman and Mullet, 1997) and prostaglandins (Groenewald and van der Westhuizen, 2001). In addition, other growth regulators, such as polyamines (Hoffmann-Benning *et al.*, 2002) and salicylic acid (Martinez *et al.*, 2004), are likely to interact with these hormones to specifically regulate plant metabolism or plant responses to biotic and abiotic stresses.

Growth retardants are a diverse group of synthetic compounds that retard stem elongation and increase the green colour of leaves. Growth retardants inhibit cell division in the subapical meristem of the shoot. The physiological effects of growth retardants can be reversed by an application of GA (Gianfagna, 1995), therefore, inhibition of GA biosynthesis is considered to be the primary effect of the action of growth retardants. In some species, flowering is promoted by growth retardants, and in others it is delayed. Table 2.4 lists some growth retardant chemicals and some of the plants they affect.

The flowering response to growth retardants is complicated, and varies with cultivar and application method. Part of the difference in response among plant cultivars is due to different growth habits, and part is due to differences in metabolism and response to GA. This may be a direct consequence of changes in concentration of GAs. or it may be a secondary effect of reducing the competition between vegetative and reproductive for sugars and mineral nutrients (Rolland *et al.*, 2006).

In *Myriophyllum spicatum*, Nelson (1996) found that flurprimidol-treated plants had up to 68% more non-structural carbohydrate. Paclobutrazol reduced leaf area and chlorophyll concentration in *Carya* but increased total carbohydrates per unit area (Wood, 1984). Paclobutrazol-treated *Oryza sativa* used less carbohydrate for shoot growth and stored more starch in crowns, although the starch-degrading activity was not altered (Yim *et al.*, 1997). The most likely cause of this accumulation of carbohydrate is decreased growth, reducing the demand for the products of photosynthesis. However, leaf carbohydrate concentrations were decreased in *Lilium* 'Nellie White' by

uniconazole (Bailey and Miller, 1989), and in *Pelargonium* 'Red Elite' by chlormequat (White and Warrington, 1984). From these examples, it can be concluded that growth retardants can alter the sugar and starch content of plants through different routes.

The effects of hormones and other compounds differ from species to species. Slight changes in hormone concentrations can radically alter their effects, and determining how a given growth regulator will influence the flowering of a particular species often requires considerable empirical investigation. There is important potential for the practical application of this knowledge because the induction of flowers often plays such an important role in agriculture. Further work with hormones and growth regulators can lead to a more complete understanding of the flowering process.

Table 2.4 Effects on flowering in some plant species of some plant growth retardants.

Effect	Plants	Chemical[1]	References
Promote flowering	*Lupinus varius*	PBZ	(Karaguzel *et al.*, 2004)
	Rhododendron hybrid 'Sir Robert Peel'	PBZ	(Wilkinson and Hanger, 1992)
	Scaevola aemula cultivars 'New Wonder'	PBZ, Uniconazole	(Starman and Williams, 2000)
	Rhododendron sp.	PBZ, Uniconazole	(Gent, 1995)
	Camellia 'Shishi-Gashira'	Uniconazole	(Keever and McGuire, 1991)
	Globularia sarcophylla	Uniconazol	(Katz *et al.*, 2003)
	Lantana camara subsp. *camara*	Chlormequat	(Matsoukis and Chronopoulou-Sereli, 2005)
Substitute for vernalisation needed for flowering	*Citrus* sp.	Chlormequat	(Srivastava *et al.*, 2000)
	Freesia hybrida 'Bailey'	Ancymidol	(Wulster and Gianfagna, 1991)
Delay flowering	*Tulipa* 'Paul Richter'	PBZ, Ancymidol	(McDaniel, 1990)
	Rhododendron obtusum	PBZ, Uniconazole	(Keever and Foster, 1991)
	Lilium sp.	PBZ, Uniconazole	(Ranwala *et al.*, 2002)
	Eustoma grandiflorum 'Yodel Blue'	Uniconazole	(Starman, 1990)
	Phalaenopsis	Daminozide	(Wang and Hsu, 1994)
	dwarf *Alstroemeria* 'Rosalina' and 'Dorotea'	Flurprimidol	(Pobudkiewicz *et al.*, 2000)
Prevent flowering	*Pharbitis nil*	Chlormequat	(Kulikowska-Gulewska *et al.*, 2000)

[1]PBZ = paclobutrazol

2.7.2 External or environmental factors

The flowering of most plant species is initiated by environmental cues, such as light, temperature, nutrient availability and stress (e.g. soil moisture). Photoperiodism and vernalisation (exposure to cold) are two of the most important mechanisms underlying such seasonal responses, enabling plants to synchronise reproduction. The synchrony has clear adaptive advantages, as it favours crossbreeding and adjusts flowering to coincide with favourable environments, particularly with respect to water and temperature (Sardans *et al.*, 2005). Other signals, such as total light radiation and water stress, can be important modifiers of the responses to day length and low temperature (Davenport, 2003).

2.7.2.1 Light

Light is one of the most important environmental factors that determines the timing of a plant's transition from the vegetative to reproductive phase. Plants perceive diverse light signals from the environment by a family of plant photoreceptors that includes phytochromes, cryptochromes, phototropins and several others (Neff *et al.*, 2000; Quail, 2002; Wang and Deng, 2003). These signals are both quantitative (photoperiod and intensity) and qualitative (spectral composition).

Phytochromes predominantly absorb the red and far-red wavelengths (600 – 750 nm), whereas cryptochromes and phototropins perceive blue and ultraviolet A (UV-A) wavelengths (320 – 500 nm), and unidentified UV-B photoreceptors absorb UV-B (282 – 320 nm) (Briggs and Olney, 2001). The induction of flowering and successful floral morphogenesis are related to phytochromes and cryptochromes, whereas the accomplishment of flowering depends on chlorophylls and carotenoids (Bavrina *et al.*, 2002). The cryptochromes and phytochromes control photomorphogenic responses to variations in the colour, intensity and diurnal duration of the irradiation (Smith, 2000; Quail, 2002), whereas the phototropins function primarily in controlling directional (phototropic) growth in response to directional light and/or intracellular chloroplast movement in response to light intensity (Lin, 2002).

The effect of light intensity, quality (colour or wave length), and daily duration (photoperiod) on flowering has been the subject of many studies and comprehensive reviews, as outlined in the following sections.

Light intensity

Morphological development of plants depends greatly on the amount of photons available during growth (Faust *et al.*, 2005). Photosynthesis increases asymptotically with instantaneous light and the response is species specific. Daily light integral (DLI), the cumulative number of photosynthetically active photons (400 to 700 nm) received in a 24-hour period, is a useful variable related to plant growth and flowering responses. Depending on species, increasing DLI improves numerous plant quality characteristics, such as lateral branching and flower number (Faust *et al.*, 2005).

Minimum DLI requirements for flowering can be very low, and low DLI can cause incomplete or delayed flowering in some potted plants. For example, chrysanthemum 'Bright Golden Anne' (Karlsson *et al.*, 1989) and African violet (Faust and Heins, 1994) required minimum light levels of only 1.8 and 2.0 mol $m^{-2}d^{-1}$, respectively, to initiate flowering. In field studies, however, the grass *Deschampsia flexuosa* did not flower at DLIs < 9 mol $m^{-2}d^{-1}$ (Foggo and Warrington, 1989). Warner and Erwin (2003) studied several *Hibiscus* species and found that most flowered even at the lowest light levels tested (< 5 mol $m^{-2}d^{-1}$). However, the herbaceous perennial *H. moscheutos* 'Disco Belle' required at least 14 mol $m^{-2}d^{-1}$ to flower consistently (Warner and Erwin, 2003). In *Pelargonium* x *domesticum*, the number of days required for floral initiation decreased as the daily light integral increased (Loehrlein and Craig, 2004).

Islam *et al.* (2005) found that photoperiod and light integral strongly influenced the subsequent growth after flower initiation in *Eustoma grandiflorum*. Short-day delayed the time to visible bud, and increased the number of nodes to first open flower compared to LD, and high DLI promoted flowering and increased several shoot characteristics and flowering compared to low light integral. The results indicate that *Eustoma* is a quantitative LD plant. Long-day, and more specifically high light, enhanced flower bud initiation, development and subsequent growth in this species. Supplemental lighting with high pressure sodium lamps provides photosynthetic and

non-photosynthetic radiation energy that increases plant temperature and developmental rate, thereby decreasing flowering time (Fausey *et al.*, 2005).

Plants that are day-neutral and unaffected by night length frequently respond to light intensity. For example, tomato (*Lycopersicon esculentum*) plants grown under high light radiation produced fruit with high sugar content (Guan and Janes, 1991), and had a marked decrease in floral abortion (Kinet, 1977). Flower buds can be a strong sink for carbohydrates, thereby promoting floral development.

Light intensity can affect the earliness of flowering of many plants. For instance, flower initiation of seed geranium (*Pelargonium* x *hortorum*) under ambient light conditions was 37 days earlier than in 60% shade, while differentiation time was reduced by seven days (Wetzstein and Armitage, 1983). High light intensity is necessary for flower induction in dihaploid *Solanum tuberosum* and diploid *S. microdontum* plants (Ward *et al.*, 1992). *Achillea millefolium* 'Summer Pastels' flowered after 57, 45 and 37 days when grown under 100, 200 or 300 μmol $m^{-2}s^{-1}$, respectively (16 hours photoperiod) (Zhang *et al.*, 1996). One of the deciduous *Rhododendron* cultivars #89132, produced more flower buds under high light intensity (150-200 μmol $m^{-2}s^{-1}$) than low light intensity (12-15 μmol $m^{-2}s^{-1}$), as reported by Vainola and Junttila (1998). Increasing irradiance from 150 to 900 μmol $m^{-2}s^{-1}$ reduced days to first open flower of *Limnanthes alba* 'Mermaid' from 104 to 73 days (Seddigh and Jolliff, 1994). Wang (1995) found that *Phalaenopsis* cv 'Joseph Hampton' grown under high light intensity (160 μmol $m^{-2}s^{-1}$) began spiking faster than under low light intensities (0, 8, 60 μmol $m^{-2}s^{-1}$). Zhang *et al.* (1995) also found that flowering of *Lysimachia congestiflora* was accelerated and dry weight production increased as irradiance levels increased from 100 to 300 μmol $m^{-2}s^{-1}$. In contrast to these observations, Pierik (1987) showed that the optimal growth and development of *Syringa vulgaris* flowers takes place at a very low irradiance of 20 – 30 μmol $m^{-2}s^{-1}$ under *in vitro* condition. In general however, high light intensity has a positive effect on flowering in most species.

Light spectrum

Visible light is the portion of the electromagnetic spectrum between 400 and 760 nm; however, plants respond to a wider spectrum of around 300 to 800 nm (Thomas and

Vince-Prue, 1997). Plants perceive changes in red (600 – 700 nm) and far-red (700 – 800 nm) light composition via phytochrome and make physiological and morphological adjustments to suit the environmental conditions. Phytochrome is synthesised in the dark as the physiologically inactive red absorbing form Pr. Absorption of red light by Pr converts the molecule to the far-red form Pfr, which initiates the biological response to light. The far-red form Pfr can be converted back to the Pr form by absorbing far-red light. Phytochrome, therefore, serves as a biological switch, being activated by red light and attenuated by far-red light. Under natural conditions of solar radiation, the relative amounts of Pr and Pfr are indicative of the ratio of red/far-red light to which the plant is exposed (Vince-Prue, 1994) and manipulating this light ratio can influence plant behaviour, including flowering time (Takano *et al.*, 2005). The light spectrum influences a variety of developmental programmes, including seed germination, regulation of stem growth, floral initiation and timing of senescence (Clack *et al.*, 1994; Quail *et al.*, 1995; Smith, 1995; Furuya and Schafer, 1996).

The flowering of some LD plants (e.g. *Campanula carpatica, Coreopsis grandiflora, Viola wittrockiana*) is delayed when plants are grown under light sources with high ratios of red to far-red light, such as cool-white fluorescent lamps (Runkle and Heins, 2001). *In vitro* bulb formation of sprouts in garlic explants was promoted slightly more under light with higher far-red content than under no far-red light (Takagi and Qu, 1995). The shade of nearby vegetation reduces the ratio of red to far-red light and can trigger shade avoidance responses, including stem elongation and the acceleration of flowering (Endo *et al.*, 2005), possibly traits to increase competitiveness.

Light duration or photoperiod

Flower induction, initiation and development of many plant species are synchronised temporally during the year with night length, i.e. flowering can be photoperiodic (Garner and Allard, 1920). Plants can be divided into five main groups according to their photoperiodic response (Thomas and Vince-Prue, 1997):

- LD plants (flowering when the day length is more than 12 hours per day),
- SD plants (flowering when the day length is less than 12 hours per day),
- intermediate-day plants (flowering only when the day length is neither too long or short),

- ambiphotoperiodic-day plants (dual day length requiring plants, require SD and LD in a sequence), and
- day-neutral plants (flower irrespective of day length).

Swainsona formosa is classified as a day-neutral annual plant (Williams, 1996). In general, day-neutral plants flower when they reach a certain morphological maturity regardless of day length and photoperiod.

Lewis *et al.* (2000) found *Lysimachia clethroides* grown in continuous SD remained as vegetative rosettes throughout their experiment (10 weeks), while LD promoted flowering. Longer day length also induced flowering in *A. thaliana*, while shorter day length delayed flowering (Meyerowitz, 1994). In *Lupinus varius*, the time to flowering decreased with an increase in the day length (Karaguzel *et al.*, 2005).

It is important to note that a key factor controlling flowering is the duration of darkness. Light interruption of the dark period can either inhibit or promote flowering, however, the timing of the night break may be a critical factor in promotion of flowering. Therefore, there is a critical length to the period of darkness for stimulation of flowering, acting primarily on the phytochrome pigment in the leaves (Runkle and Heins, 2003).

2.7.2.2 Temperature

Many plants require exposure to low temperatures (vernalisation) before they initiate flowering. Vernalisation is an adaptive response of plants ensuring that flowering occurs at a propitious time in the annual seasonal cycle (Amasino, 2004). Susceptibility to this treatment can differ markedly between varieties of a species (Amasino, 2004). For example, many naturally occurring *A. thaliana* varieties will flower very late if they are not exposed to a vernalisation treatment, but flower early if exposed to low temperatures for four to eight weeks (Michaels and Amasino, 2000). In pea, the early varieties commonly flower at the ninth or tenth node and are insensitive to vernalisation and photoperiod. The late varieties, which commonly do not flower below the 15th node, usually behave as quantitative LD plants and are vernalisable (Moore and Bonde, 1962). It suggests that the cold treatment has a distinct and separate effect on the vegetative development of the plant as well as on flowering.

DNA methylation plays a key role in the control of flowering initiation in plants responding to cold treatment. Plants with decreased methylation displayed a number of abnormal phenotypes, including reduced apical dominance, smaller plant size, altered leaf size and shape, decreased fertility, abnormal floral development, and altered flowering time (Finnegan *et al.*, 1996). The significant effect of decreasing DNA methylation in plants suggests that it plays an integral role in regulating gene expression during development, either directly or by maintaining the appropriate chromatin structure (Finnegan *et al.*, 2004).

Irradiance before or during vernalisation affects vernalisation responses in some plants. For example, in *Triticum aestivum*, vernalisation was more effective under long days than under short days when light intensities were low, and the influence of photoperiod was reduced when light intensity was increased; vernalisation did not take place in the dark unless 2% sucrose was added to the culture medium (Krekule, 1987). These results suggest that some minimal amount of carbohydrate is necessary for vernalisation.

It is important to note that for vernalisation to occur, active metabolism is required during the cold treatment. Both sugars and oxygen are required for the effect of low temperature, so it appears unlikely that low temperatures simply arrest some metabolic reactions that inhibit flowering. The sugar and oxygen requirements are more consistent with the activation of an aerobic metabolic reaction that is essential for flowering. On the other hand, the rates of most metabolic reactions decrease with temperature, so the induction of flowering by low temperatures is likely to be more complex than the simple activation of a metabolic pathway. Furthermore, cell division and DNA replication also appear to be required (Amasino, 2004).

2.7.2.3 Stress

Flowering has been stimulated under conditions of stress. A variety of stress factors are able to promote flowering, including drought (Wery, 2005), mechanically induced stress (Davenport, 2003), root overcrowding (Xu *et al.*, 2001), poor nutrients (see section 2.7.1.2), high temperature (Rhoads *et al.*, 2005) and high light intensity (see section 2.7.2.1). Stress may cause the plant 'to think it may die' and flower prematurely to produce seeds for species survival and succession. Many stresses can affect flowering by affecting the C pools (Hermans *et al.*, 2006).

The adverse effects of high temperature stress on plant reproduction have implications for crop production. In indeterminate plants, such as white clover (*Trifolium repens*), a moderate drought stress increased inflorescence and flower development (Bissuel-Belaygue *et al.*, 2002). Rhoads *et al.* (2005) suggested that, because heat stress can induce flowering, the early flowering phenotype of the transgenic lines of *Arabidopsis* may be the result of the sensing of the high accumulation of a stress response-related protein, ZmHSP22, in the mitochondria. The sensitivity of reproductive development to high temperature stress is not well understood (Young *et al.*, 2004).

Mechanically induced stress when reorienting the shoot to the horizontal position is known to increase the number of flowers in apple and pear (*Pyrus pyrifolia*) (Ito *et al.*, 2004). The reorientation may increase the sink capacity of the bud relative to shoot tissue, thus stimulating bud growth. Girdling has been used to promote flowering and fruiting in a wide range of tree crops, such as litchi (Ramburn, 2001) and citrus (Rivas *et al.*, 2006). Girdling brings about the accumulation of assimilates (carbohydrate) in the leaves and shoot bark above the girdle. However, the effects of girdling are often unpredictable and have been associated with tree decline when repeated over successive years (Li *et al.*, 2003). Davenport *et al.* (2001) interpret the response to girdling in terms of both a reduced supply of assimilates and auxins to the roots and, as a consequence of reduced root activity, a reduction of cytokinin to the shoots. Such a disruption to these endogenous hormone and assimilate levels is associated with a delay in vegetative flushing. As leaf age appears to be an important factor in floral induction, this delay in vegetative flushing increases the possibility of flowering in marginally inductive conditions (Davenport *et al.*, 2001). Both examples of gross manipulation of resources are human-induced effects on resource allocation.

The flowering times of many fruit trees and cut flowers, e.g. Proteaceae (Gerber *et al.*, 2001), can be manipulated by pruning. The primary effect of pruning is to control and direct shoot growth. Pruning plants at different times of the year may be an effective method to indirectly manipulate time of flowering by controlling shoot growth. Starman (1991) found that manual and chemical pinch methods delayed the flowering of *Impatiens* hybrids (Kientzler New Guinea impatiens) by approximately three days compared with non-pinched controls. It is clear that the leaves and other organs of different species may have different roles in the control of flowering.

2.8 *In vitro* flowering

An *in vitro* plant production system is considered a convenient tool to study the flowering mechanism, as the conditions can be manipulated and so provide a controlled system. It is possible to use *in vitro* flowering in genetic studies, biochemistry and studies of environmental factors affecting flower formation. It can also be applied as a tool to shorten breeding programmes or can be adjusted to commercial production of specific compounds (Lin *et al.*, 2003). For more general reviews of *in vitro* flowering include Lakshmanan and Taji (2004) and Ziv and Naor (2006).

In vitro flowering has been reported from both monocotyledonous and dicotyledonous families (Lakshmanan and Taji, 2004), however, a limited number of reports are available regarding legumes, such as soybean (*Glycine max*) (Jullien and Wyndaele, 1992), *Arachis hypogeae* (Narasimhulu and Reddy, 1984), *Vigna mungo* (Ignacimuthu and Franklin, 1999) and *P. sativum* (Franklin *et al.*, 2000).

Several factors, such as genotype, culture medium, physical and environmental growth conditions, and factors inherent to the explants, affect *in vitro* flowering (Ziv and Naor, 2006). However, no favourable set of environmental and nutritional conditions exist that could be universally applied to *in vitro* floral induction among species (Lakshmanan and Taji, 2004).

2.8.1 Carbohydrates

Sucrose has long been recognised as a flower-promoting compound (Rastogi and Sawhney, 1987). Work done *in vitro* has demonstrated that culture media intended to produce flowers contain higher sucrose levels than media used to grow plants vegetatively (Dickens and van Staden, 1988b; Ishioka *et al.*, 1991) and treatments or plant manipulations which result in increasing assimilate supply to the apex promote floral transition in various species.

In vitro flowering of *Pisum sativum* was observed only when the sucrose concentration was at 3 – 6% w/v (Franklin *et al.*, 2000). The frequency and efficiency of flowering was higher in the presence of 3% w/v sucrose in the medium; this result coincides with earlier reports in potato (Al-Wareh *et al.*, 1989), *Vigna mungo* (Ignacimuthu *et al.*,

1997) and in *Lycopersicon esculentum* (Rastogi and Sawhney, 1987). In *Oncidium varicosum* (Barbante-Kerbauy, 1984), however, 1% w/v was optimal. The development of spikelets of cultured tassels of *Zea mays* required 0.1% w/v sucrose (Pareddy and Greyson, 1985). An increase of sucrose in the medium (1, 3, 5 and 7%) resulted in an increase in percentage of buckwheat (*Fagopyrum esculentum*) explants producing flower buds and flowers per explant (Kachonpadungkitti *et al.*, 2001). In an experiment with tomato, *Lycopersicon esculentum*, Dielen *et al.* (2001) found that a medium containing 3% w/v sucrose gave a similar flowering percentage to a medium with 1.5% w/v sucrose. Higher sucrose levels (4.5, 6 and 9% w/v) were found to impair growth and inhibit flowering (Dielen *et al.*, 2001). All these results indicate that sucrose is required at an optimal concentration for *in vitro* flowering to be maximised.

2.8.2 Mineral nutrients

Mineral nutrient plays a role in flowering response *in vitro*. Nitrogen in the form of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) promoted *in vitro* flowering in *Kalanchoe blossfeldiana* (Dickens and van Staden, 1988a). Studies conducted by Vaz and Kerbauy (2000) on *Psygmorchis pusilla* (Orchidaceae) *in vitro* grown cultures found that a low N concentration stimulated flower formation. Franklin *et al.* (2000) found that increasing the concentration of NH_4NO_3 in the medium tended to reduce the *in vitro* flowering response. In *Torenia*, the absence of NH_4NO_3 enhanced floral bud differentiation from the internodal segments on Murashige and Skoog (MS) medium (Tanimoto and Harada, 1981). Hirai *et al.* (1993) also reported that *P. nil* cv. Violet can be induced to flower by culture under poor nutritional conditions. The above reports support the view that low concentrations of mineral nutrients, especially reduced N concentration, promote *in vitro* flowering.

2.8.3 Phytohormones

The most commonly used phytohormones for flowering *in vitro* are auxins, cytokinins and gibberellins (Naor *et al.*, 2004). Auxins form one of the key factors without which flowers cannot be induced in *P. sativum*. Among the auxins tested by Franklin *et al.* (2000), IBA supplementation was found to be more efficient than NAA in induction and maturation of *in vitro* flowers. *Pisum sativum* flower buds arose on MS medium with NAA, and GA_3 enhanced flowering. In *P. sativum*, it is essential to add NAA or IBA to

the medium for flower induction (Franklin *et al.*, 2000). Franklin *et al.* (2000) found that *P. sativum* shoots without roots did not produce flowers. It might be suggested that the induction of roots is an important prerequisite for *in vitro* flowering in this plant by possibly improving the absorption of the phytohormones by the plants from the medium, depending on the carbohydrate and other nutrition requirement of the species.

2.9 Chapter summary

The flowering process involves changes in the development of shoot apices triggered by the interaction of signals under the control of environmental factors, mainly temperature and day length. In species that require low temperatures before flowering, cold stimulus may act directly on the shoot apex tissue or stimulated tissue may produce a flower-inducing stimulus that is transported to other apical tissues causing conversion from vegetative to floral apices. Photoperiodic control of flowering, on the other hand, depends on the length of the uninterrupted dark period affecting the critical concentration of the Pfr form of phytochrome. Whatever the nature of these stimuli, they are transmitted to the apical meristem, where they initiate biochemical and morphological changes.

Recent studies indicate that flower development can be divided into four steps that occur in a temporal sequence, all regulated by various genes. First, in response to both environmental and endogenous signals, the plant switches from vegetative growth to reproductive growth; this process is controlled by a large group of flowering-time genes. Second, signals from the various flowering-time pathways are integrated and lead to the activation of a small group of meristem-identity genes that specify floral identity. Third, the meristem-identity genes activate the floral-organ-identity genes in discrete regions of the flower. Fourth, the late-acting genes activate downstream organ-building genes that specify the various cell types and tissues that constitute the four floral organs.

Underpinning these developmental steps is a corresponding series of biochemical processes affecting flowering. Although there have been numerous efforts to describe the morphological, biochemical and molecular events associated with flowering, a complete and systematic description of the changes that accompany the conversion of a vegetative shoot apex to a floral apex in a single species has not been attempted.

Nutrient status affects plant development and flowering. In particular, alterations in the availability of carbohydrate and nitrogen help regulate a diverse array of flowering processes. Proteins are also involved in the regulation of floral formation, and protein analysis has been employed to generate reference maps of the most abundant soluble proteins at a defined developmental stage. Flowering *in vitro* is not a widespread phenomenon. A number of endogenous factors, including differences in carbohydrate transport and accumulation within tissues, and the interaction between sugars and other components of media, can influence floral induction and development.

Numerous research gaps need to be filled for an in-depth understanding of the entire flowering mechanism. Such understanding will not only open up new questions for science, but will also provide us with a better ability to manipulate the process of flowering in commercial plant production.

The research described in the following chapters is focused on addressing key knowledge gaps in the flowering mechanisms of *S. formosa*, including morphological development and nutritional status.

Chapter 3 General Materials and Methods

The procedures outlined in this chapter were those commonly used throughout the experiments reported in Chapters 4 – 8. All experiments were conducted using facilities at the School of Rural Science and Agriculture and the School of Biological, Biomedical and Molecular Sciences, University of New England (UNE), Armidale, NSW.

3.1 Seed germination

Swainsona formosa seeds used in this study were supplied by the School of Rural Science and Agriculture at UNE. The seeds were obtained from plants routinely grown in greenhouses at the School of Rural Science and Agriculture at UNE. The seeds were germinated in Petri dishes using the Taji and Williams (1989) procedure. Large, uniformly-sized seeds were selected and surface-sterilised by being soaked in 70% ethanol for 10 seconds, then soaked in 1.0% w/v sodium hypochlorite (NaOCl obtained by diluting 25 mL commercial bleach, containing 4.0% available chlorine, in 75 mL deionised water) for 10 minutes. Seeds were then rinsed twice in sterile water, followed by soaking in hot sterile water (80 °C, 100 mL) for 10 seconds. The hot water treatment is necessary to break the dormancy of the seed owing to the impermeable seed coat. The hot water was decanted and seeds were soaked overnight in cool sterile deionised water. The following day, the seeds were transferred to Petri dishes and placed on moist filter paper. These dishes were stored in a growth room at a temperature of 25 ± 2 °C and constant light intensity of 60 ± 10 μmol $m^{-2}s^{-1}$ for 72 hours. Light intensity was measured using a Licor® light meter, model LI-250.

3.2 Seedling establishment

Almost 100% of the seeds germinated within 48 hours and any ungerminated seeds were discarded. The testae were removed soon after radicle formation to aid cotyledon development and to obtain a high growth rate and uniform seedlings. Seedlings were then transplanted into Jiffy pots (Yates and Co Jiffy-7™ compressed peat pots). The Jiffy pots were prepared by soaking in water for 15 minutes. The seedlings were transferred to a greenhouse with a natural light intensity of approximately 100 – 170

μmol $m^{-2}s^{-1}$ and placed under intermittent misting for 10 days for further growth. The temperature of the greenhouse was maintained at 25 °C during the day and 18 °C during the night, with a relative humidity of approximately 80%.

Within 2 weeks after germination, the well-established and uniform seedlings were transferred to 20 cm diameter black plastic pots (Pite-GRO 200 mm, 5L capacity produced by Yates and Co) containing pasteurised potting medium. The potting medium was a mixture of washed river sand and peat (3:1). Prior to potting the plants, the potting mix was thoroughly mixed, wetted down, pasteurised at a temperature of 80 °C for 30 minutes to eliminate pathogenic micro-organisms and weed seeds, and left to cool overnight. The pots used in the experiments were also washed in water containing 1.0% w/v sodium hypochlorite solution, rinsed in clean water and dried in the sun.

Once potted, the plants were kept in the growth cabinets (Thermoline Australia) (Figure 3.1). The growth cabinet was set to a 16-hour photoperiod. Light intensity was dependant on the experimental design, but was normally 800 ± 50 μmol $m^{-2}s^{-1}$ (high light intensity) and 150 ± 10 μmol $m^{-2}s^{-1}$ (low light intensity) unless otherwise stated. The day/night temperatures were set at 30 °C (day) and 18 °C (night).

Figure 3.1 *Swainsona formosa* plants growing in the growth cabinet.

Plants were watered manually with approximately 250 mL pot^{-1} day^{-1} or according to need. Two types of fertiliser were used during the experiments. Plants were fertilised

once per week for the full trial period using about 250 mL of Aquasol™ solution (0.888 g/L), which contains 23% N, 4% P, 8% K, 0.05% Zn, 0.06% Cu, 0.0013% Mo, 0.15% Mn, 0.06% Fe and 0.11% B. The second fertiliser, Nutricote™ [a slow release fertiliser (16% N, 4.4% P, 8.3% K and 4% Ca)], was surface applied to the pots approximately 2 weeks after germination at the rate of 4 g pot^{-1}. Both fertilisers were obtained from the Yates and Co. (NSW, Australia).

For the control of *Phytophthora* and other soil borne fungi, the systemic fungicide Fongarid® (active constituent is 250 g kg^{-1} furalaxyl 250 WP) was applied at a concentration of 1 g L^{-1} when plants were 4 weeks and 8 weeks old. Pirimor® (active constituent is 500 g kg^{-1} Pirimicarb) was applied at the rate of 1 g L^{-1} for aphid infection whenever necessary. Weed control was achieved manually when weeds appeared.

To control uniform growth, any unwanted axillary branches were removed and only two branches growing close to the soil surface were left. Each plant, therefore, consisted of one upright main stem and two axillary branches. These stems were staked using bamboo rods (approximately 1 cm in diameter) to support the upright growth (Figure 3.2).

Figure 3.2 *Swainsona formosa* stems were staked using bamboo rods to support upright growth growing.

3.3 Statistical analysis

Data were statistically analysed using t-tests or analysis of variance (ANOVA) at a 5% level of significance. The SigmaStat statistical software (SPSS Inc., Chicago) version 12.0.1 was used for the analysis.

Chapter 4 Conversion from Vegetative Stage to Reproductive Stage and Floral Ontogeny of *Swainsona formosa*

4.1 Introduction

The transition from vegetative to reproductive growth is a critical event in the life cycle of plants. Williams (1996) reported that *Swainsona formosa* may start to flower within 70 – 84 days after seed germination and plants flower all year round. Yusuf *et al.* (2002) gave a more precise figure on the relationship between photoperiod and first flower appearance on *S. formosa*, based on visual examination. They reported that plants receiving a 16-hour photoperiod flowered within 61 days after seed germination, whereas, plants grown under 12- and 8-hour photoperiods started to flower 66 and 71 days after seed germination, respectively. Histological investigation on floral buds of *S. formosa* reported by Zulkarnain (2003) suggested that the period between 56 and 65 days after germination is critical for the conversion from vegetative to reproductive growth under a 12- to 16-hour photoperiod. Physiological and genetic analysis of flowering has shown that multiple environmental and endogenous inputs influence the timing of the switch (Boss *et al.*, 2004). During the last few years, the data relevant to a molecular understanding of the controls affecting flowering time have increased rapidly as reviewed in Chapter 2. In addition, there have been numerous excellent recent reviews on various aspects of flowering time (Simpson and Dean, 2002; Henderson *et al.*, 2003; Valverde *et al.*, 2004; Simpson, 2005).

Previous investigations and reviews of floral structure development in *S. formosa* include those of Jusaitis (1994), Tade (1992), Williams (1996) and Zulkarnain (2003). Most of these studies focused on morphological characteristics, such as stem elongation, number of nodes formed prior to flowering, axillary bud development, and floral structure and development, rather than events occurring at the shoot apex. The acceleration of the cell division cycle in the shoot apical meristem is a very early event in the floral transition, which precedes, by many hours, sometimes by days, the earliest growth changes affecting various apical parts as well as the initiation of the first reproductive structure (Jacqmard *et al.*, 2003). Knowledge of the morphological changes occurring before and during flower initiation is essential to understand

flowering in *S. formosa* and to use this knowledge in managing production in a commercial setting.

The flowering process is a particularly important aspect of a floriculture crop such as *S. formosa*. The aim of this chapter is to: (1) identify the time of conversion from the vegetative stage to the reproductive stage and later stages of floral development in *S. formosa*, and (2) determine the complete ontogenetic sequence of floral meristem development in *S. formosa*. Accurate identification of the growth stages of plants is critically important in making management decisions about floricultural plants.

4.2 Materials and methods

4.2.1 Plant materials

Seeds were germinated as described previously in Chapter 3. After transplanting to the plastic pots, 80 plants were raised in a growth cabinet. The growth cabinet was set with a 16-hour photoperiod and light intensity of 800 ± 50 μmol $m^{-2}s^{-1}$. Day/night temperatures were set at 30 °C (day) and 18 °C (night).

Plants were sampled 20, 30, 32, 34, 36, 38, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 and 54 days after seed germination and shoot tips were dissected under a stereomicroscope to determine the transition from the vegetative to the reproductive stage. Plants were considered as floral when at least one flower primordium was present. Four plants were sampled for each date. This experiment comprised 80 plants that were grown and examined for the number of nodes before the first flower. Samples were taken from the central stem and two axillary branches growing from the base. Results presented in this study are means ± SE from four replications.

The date of floral initiation for both main stems and branches was determined. Time was then expressed in cumulative degree-days from emergence to floral initiation using 5 °C as the base temperature [Ash *et al.* (1999) for *P. sativum* = 4.4 °C; Peet (1996) for *P. sativum* = 5.5 °C]. The growing degree-days (GDD) were calculated from the equation by Herms (1998):

$$GDD = t \times \left(\frac{T_{\min} + T_{\max}}{2} - T_{base} \right)$$

where:

t is the time (number of days) required to complete a given developmental stage

T_{min} is the minimum daily temperature

T_{max} is the maximum daily temperature

T_{base} is the base temperature.

4.2.2 Scanning electron microscopy

Morphogenic responses were observed and recorded using a Scanning Electron Microscope (SEM). Terminal buds were sampled at the designated times and prepared for SEM investigation using the Chen *et al.* (2003) method. The outer and inner stipules and leaflets were carefully removed under a stereomicroscope to expose the shoot apex. The meristems were excised and placed in a 2.5% glutaraldehyde solution (in 0.1 M phosphate buffer, pH 7.0) for 2 hours. The specimens were rinsed with 0.1 M phosphate buffer three times (15 minutes each), followed by a dehydration series in ethanol (30%, 50%, 70%, 80%, 90% and 95%) for 10 minutes each. After three changes in 100% ethanol, the samples were dried with a critical point dryer (Polaron E3000). Samples were then mounted on aluminium stubs and coated with gold, at a 20 – 40 nm thickness by an ion cater (Polaron E5000 sputter coater). All samples were observed and photographed with the SEM (JEOL JSM-5800LV) at an accelerating voltage of 15 kV. Using SEM, the early development of the flowers was characterised, and selected morphological characters or markers were used to divide the developmental process into distinct stages. Photos were processed and labelled using the Photoshop 7.0 program.

4.3 Results

4.3.1 The time of transformation from the vegetative stage to the reproductive stage

The sampled shoot apices were in a vegetative stage when sampled up to 40 days after seed germination. The conversion from the vegetative into the reproductive stage started at 40 days after seed germination, when one out of four samples showed the floral apex had been initiated. At 52 days after seed germination, all plants had entered their reproductive stage (Table 4.1).

Table 4.1 Developmental stages of *Swainsona formosa* in relation to time after seed germination.

Plant age (days after germination)	Growing Degree-days (d °C)	Reproductive stage (%)[A]	
		Central stem	Axillary branches
20 – 36	380 – 684	0	0
38	722	0	0
40	760	0	25
41	779	0	25
42	798	0	50
43	817	0	50
44	836	0	75
45	855	0	75
46	874	25	100
47	893	25	100
48	912	50	100
49	931	50	100
50	950	75	100
51	969	75	100
52	988	100	100
54	1026	100	100

[A] Calculated from four plants for each age

The floral transition periods of the axillary branches were considered to be between 40 and 46 days after seed germination, while for the central stem the floral transition periods were considered to be between 46 and 52 days after seed germination. At the temperature and light conditions used in this trial, plants required a GDD of 807.5 d °C for the axillary branches and 921.5 d °C for the central stem to reach 50% flowering (Table 4.1). The central stems grew more nodes (11.08 ± 0.97) before the initiation of the first flower, compared to the axillary branches (7.21 ± 0.93).

4.3.2 Morphology and ontogeny of the *S. formosa* flower and inflorescence

Swainsona formosa apical meristems were observed using scanning electron microscopy throughout floral initiation. To our knowledge this is the first report of the complete floral ontogeny of a member of the tribe Galegeae (Tapingkae *et al.*, in press). The sequence of the *S. formosa* flowering process was divided into eight stages (Table 4.2 and Figures 4.1 – 4.8).

Table 4.2 Sequence of stages in the flowering process of *Swainsona formosa*.

Stage	Apex development (corresponding figures)	Days after seed germination
0	Vegetative (Figures 4.1*A-B*)	< 40
1	Transitional (Figures 4.2*A-B*)	40 – 45
2	Flower bud initiation (Figures 4.3*A-D*)	45 – 50
3	Sepal initiation (Figures 4.4*A-H*)	50 – 55
4	Carpel and petal initiation (Figures 4.5*A-B*)	50 – 55
5	Stamen initiation (Figures 4.6*A-F*)	55 – 60
6	Further development of floral organ (Figures 4.7*A-B*)	60 – 80
7	Full bloom flower: keel and wings fully open (Figures 4.8*A-B*)	80

At less than 40-days-old, the terminal bud was in the vegetative stage (stage 0) and the apex was covered by the leaf primordia (lp) with its paired stipules (st) (Figure 4.1*A*). After the upper pair of leaf primordia separated, the apex could be seen clearly, adjacent to the youngest leaf primordium and its paired stipules (Figure 4.1*B*). The diameter of the vegetative apex 40 days after seed germination was ~100 – 200 μm.

At the early transitional stage (stage 1), 40 – 45 days after seed germination, the shoot apical meristem increased in size and later initiated a floral primordium (fp) in the axil of a leaf primordium (Figure 4.2*A*). The floral primordium was initially roundish but soon became somewhat irregular; the shoot apical meristem was ~250 – 300 μm in diameter (Figure 4.2*B*). In *S. formosa*, the flowers are arranged in umbelliform racemes with an indeterminate flowering pattern. On the inflorescence axis, the flowers were crowded around the apex (Figures 4.3*A-D*). At this stage, the bracts (Br) elongated to cover the rest of the floral organ primordial; they had to be removed during further investigations.

Once the floral apex flattened and the flower bud was ~150 – 200 μm in diameter (Figure 4.4*A*), bracteoles (Br) had been initiated at the sides of floral bud (Figure 4.4*B*). After that, the sepals were initiated. Initially a single sepal primordium (Sab) was produced at the abaxial position of the floral meristem (Figure 4.4*C*). Next, two lateral sepal primordia (Sl), which were laterally convergent with the abaxial sepal, were initiated (Figure 4.4*D*).

After the stamens were completely differentiated, the floral centre became dome-shaped, and the carpel primordium was initiated at the centre of the floral apex (about Day 50 – 55 after seed germination) (Figure 4.5*A*). The carpel (C) was first visible at the same time as the five petal primordia (P) (Figure 4.5*A*). The petal primordia were apparently simultaneous in origin and whorled from initiation (Figure 4.5*A*).

At stage 5, the sepals elongated to cover the rest of the floral organ primordia, so they were removed in further investigations (about Day 55 – 60 after seed germination). The stamen primordia were initiated from two successive whorls; antesepalous (A) or outer stamen primordia and antepetalous (a) or inner stamen primordia, each whorl unidirectional (Figures 4.6*A-C*). The first outer stamen primordium was median abaxial (Figure 4.6*A*). The inner stamen primordia were initiated on the same radii as the petals (Figures 4.6*C-D*). Shortly after, the carpel developed a cleft, which ultimately formed the locule of the ovary (Figure 4.6*E*). The stamens elongated and began to differentiate anthers and filaments (Figure 4.6*F*).

At stage 6 (~60 – 80 days after seed germination), all flower parts of the meristem had completely formed. The pistil (pt) had completely differentiated (Figures 4.7*A-B*) and so had the filaments and the anthers (at). Flowers were tightly clustered in bud and petals were completely enclosed by the calyx.

By stage 7, the flowers were fully formed and the keel and wings were fully open (Figures 4.8*A-B*). The change between stages 6 and 7 took about 20 days to complete. Individual flowers are about 9 cm long and are borne in groups of five or eight.

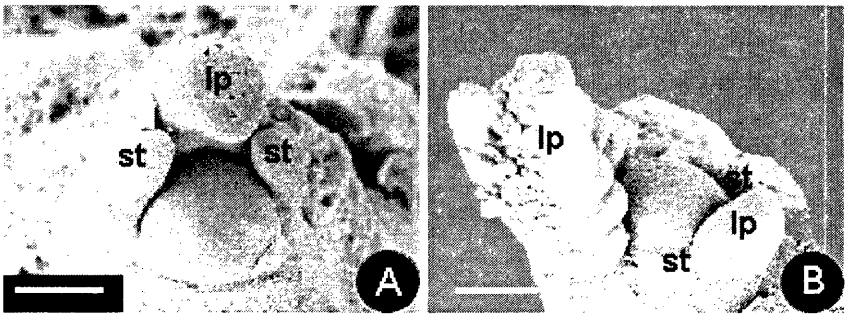

Figure 4.1 Stage 0: *(A)* vegetative apex showing one leaf primordium, with its paired stipules on either side (< 40 days after seed germination). *(B)* Showing two leaf primordia (the younger with two stipule primordia). st = stipule primordium; lp = leaf primordium. Scale bar = 100 μm.

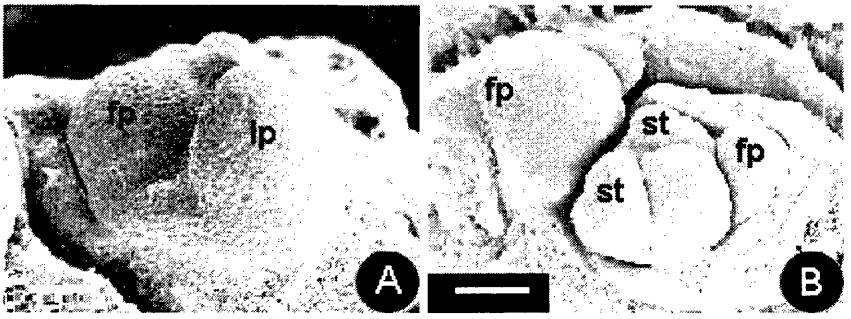

Figure 4.2 Stage 1: (*A*) early transition stage (~ 40 – 45 days after seed germination) showing a floral primordium in the axil of a leaf primordium. (*B*) General view of apex in which the floral primordia can be distinguished. st = stipule primordium; lp = leaf primordium; fp = floral primordium. Scale bar *A* = 50 μm; *B* = 100 μm.

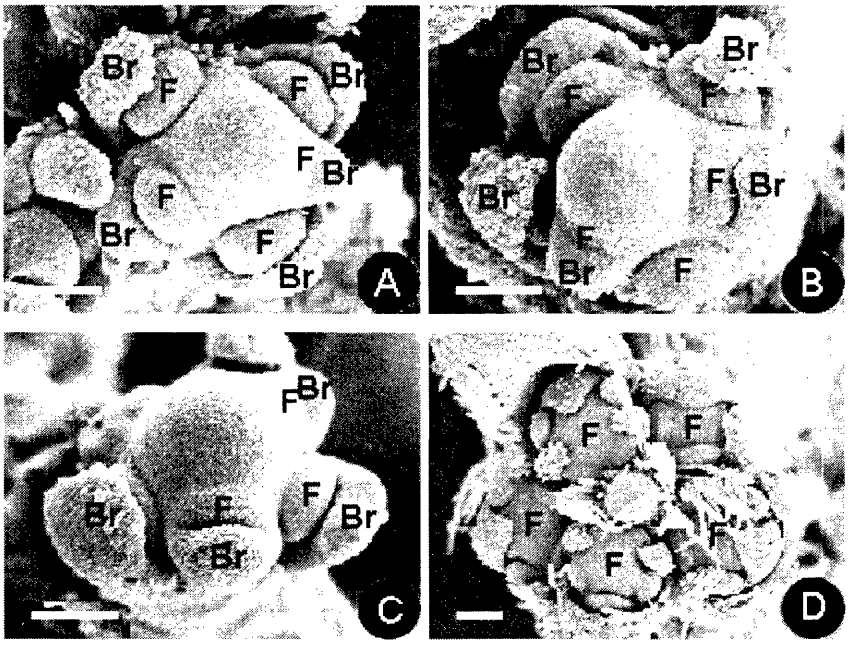

Figure 4.3 Stage 2: (*A*) inflorescence meristem showing five floral buds (~45 – 50 days after seed germination). (*B*) General view at the floral initiation stage. (*C*) Lateral view of (*B*). (*D*) Top view showing five floral buds. Br = bract; F = floral bud. Scale bar = 100 μm.

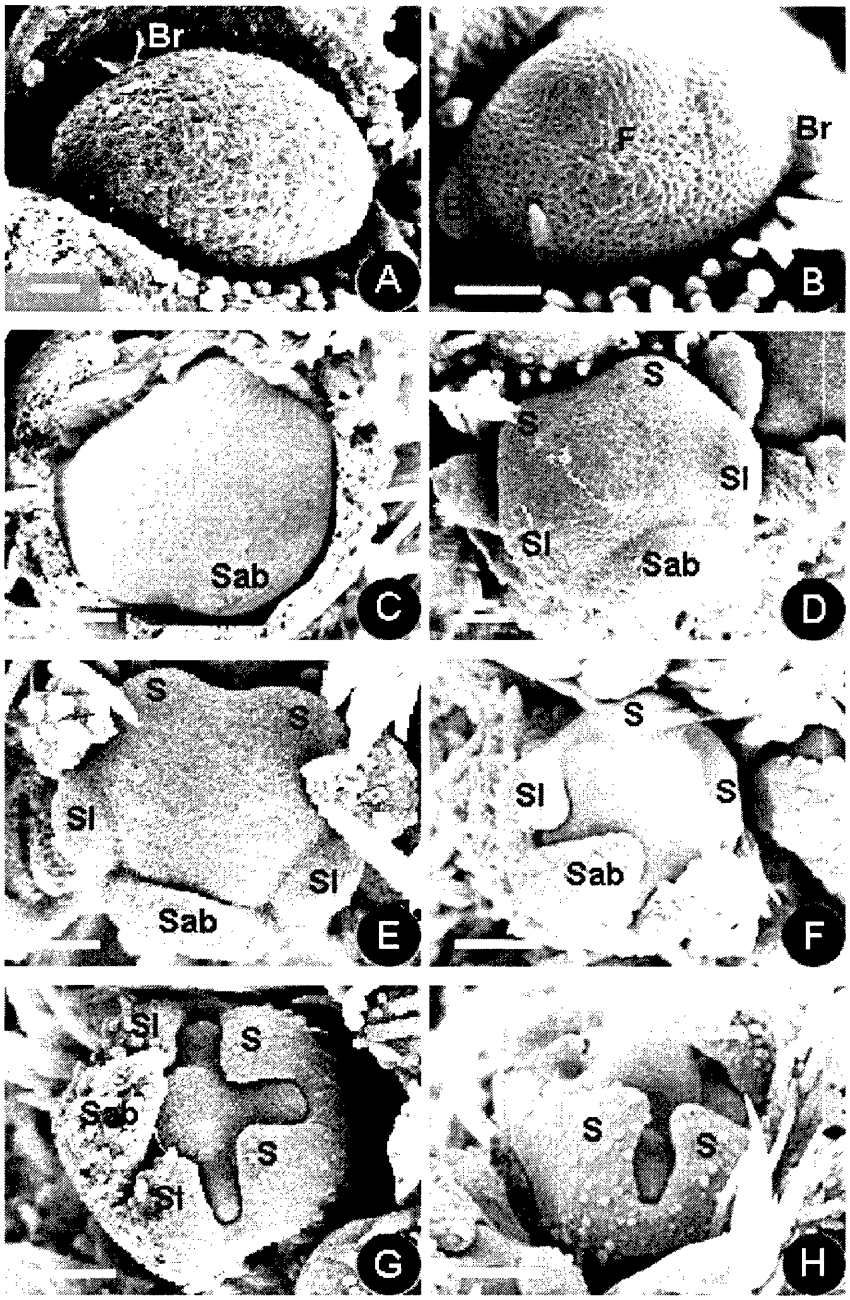

Figure 4.4 Reproductive meristem showing continued development of individual floral buds. Bracts removed. (*A*) Floral apex prior to bracteole initiation, showing a bare floral apex in the axil of a bract or leaf, before any organs form on it. (*B*) Showing a floral apex after two bracteoles form on the two sides. (*C*) Stage 3: the first sepal primordium visible (~50 – 55 days after seed germination). (*D*) Showing completion of sepal initiation. (*E*) Continued development of abaxial sepal primordium and two lateral sepal primordia. (*F*) All sepals visible. (*G*) Sepals cover the rest of the floral primordia. (*H*) Lateral view of (*G*). Br = bracteole; Sab = abaxial sepal primordium; Sl = lateral sepal primordia; S = sepal. Scale bar *A* = 20 μm; *B-E* = 50 μm; *F-H* = 100 μm.

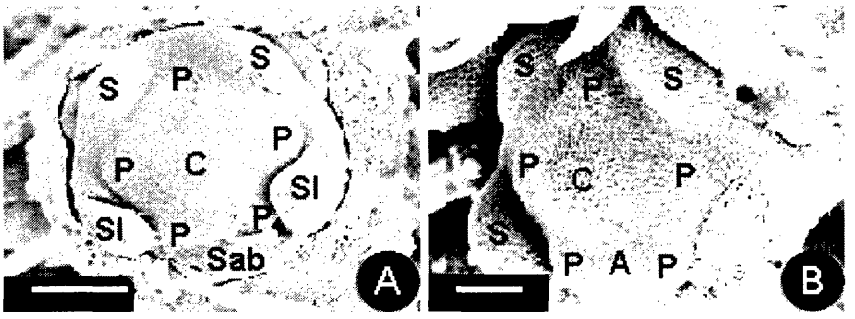

Figure 4.5 Stage 4: (*A*) a carpal primordium and all five petal primordia are initiated (~50 – 55 days after seed germination). (*B*) Appearance of first antesepalous stamen between the two petal primordia at the bottom of the micrograph. Bracts were removed. C = carpel primordium; P = petal primordium; S = sepal; A = antesepalous stamen. Scale bar *A* = 100 μm; *B* = 50 μm.

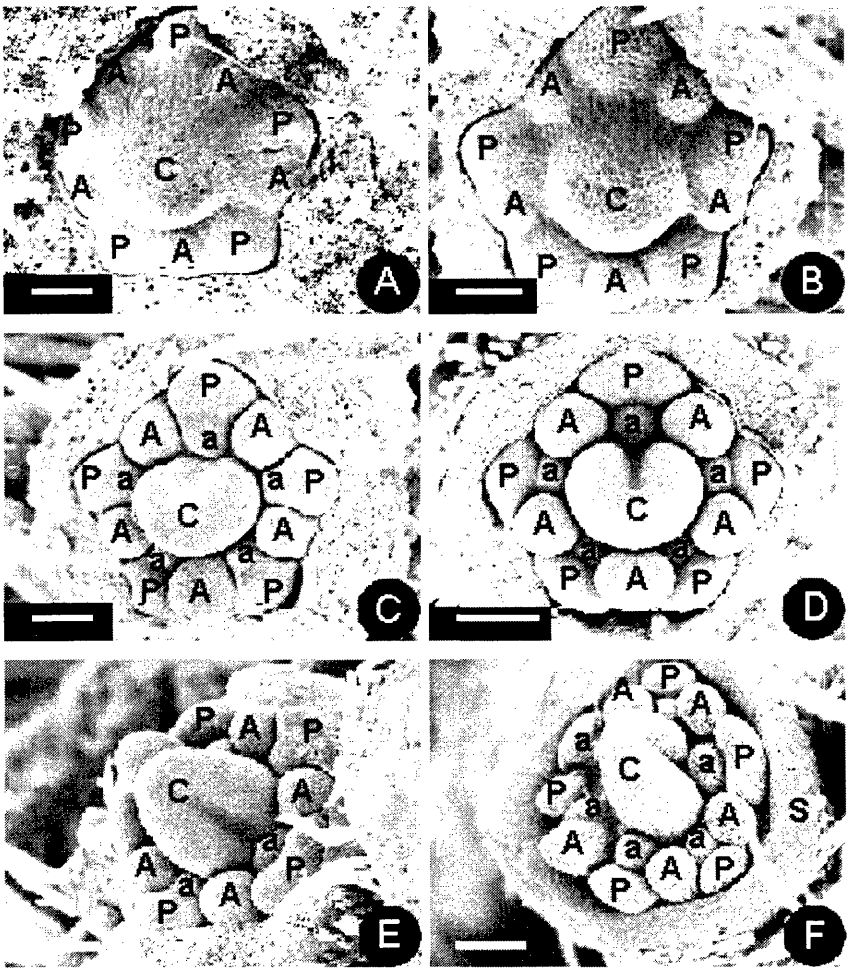

Figure 4.6 Stage 5: Sepals removed. (*A*) all five antesepalous or outer stamen primordia, alternate with petal primordia (~55 – 60 days after seed germination). (*B*) Showing all five antesepalous primordia at a slightly later stage. (*C*) Well-formed antepetalous or inner stamen primordial. (*D*) Completely differentiated floral primordia. (*E*) Carpel with cleft which will form the locule of the ovary. (*F*) Stamens elongate and begin to differentiate anthers and filaments. (*A-D*) apical view of single flower bud. (*E-F*) lateral view of single flower bud. P = petal; C = carpel; S = sepal; A = antesepalous stamen (outer); a = antepetalous stamen (inner). Scale bar *A-B* = 50 μm.; *C-H* = 100 μm.

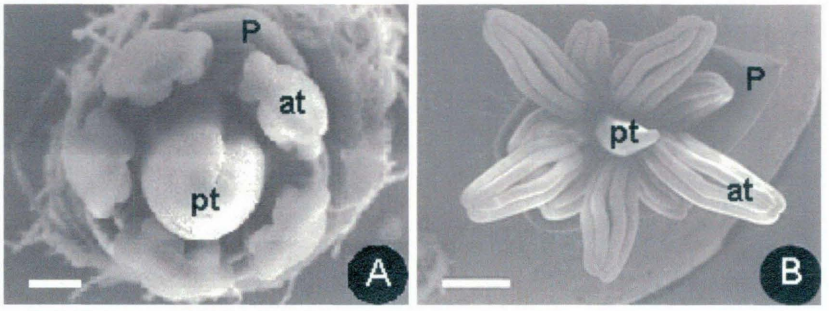

Figure 4.7 Stage 6: (*A*) all floral parts are present and identifiable (about 60 – 70 days after seed germination). (*B*) Anthers and pistil fully differentiated. All sepals were removed to facilitate observation. P = petal; pt = pistil; at = anther. Scale bar *A* = 100 μm; *B* = 1 mm.

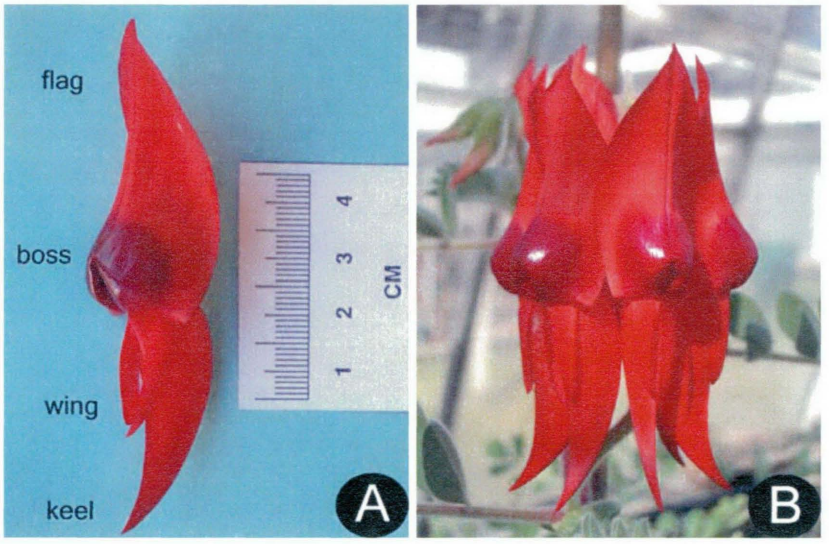

Figure 4.8 Stage 7: (*A*) showing individual flower. (*B*) Inflorescence (umbel) showing five to eight flowers (about 80 days after seed germination).

4.4 Discussion

4.4.1 The time of transformation from the vegetative stage to the reproductive stage

Generally, the first *S. formosa* flower can be observed visually between 61 and 84 days after seed germination (Williams, 1996; Yusuf *et al.*, 2002), depending on the temperature and light conditions. Histological studies on *S. formosa* floral buds by Zulkarnain (2003) concluded that the period between 56 and 65 days after germination is critical for the conversion of the vegetative to the reproductive apex under a 12- to 16-hour photoperiod. However, in Zulkarnain's (2003) experiments, plants were grown in a greenhouse where the light and temperature could not be accurately controlled.

Under a 16-hour photoperiod with light intensity at 800 ± 50 μmol $m^{-2}s^{-1}$ and temperatures at 30 °C (day) and 18 °C (night), the transformation from the vegetative apex to the floral apex in *S. formosa* began within 40 – 46 days after seed germination for the axillary branches and 46 – 52 days for the central stems. The difference in time taken to flower for the axillary verses the central stems may be due to the angle of the axillary stems since the axillary stems were staked at a 45° angle. The angle of the stem may have an effect on the rate of photosynthesis, with better light penetration and capture by the leaves resulting in a better rate of growth (Kikuzawa, 2003). Furthermore, the angle of the stems affects the hormone distribution and/or balance within the stem and thus gene expression resulting in the faster time to flowering (Wilson, 2000). Blake *et al.* (1980) suggested that ethylene was involved in the plagiotropic growth of branches in *Cupressus arizonica*, which in turn could influence changes in auxins or other compounds in the branch. A hormonal process involving auxins within the plant is responsible for both cell division in the apical meristem and the suppression of cell division in lateral meristems (Tamas, 1995).

The variation in time to flowering in this experiment, compared to that of other workers, such as Williams (1996), Yusuf *et al.* (2002) and Zulkarnain (2003), may be due to external factors, such as light quality, light quantity and temperature. The provenance of plant materials may also be important, as the critical node number and the timing of floral development are genetically determined and may vary considerably on the basis of place of origin; in work reported here, however, all seed originated from plants grown in the greenhouses at the School of Rural Science and Agriculture at the University of New England.

To complete a developmental process, many plants must experience a specific number of units of degree-days (or thermal time) above the base temperature characteristic of that process (Roberts and Summerfield, 1987). In addition, the effect of thermal time is a result of the integrated effect of genes that partially control the rate of development to the reproductive stage (Yan and Wallace, 1996). In this experiment, plants required 807.5 d °C for the axillary branches and 921.5 d °C for the central stem to reach 50% flowering. Similarly, Sureja *et al.* (2002), using 30 garden pea (*P. sativum*) genotypes of indigenous and exotic origins, found that the GDD required to reach the first flowering was 755 – 832 d °C for most of the genotypes. Moreover, those genotypes requiring the

lowest GDD had significantly higher yield than those requiring the higher GDD. Variation in the GDD may relate to the accumulation of certain amounts of stored nutrients (carbohydrates) before conversion can take place (Poethig, 2003).

The formation and development of floral primordia occur at the shoot apex, as a result of biochemical and cellular changes leading to the production of the floral organs (Bernier, 1988). One of the internal stimuli considered by Schwabe (1987) as a possible activator of the transition of the shoot apex from the vegetative to the reproductive phase is a quantitative change in carbohydrates. It has been shown that there is a strong relationship between flower development and carbohydrates in woody plants, such as grape (Lebon *et al.*, 2005). Roh *et al.* (1992) also found increased quantities of saccharose in the apex of the flowering bulb *Eucrosia bicolour*, during its transition from the vegetative to the reproductive stage. The complete role of carbohydrates in floral induction in *S. formosa* is yet to be determined.

In addition to biochemical processes, many studies report the effects of environmental factors on flower induction and development. Photoperiod and light intensity often have other effects on plants in addition to flower initiation and development, including the number of nodes formed prior to flowering, axillary bud development, stem elongation, rooting, storage organ formation, and induction or breaking of dormancy. For example, placing *Helianthus annuus* (a determinate plant) under a short-day photoperiod not only decreases the number of days to flowering, but also decreases the number of nodes and plant height compared to plants under a long-day photoperiod (Pallez *et al.*, 2002). However, Yusuf *et al.* (2002) found the opposite with respect to *S. formosa*; plants grown under a short-day photoperiod showed increased number of days to flowering and significantly increased height of plants compared to plants grown under a long-day photoperiod. In addition, the number of internodes between treatments were not significantly different in the study by Yusuf *et al.* (2002), the increase in plant height possibly caused by cell expansion rather than cell division as a result of lower quantities of light.

Swainsona formosa is classified as a day-neutral annual plant (Williams, 1996). In general, day-neutral plants flower when they reach a certain morphological maturity, regardless of day length and photoperiod. In some species, flowering occurs after the

apex has produced a particular number of leaves or has attained some minimal node number, for example *Campanula* 'Champion' flowers once 8 – 9 leaves are formed (Cavins and Dole, 2001), hybrid geraniums at 15 nodes (Armitage, 1984), *Antirrhinum majus* at 18 to 22 leaves (Cockshull, 1985) and the Melrose field pea flowers when 15 nodes are attained (Auld *et al.*, 1978).

Usually, the appearance of visible flower buds and time to first open flower, along with the number of nodes to first flower, are used as indicators of environmental effect on the flowering process (McDaniel *et al.*, 1996; Clapham *et al.*, 2000; Garcia-Maroto *et al.*, 2000; Munier-Jolain and Salon, 2003). Adkins and Dirr (2003) found that the stage of meristem development of *Hydrangea macrophylla* was highly correlated with the number of nodes, but not with shoot length. Islam *et al.* (2005) reported that short-day conditions delayed flower bud initiation and increased the number of nodes to first open flower in the day-neutral plant *Eustoma*. However, Halevy and Kofranek (1984) found that photoperiod had no effect on flowering in *Lisianthus*, a day-neutral plant. The link between environmental conditions, number of nodes, length of shoot and flowering is clearly species specific.

The indeterminate growth habit of *S. formosa* must also be considered. Indeterminate plants generally produce inflorescences only in the leaf axils; the main stem remains vegetative and continues to grow indefinitely. Flowering of indeterminate plants is a continuous process and the production of axillary flowers is dependent on continued leaf production by the apex. Any factor, such as low light or unsuitable temperature that slows the growth rate of the main stem, will reduce flower number. Flower initiation of indeterminate plants, such as *Hibiscus rosa-sinensis* does not end vegetative growth and has little effect on node number or height to first flower appearance (Warner and Erwin, 2003). However, Yusuf *et al.* (2002) reported that neutral and short-day photoperiods in *S. formosa* produced taller plants (13.5 cm and 13.7 cm respectively) than long-day photoperiods (9.95 cm), possibly related to stem elongation as a result of etiolation.

The central stem in this study produced more nodes than the axillary branches at the first flower initiation and this is consistent with the observations of Zulkarnain (2003) on plants grown in the greenhouse. Zulkarnain (2003) suggested that the reason fewer nodes formed on axillary branches compared to the central stem was due to the sturdier

growth of the central stem. Generally, a strongly growing main shoot apex represses the growth of lateral buds by exerting 'apical dominance' (Emery *et al.*, 1998). Quiescent axillary buds are regarded as 'replacement apices', being called upon following loss of the strongly growing shoot apex. While this scenario may apply to many plant species, the hypothesis has limitations and cannot readily explain branching patterns in plants with weak apical dominance, such as *Coleus blumei* and *Arabidopsis thaliana* (Cline, 1996). Although *S. formosa* has a predictable branching pattern, it does not adhere to the classical apical dominance theory. In addition, water and nutrient transport to the axillary branch may be restricted relative to transport to the central shoot and therefore axillary branch growth can be reduced relative to the central branch (Lomax *et al.*, 1995). Another possible cause of fewer nodes formed on axillary branches compared to central stem is the difference in hormonal balance in plagiotropic shoots (Blake *et al.*, 1980).

This study showed that node number at the conversion from vegetative to reproductive stage in plants cultivated under growth cabinet conditions (node 11.1 on the central stem and node 7.2 on the axillary branches) was lower than that reported by Zulkarnain (2003), but it is similar to plants grown under greenhouse conditions. Zulkarnain (2003) reported that the critical node for vegetative to reproductive conversion growth was node 12.0 on the central stem and node 10.5 on the axillary branches for plants cultivated under growth cabinet conditions (16-hour photoperiod and at 150 – 1000 μmol $m^{-2}s^{-1}$ light intensity range with day/night temperatures set at 30/20 °C). Under greenhouse conditions of 12- to 16-hour photoperiods and 650 – 1200 μmol $m^{-2}s^{-1}$ light intensities with day/night temperatures of 30/20 °C, Zulkarnain (2003) observed that the critical node for change from vegetative to reproductive growth was node 10.6 on the central stem and node 8.1 on the axillary branches. It appears that photoperiod does not affect critical node number for the conversion from the vegetative to the reproductive stage. The effective factors seem to be light intensity and temperature.

4.4.2 Morphology and ontogeny of the *S. formosa* flower and inflorescence

The transition from the vegetative to the reproductive phase involves a profound change in the structure of the shoot apical meristem. In many species, the arrival of the flowering stimulus leads to an immediate increase in mitotic activity, and nucleus size often increases, as does the size of the nucleolus. The number of ribosomes and

mitochondria and the amount of RNA in the apical cells frequently increases (Wareing and Phillips, 1978). From histological studies of the apical meristem of *S. formosa*, it is apparent that tissues progressively differentiate through changes in cell size, degree of vacuolation, and rate and orientation of mitoses (Zulkarnain, 2003). Morphological changes may be caused by an increase in mitotic activity within the central zone of the shoot apex at the transition stage; changes in the shoot apical meristem shape and size should be the first morphological sign of commitment to flowering.

In higher dicotyledonous plants, five classes of homeotic genes relate to the formation of the sepals, petals, stamens, carpels and ovules (Weigel and Meyerowitz, 1994; Pelaz *et al.*, 2000; Theissen *et al.*, 2000). The homeotic transformations observed in pea floral mutants by Ferrandiz *et al.* (1999) suggest that the identity and developmental pattern of the four organ types (sepals, petals, stamens and carpels) in pea flowers is governed by at least the same developmental functions proposed for the plant model systems *Arabidopsis thaliana* and *Antirrhinum majus*. However, the number and molecular characteristic of the genes responsible for the organ characteristic functions in pea have not yet been established (Berbel *et al.*, 2001).

Extensive studies of flowering plants show that the genetic functions controlling inflorescence and flower development are highly conserved among different species within a genus (Theissen *et al.*, 2000). The control of floral organ identity of *S. formosa* is similar in many respects to other legume flowers, such as soybean, pea, *Thermopsis lanceolata* and *Baptisia australis* (Carlson and Lersten, 1987; Ferrandiz *et al.*, 1999; Prenner, 2004). The order of floral organ initiation in *S. formosa* within each whorl is unidirectional, except for the petal whorl, which is simultaneous; the flower is organised into five whorls and shows a pentamerous arrangement of sepals and petals, ten stamens in two whorls and a central carpel. Using scanning electron microscopy, the early development of the flowers was characterised and selected morphological characters or markers used to divide the developmental process into eight different stages.

However, the ontogeny of *S. formosa* flowers, as in many legumes, proceeds through a very different sequence of events from the process in model plants, such as *Arabidopsis* and *Antirrhinum* (Ferrandiz *et al.*, 1999; Tucker, 2003). In these model plants, primordial initiation of the floral organs is a centripetal and sequential process. First, the

sepal primordia are formed, then the primordia that will form petals appear, immediately followed by the stamen primordia and finally the residual central part of the floral meristem, which forms the carpel primordium (Schwarz-Sommer *et al.*, 1990). The order of floral organ initiation in pea (*Pisum sativum*), differs from the model species *Arabidopsis* and *Antirrhinum*. Petal and stamen primordia are formed indirectly from common primordia in pea flower ontogeny. Once the sepal and carpel primordia have emerged, four structures called common primordial, made up of proliferating cells, are formed. Proliferation and differentiation processes within each of these common primordia ultimately give rise to second- and third-whorl floral organs in pea flowers (Figure 4.9).

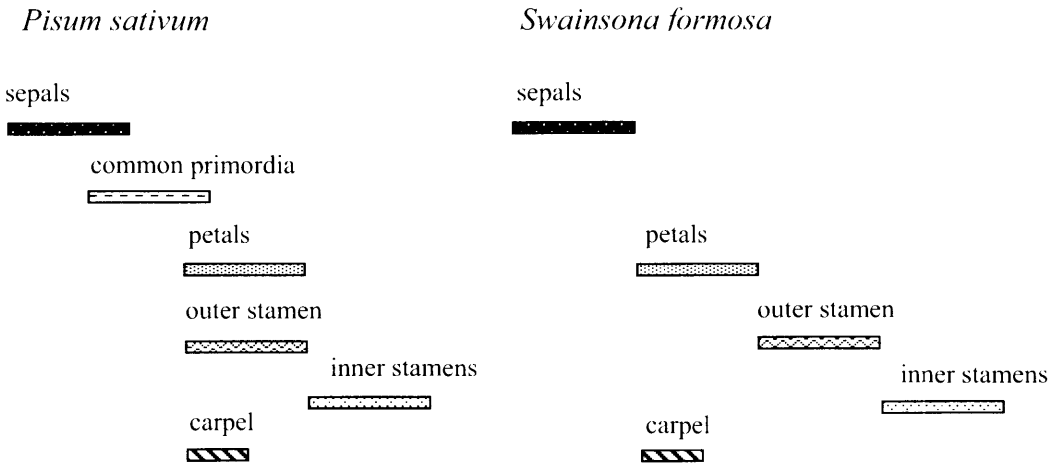

Figure 4.9 Diagram showing proportional overlap among whorls of floral organs in *Pisum sativum* and *Swainsona formosa*. Ontogeny of *P. sativum* is detailed in Tucker (1989).

The formation of common primordia, as opposed to discrete petal and stamen primordia, has been described in *P. sativum* (Ferrandiz *et al.*, 1999) and a few other legumes, but does not occur in *Swainsona*. In *S. formosa*, the order of floral organ initiation is unidirectional in all whorls; sepals arise first and then the carpel primordia initiates at the same time as the petal primordia (Figure 4.9). Overlap in time of initiation among different whorls is common in papilionoid legumes (Tucker, 1989).

4.5 Conclusion

Under the growing conditions used, the conversion of the apex in *S. formosa* from the vegetative to the floral stage started to occur 40 – 46 days after seed germination for the axillary branches and 46 – 52 days for the central stems. The numbers of nodes

produced before first flower initiation is closely related to the transformation from the vegetative to reproductive stage and may be a useful predictive indicator given a good quantitative understanding of the effect of light and temperature on flowering.

Based on scanning electron micrographs of dissected shoot apices and the progression of development from the vegetative to the floral stage, eight morphologically distinct stages of shoot apex development were defined. The floral ontogeny of *S. formosa* conforms to the common model of flower development in legumes; the order of floral organ initiation within each whorl is unidirectional, except for the petal whorl, which is simultaneous; the flower is organised into five whorls and shows a pentamerous arrangement of sepals and petals, ten stamens in two whorls and a central carpel. This chapter describes the complete floral ontogeny in a member of tribe Galegeae and provides a framework for the interpretation of future chapters in this thesis.

Chapter 5 Determination of Sugar Content in *Swainsona formosa* Shoot Apices during the Transition to Flowering

5.1 Introduction

The formation and development of floral primordia occur at the shoot apex as a result of biochemical and cellular changes (Bernier, 1988). One modification exhibited by evoked apices is an increased carbohydrate content (Ito *et al.*, 2004). Soluble sugars, such as glucose and sucrose, have been postulated to act as signalling molecules that help regulate flowering (Rolland *et al.*, 2006).

The processes of sugar production, transport, consumption, and storage are dynamic and tightly linked to cellular physiology, organ identity, environmental inputs, and developmental stages (Rolland *et al.*, 2006). A plant's ability to monitor and respond to sugar levels may serve as a control mechanism to integrate external environmental conditions, including light, other nutrients, and abiotic and biotic stresses, with intrinsic developmental programmes directed by multiple plant hormones. In plants, sugars have conventionally been viewed as resources for respiration and metabolic intermediates, as well as structural or storage components (Rolland *et al.*, 2002). When there is an excess supply of carbohydrates, as in the case of vegetative shoot apices of plants grown under low light intensity, sucrose can be converted into more complex compounds, such as starch (Kossmann and Lloyd, 2000).

Swainsona formosa is an Australia native legume which has potential as an ornamental pot plant and is also suitable for hanging baskets and as a cut flower. One of the impediments to the commercialisation of *S. formosa* as a pot plant is its inability to produce flowers under low light conditions. The aim of the research reported in this chapter was to investigate the effect of high light intensity (800 ± 50 μmol $m^{-2}s^{-1}$) and low light intensity (150 ± 10 μmol $m^{-2}s^{-1}$) on the flowering of *S. formosa*, with particular emphasis on the changes of sugar content in shoot apices from the vegetative stage to the floral stage.

5.2 Materials and methods

5.2.1 Plant materials

Seeds were germinated as described previously in Chapter 3. After transplanting to the plastic pots, plants were raised in growth cabinets. The growth cabinet was set with a 16-hour photoperiod. Day/night temperatures were set at 30 °C (day) and 18 °C (night). There were two treatments: high light intensity (800 ± 50 μmol $m^{-2}s^{-1}$) and low light intensity (150 ± 10 μmol $m^{-2}s^{-1}$).

For soluble sugar analyses, plant material was sampled 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 days after seed germination for plants grown under high light intensity. The experiment was terminated at Day 60 for the low light intensity treatment because no flowers were initiated during this time. Samples were taken from the two axillary branches. Shoot tips were examined microscopically, and apical meristem development was ranked according to five simplified stages as per the photographs in Table 5.1 (for more details on the developmental stages see Chapter 4).

Table 5.1 Shoot apex development of *Swainsona formosa* plants grown under high light conditions (800 μmol $m^{-2}s^{-1}$).

Shoot apex development stage	Days after seed germination	
Vegetative stage	< 40	
Transitional stage	40 – 45	
Floral initiation and differentiation	45 – 60	
Floral bud	60 – 75	
Full bloom flower	75 – 80	

5.2.2 Extraction and quantification of sugar content

Carbohydrate extraction was carried out using the Glyad (2002) method. Shoot apices (1 cm) were collected at sampling time. Fresh plant material (0.5 g) was soaked in 5 mL of 80% ethanol in a 10 mL polypropylene tube and heated in an 80 °C water bath for 40 minutes to stop the enzyme activity. Samples were then transferred to a mortar, ground with a pestle, and returned to the same polypropylene tube. One mL of 80% ethanol was poured into a mortar for rinsing, and was added to the initial extract. The mixture was centrifuged in a bench top centrifuge at 1000 g for 15 minutes, and the supernatant was pipetted into a glass tube. The residue was re-extracted using 2 mL 80% ethanol at 80 °C for 15 minutes, centrifuged at 1000 g for 15 minutes, and the supernatant added to the previous supernatant. This process was repeated one more time.

The combined supernatants were evaporated using a freeze-dryer (Savant Instruments, Inc. Farmingdale, NY) until dry. The dry residue was dissolved in 1 mL of distilled water. To remove solid impurities, the supernatant was filtered through a 0.45 μm membrane filter. The sugar content was characterised and quantified by high-performance liquid chromatography (HPLC) (Shimadsu LC-10A) with an RI detector, using a Inertsil NH_2 (GL Science) column (250 x 4.6 mm, Waters Associates) at 35 °C, using deionised distilled water (1 mL min^{-1}) as the eluant. Sucrose, glucose and fructose were identified by their retention times compared to pure standards and quantified by integrating peak areas. All results were calculated on a fresh weight (FW) basis. The analysis was replicated at least four times.

5.2.3 Statistical Analysis

The data were analysed using the independent – sample t test at a 5% level of significance with SigmaStat statistical software (SPSS Inc., Chicago) version 12.0.1.

5.3 Results

Microscopic examination of the apices showed that plants grown under the high light intensity treatment initiated flowers. Plants grown under low light intensity, however, remained vegetative and had initiated no flowers when the experiment was terminated on Day 60. It is known that *S. formosa* will not flower under low light for a period up to

121 days (Yusuf, 2000), so it is very unlikely that transition could have occurred after the last measurement on Day 60.

The sugar content changed markedly during the development of the shoot apices. From Figure 5.1 it can be seen that during the first 20 days after seed germination, fructose was the only major sugar detectable in the shoot apices and all apical meristems were in a vegetative stage. In both the high and low light intensity treatments, sucrose first appeared after 20 days from seed germination. There was almost no difference in the concentration of fructose, glucose and sucrose between the treatments during the first 35 days. Fructose concentration of the plants grown under high light was fluctuating (peaking on day 35), but both high and low light intensity treatments produced a gradual increase in fructose concentration over time (Figure 5.1), although the increase was greater under high light intensity.

Trace amounts of glucose were detected when shoots were 40 days old (transitional stage) in plants grown under high light intensity. After that, glucose concentration in the shoot apices increased gradually, and then increased rapidly after Day 65 (floral buds) until Day 80 (Figure 5.2), when the plants were in full bloom.

Sucrose was the most abundant sugar in the plants grown under low light intensity (Figure 5.1). The sucrose concentration in the shoot apices of the plants grown under the low light intensity treatment was twice as high as the high light intensity treatment from Day 40 to Day 50 (Figure 5.1), whereas, the sucrose level in the shoot apices of plants grown under high light intensity was stable from Day 25 to Day 65 (Figure 5.2).

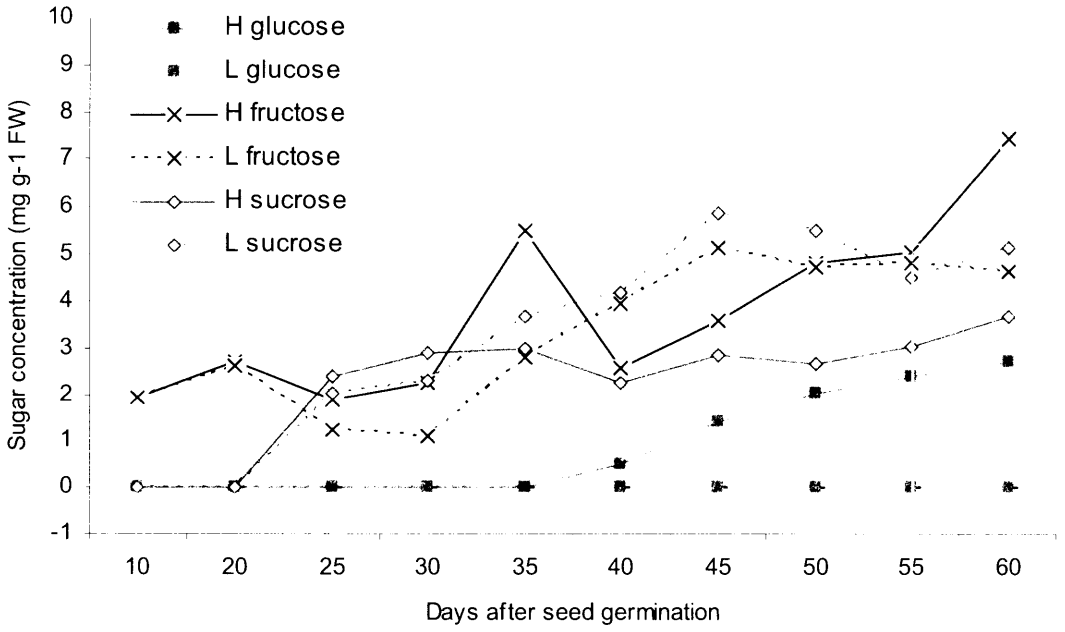

Figure 5.1 Changes in sugar content (sucrose, glucose and fructose) in the shoot apices of *Swainsona formosa* plants grown under high light intensity (H, 800 μmol $m^{-2}s^{-1}$) and low light intensity (L, 150 μmol $m^{-2}s^{-1}$) during the study period of 60 days. Results are the means ± standard error from at least four repetitions.

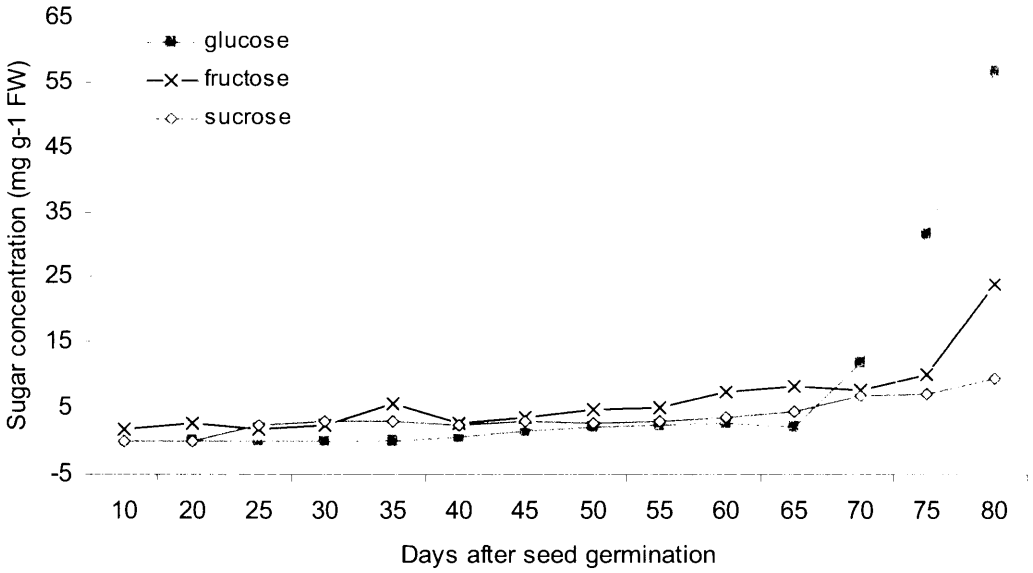

Figure 5.2 Changes in sugar content (sucrose, glucose and fructose) in the shoot apices of *Swainsona formosa* plants grown under high light intensity (800 μmol $m^{-2}s^{-1}$) during the study period of 80 days. Results are the means ± standard error from at least four repetitions.

A change in the sucrose:glucose ratio occurred between Day 35 and Day 45 (Figure 5.3), after which the sucrose:glucose ratio remained constant.

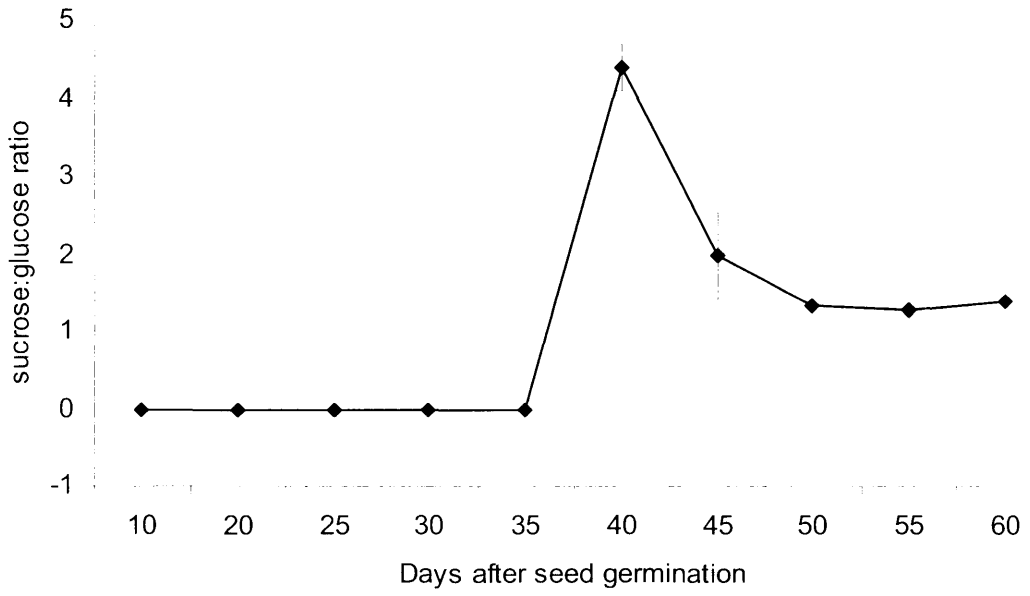

Figure 5.3 Changes in sucrose:glucose ratio in the shoot apices of *Swainsona formosa* plants grown under high light intensity (800 μmol $m^{-2}s^{-1}$) during the first 60 days of the study period.

5.4 Discussion

During the 60-day growing period, the most abundant sugar in the shoot apices of *S. formosa* was fructose, followed by sucrose. The glucose content was much less than either the fructose or sucrose, and the glucose was not detected in plants grown under low light intensity employing the present method. Before the transition stage, the fructose content in the shoot apex increased rapidly, then dropped by 50% but rose again after the transition stage with the formation of the floral primordia. Whether the increase in the fructose levels of the shoot apices of *S. formosa* has only a supportive role, providing energy for the stimulation of the meristematic activity occurring at evocation, such as the stimulation of cell proliferation (Fletcher, 2002) and of RNA (Shinano *et al.*, 2006) and protein synthesis (Rolland *et al.*, 2006), or whether it has a more specific role in the mechanisms controlling the shift of the meristem activities from the vegetative stage to the floral bud needs to be considered. However, it is more likely that this fluctuation should not be considered to be as important as the overall rise, as shown in the graphs, during the study period.

Plants adjust to a changing environment by altering their acquisition and allocation of carbon and nutrients in order to maintain homeostasis between carbon-based (carbohydrates and lipids) and nitrogen-based compounds (nucleic acids, amino acids, proteins), and secondary metabolites (terpenoids, alkaloids, phenolics) (Andrews, 2002). These alterations may enable plants to acclimate to environmental stress, such as low light intensity, resulting in responses that maintain adequate growth, whether vegetative or floral.

Swainsona formosa plants grown under the high light intensity treatment initiated flowers within 45 days after seed germination, while plants grown under the low light intensity treatment remained vegetative and initiated no flowers during the 60-day experimental period. These results are similar to those obtained by Yusuf (2000), where *S. formosa* plants grown under low light intensity (140 μmol $m^{-2}s^{-1}$) produced no flowers during the experimental period of 121 days.

Light affects the speed of photochemical and electron transport reactions in the thylakoid membranes of chloroplasts. It may affect photosystem I (PSI) and photosystem II (PSII) in the thylakoids. Both PSI and PSII are proteins with some chlorophyll and carotenoid molecules that absorb photons of light (Paul and Foyer, 2001). The carboxylating enzyme ribulose-1,5-bisphosphate carboxylase (RuBPC) is not fully activated at low irradiance and thus the degree of activation may regulate the flux of carbon through the photosynthetic pathway (Bertamini *et al.*, 2006). In other words, light may affect the nature, type and concentration of these molecules and thus affect photosynthesis.

Plants may readily adapt to low light conditions, such as shady environments, and in doing so increase light efficiency by lowering the rates of assimilate production by altering chloroplast protein and pigment composition to optimise light capture in the leaves (Paul and Foyer, 2001). The inability to establish a new sink capacity (flower) results in the accumulation of assimilates in the leaves.

Plants, as photoautotrophic organisms, are able to produce the carbohydrates they require and have developed mechanisms to coordinate their carbohydrate production and metabolism. Carbohydrate-derived signals regulate the expression of genes

involved in both photosynthesis and metabolism, and control carbohydrate partitioning (Rolland *et al.*, 2006). This reflects the essential role of sugars in the growth and developmental processes. An adequate supply of carbohydrates is needed both to energise metabolism and to make cellular structures and cell walls (Rook and Bevan, 2003). The high fructose and sucrose concentrations in the *S. formosa* shoot apices of plants grown under low light apparently might provide an extra supply of energy for vegetative growth.

There is strong experimental evidence that leaves produce agents that promote or inhibit flowering, when exposed to favourable or unfavourable conditions, respectively (Bernier *et al.*, 1981a, b). Bernier *et al.* (1998) analysed carbohydrates in several photoperiodic plants which can be synchronously induced to flower by exposure to a single LD or SD. In all cases, an increase in sucrose export out of the leaves was observed during the transition to flowering (see Chapter 2, section 2.7.1.2). Miller and Langhans (1989) found irradiance reductions of 50% to 85% in greenhouses significantly reduced total carbohydrate concentration in the leaves and floral buds of Easter lilies (*Lilium longiflorum* 'Nellie White'). Wang (1995) reported *Phalaenopsis* plants did not flower in response to low temperature under low-intensity light or in complete darkness.

In many species, sucrose was found to be the major sugar in the shoot apical meristem and phloem sap during flower induction (Bodson and Outlaw, 1985; Corbesier *et al.*, 2002). The sucrose content in *Phalaenopsis* leaves correlated significantly with the days to spiking, but the contents of other soluble sugars in the leaves did not (Kataoka *et al.*, 2004). King and Ben-Tal (2001) found that the sucrose content of the shoot apices of *Fuchsia hybrida* increased as the photosynthetic irradiance increased, reaching saturation at about 500 μmol $m^{-2}s^{-1}$. These same plants flowered at higher irradiances, remaining vegetative at an irradiance of 230 μmol $m^{-2}s^{-1}$. However, it was found that *F. hybrida* and *Lolium temulentum* can flower with no increase in sucrose at the shoot apex following a low-irradiance LD extension of 10 – 15 μmol $m^{-2}s^{-1}$ (King and Evans, 1991). Thus, it is clear that sucrose is not a component of the true LD florigen(s) of *F. hybrida* and *L. temulentum* but acts as an effective florigenic synergist.

Changes in the amounts as well as the composition of soluble sugars during flower development in the floral organs of *S. formosa* were consistent with changes in sugar contents of *Lilium longiflorum*, as reported by Ranwala and Miller (1998). Banon *et al.* (1997) suggested that the soluble saccharide concentration in the *Liatris spicata* cv. Callilepis corm reached a maximum after sprouting, probably due to rapid growth of the shoot. There was no change in the concentrations of soluble saccharides immediately prior to flower initiation, which suggests that quantitative changes in these saccharides were not responsible for activating flower initiation (Banon *et al.*, 1997). Judel and Mengel (1982) reported that the non-structural carbohydrate present in the highest quantity in the leaves and culms of spring wheat (*Triticum aestivum* 'Kolibri') vegetative plant material was sucrose. The conflicting results on the effect of sugars on these plants are probably due to species differences in carbohydrate transport and accumulation within tissues, and plant metabolism. In many cases the effects of sucrose may be completely substituted by hexoses, such as glucose and fructose. For instance, sucrose repression of photosynthesis genes can be mimicked by a lower concentration of hexoses (Jang and Sheen, 1997). These results indicate that sucrose is unlikely to be the direct signalling compound in *S. formosa*, but may act as a supporting compound, providing biochemical energy for floral initiation and development.

Sucrose concentration under high light remained stable during the trial period (possibly suggesting as equilibrium between sucrose transport to the apices and hydrolyse at the apices), whereas sucrose concentration under low light increased with time and did not appear to be used as a resource for glucose production.

The differences reported in the present study to other studies on light intensity and sugar might result from the different plant species used, organ type examined and sample collection techniques. Shoot apices were chosen for use in this experiment because they are the site of floral transition. In *S. formosa*, trace amounts of glucose were detected under high light intensity at the beginning of the transition stage and these amounts then increased with progressive floral development. It is possible that such high levels of glucose may be indicative of a high rate of sucrose mobilisation preceding any increase in the sink activity of the shoot apical meristem, suggesting that extra sucrose may act initially as a substrate and glucose is acting as a signal; glucose may trigger a chain reaction leading into flower induction. Investigations on *Arabidopsis thaliana*, using a

starchless mutant and various photoperiodic treatments either causing flowering or maintaining the plants at the vegetative stage, indicated that a large percentage of induced plants is always associated with a large, early, and transient increase in sucrose export from the leaves (Corbesier *et al.*, 1998). Alternatively, under high light, plants may produce a higher level of enzymes to cleave the sucrose into the monosaccharides, glucose and fructose, which can then be incorporated either in the metabolic or biosynthetic reactions (Ranwala and Miller, 1998).

Sucrose is one of the main end products produced during photosynthesis and is also the main form of translocated sugar in most plants. Sucrose was present in shoot apices of *S. formosa* grown under low light intensity, indicating that a source for glucose and fructose production was available. However, glucose was not detectable (measurable) at any stage in the shoot apices of the plants grown under low light conditions. This suggests that the amount of glucose may be very low and could not be measured using the present method. It is also possible that the shoot apices of plants grown under low light may be incapable of utilising glucose.

Sucrose is the major sugar in both leaf and apical exudates (Lejeune *et al.*, 1993). Corbesier *et al.* (1998) reported that sucrose was the only carbohydrate detected in the phloem sap in *Arabidopsis thaliana*. This indicated that sucrose can be hydrolysed to glucose and fructose in the shoot apices (sink organs) of *S. formosa* rather than transported to the apices from elsewhere in the plant. Two major enzymes are involved in the cleavage of sucrose in plants, invertase and sucrose synthase, and they are paramount in sugar metabolism after phloem unloading. Invertase catalyses irreversible hydrolysis of sucrose to its hexose moieties, glucose and fructose. Invertase exists in several isoforms with different biochemical properties and subcellular locations (Sturm *et al.*, 1999). Invertases with acidic pH optima are found ionically bound to the cell wall (cell-wall invertases) or as soluble proteins in the vacuole (vacuolar invertases). Both enzymes are expressed in sink tissues (shoot apices), but the predominant sucrolytic activity varies, depending on the species, tissue type, and developmental stage (Krapp *et al.*, 2005).

The balance between glucose, fructose and sucrose may be important for flowering to occur. If irradiation is inadequate, the balance of sugars in the shoot apices becomes

unfavourable to regulate flowering. In this present study, a rapid increase in the sucrose:glucose ratio coincided with the switch from the vegetative to the reproductive stage in *S. formosa*. This might be due to an increase in sucrose transport to the apices in response to signals to begin flowering development. The subsequent decrease in the sucrose:glucose ratio after Day 40 indicates that sucrose hydrolysis has begun and glucose is being produced. There is little published data on the sucrose:glucose ratio. In *Vicia faba*, an increase in sucrose levels and a decrease in glucose levels in developing cotyledons caused a steep rise in sucrose synthase activity and the onset of starch biosynthesis (Weber *et al.*, 1996).

The increase in glucose may be due to the production of secretions (nectar) in the flower, however Mallick (2001) found that bud-stage *Eucryphia lucida* flowers produced no nectar. Nectar was first detected in early male flowers as the anthers began to dehisce, and continued to be produced until the petals started to senesce. Carter *et al.* (2006) indicated that the secretion of nectar is under developmental control, beginning just before anthesis.

There is a strong relationship between flower development and carbohydrates (Iglesias *et al.*, 2003). Waithaka *et al.* (2001) reported that the opening of gladiolus flowers was accompanied by a substantial increase in the carbohydrate content of the perianth. The principal soluble carbohydrate was fructose, with substantially lower concentrations of glucose and sucrose. Yamane *et al.* (1991) showed that the predominant sugars in the gladiolus perianth were fructose and glucose, and considered that starch in the flowers was the primary source of soluble carbohydrate, which contributes to the early stages of flower expansion. They also demonstrated a high acid invertase activity in the opening flowers, indicating the possibility that the osmotic changes required to drive cell expansion might be achieved by hydrolysis of sucrose in the buds, or sucrose translocated to the developing bud. In *S. formosa*, the amount of carbohydrates increased greatly after all floral parts had been fully differentiated (after Day 60), presumably to support the next stages of floral development.

Recent studies by Rolland *et al.* (2006) indicate that, in a manner similar to classical plant hormones, sugars can act as signalling molecules that control gene expression and developmental processes in plants. Crucial evidence includes uncoupling glucose

signalling from its metabolism (Rolland *et al.*, 2002), identification of glucose sensors e.g. hexokinase (Moore *et al.*, 2003), and the isolation and characterisation of mutants and other regulatory components in plant sugar signal transduction pathways (Price *et al.*, 2004). The emerging scenario points to the existence of a complex signalling network that interconnects transduction pathways from sugars and other hormone and nutrient signals (Sheen *et al.*, 1999). In *Citrus*, while changes in the carbohydrate concentration are unlikely to trigger flower initiation, the carbohydrate concentration is thought to influence cytokinin production and subsequently the number of flowers produced (Davenport and Nunez-Elisea, 1990). Assimilate distribution of sugars in *S. formosa* appears to be under sink control and may be hormonally and genetically regulated.

5.5 Conclusion

No glucose was detected (using HPLC) in the shoot apices of plants grown under low light intensity. The availability of glucose in the apical meristem may, therefore, be related to the initiation of floral development. The timing of changes in sucrose quantity matched the timing of morphological changes (see Chapter 4), indicating a key role for sucrose in flower development. The changes in the sugar composition of the shoot apices are associated with quantitative changes of sugar translocation and changes of enzyme activities related to the floral transition. These effects could be due to sugar signalling, metabolism or both. If floral induction in *S. formosa* is triggered by a threshold level of photosynthate and other saccharide compounds, it may be possible to control the flower induction and development by increasing photosynthesis or by the external application of sugar.

Consequently, further study (Chapter 7) is focused on the responses of the *S. formosa* plants grown under low light intensity to an external application of sugar, to determine whether phase change can be induced under *in vitro* and *in vivo* conditions. Changes in C:N ratio (Chapter 6) and protein expression (Chapter 8) were also investigated.

Chapter 6 The Relationships of Carbon and Nitrogen in the Shoot Apices of *Swainsona formosa* at Different Stages of Development

6.1 Introduction

The carbon:nitrogen (C:N) ratio in plants has often been associated with bud formation, flowering and fruiting, but the hypothesis regarding this has also been found to vary with species (see Chapter 2, section 2.7.1.2). According to the nutrient diversion theory (Sachs, 1977), vegetative development is maintained as long as the labile pools of carbohydrate ($[CH_2O]_n$) and N in the meristem remain in a constant proportion. The apical meristem is unable to sustain vegetative development when the relative proportion of these pools in the meristem is either increased or decreased (Rideout *et al.*, 1992). As a consequence, floral development is stimulated as a default state.

While it is not known with certainty if relative changes in $[CH_2O]_n$ and N availabilities result in floral transition or if floral transition results in a change in $[CH_2O]_n$ or N availability, it appears that the two events are related (Rideout *et al.*, 1992). As an energy reserve, $[CH_2O]_n$ are necessary for floral organ development. Flowering of *S. formosa* can be inhibited by insufficient $[CH_2O]_n$ (see Chapter 5). Flowering of *Hesperaloe funifera* required stored $[CH_2O]_n$, as well as currently assimilated $[CH_2O]_n$ and considerable amounts of N (McLaughlin *et al.*, 2000). A recent study using global transcription profiling demonstrated that much of the regulation by N depends on the availability of C, but regulation by C is modulated by the presence of N (Price *et al.*, 2004). Price *et al.* (2004) work provides a molecular basis for the importance of the C:N ratio in the control of plant growth and development.

King and Ben-Tal (2001) found that sucrose levels changed at the apex at the time of flowering in *Fuchsia hybrida*, regardless of whether or not an inductive photoperiod occurred. It is probable that changes in sucrose concentrations or other nutrients are a result of floral transition and not the signal that induces flowering. Indeed, when long-day inductive signals at low light levels were applied to *F. hybrida*, the amount of sucrose at the apex did not increase, while plants still flowered in response to the day

length changes (King and Ben-Tal, 2001). While the results above, and the work of Stutte and Martin (1986) indicate that nutrient changes at the apex are not required for floral evocation, it is not known if nutrient changes are involved in the flowering of *S. formosa*.

Swainsona formosa is an annual plant whose requirements for floral initiation and development are not fully understood. Results from Chapter 5 showed that changes in sugar content (carbohydrate) were associated with floral transition in the shoot apices. The changes in the sugar content only reflect a portion of the total carbon in the shoot apices, so the %C of the shoot apices needs to be investigated. Experiment 6 provides a basis for an improved understanding of the C:N ratio, %C, %N and their involvement in the floral transition of the apical meristem in *S. formosa*.

6.2 Materials and methods

6.2.1 Plant material and growth conditions

Seeds were germinated as described previously in Chapter 3. After transplanting to plastic pots, the plants were raised in growth cabinets. The growth cabinets were set with a 16-hour photoperiod, with light intensity at 800 μmol $m^{-2}s^{-1}$ (high light treatment) or 150 μmol $m^{-2}s^{-1}$ (low light treatment). Day/night temperatures were set at 30 °C (day) and 18 °C (night). Samples were taken at different developmental stages, according to the previous study in Chapter 4, to represent vegetative, floral transition, initiation and differentiation, and flower (flower buds can be seen with the naked eye) stages, as indicated in Table 6.1. As the apices of the plants grown at low light intensity remained vegetative, those plants grown under low light intensity were only sampled at Day 60. This was to avoid high numbers of analyses and also the limitation of the space in the growth cabinets. Plants grown under high light intensity were sampled on Days 30, 35, 40, 45, 50, 55 and 60. For both treatments and all sampling times, the samples were taken from the apices of the two axillary branches in each experimental plant.

Table 6.1 Sampling development stages (Days after seed germination) for Carbon:Nitrogen ratio analysis of the shoot apices of *Swainsona formosa* plants grown under high or low light intensity.

Light intensity	Days after seed germination	Apex developmental stages
High light (800 μmol $m^{-2}s^{-1}$)	30 – 35	Vegetative
	40 – 45	Transitional
	50 – 55	Floral initiation and differentiation
	60	Flower
Low light (150 μmol $m^{-2}s^{-1}$)	60	Vegetative

6.2.2 Carbon:Nitrogen ratio analysis

Shoot apices (1.0 cm) were collected at sampling time. The shoots were dried in an oven at 80 °C for 2 days. The dried samples were then ground finely using a mechanical grinder to pass through a 0.5 mm sieve.

Samples were analysed using a Carlo Erba NA 1500 Solid Sample Analyser coupled to a Tracer Mass Stable Isotope Analyser (Europa Scientific). Approximately 4 mg of ground plant sample was weighed into a 5 x 8 mm tin cup, crimped and loaded into the autosampler for the analysis. Both elemental N and C were recorded and results given as %N, and %C. The C:N ratio was calculated from %C divided by %N.

6.2.3 Statistical analysis

The experiment was carried out with four replications. Data were statistically analysed using analysis of variance (ANOVA) at a 5% level of significance. The SigmaStat statistical software (SPSS Inc., Chicago) version 12.0.1 was used for the analysis.

6.3 Results

The C:N ratio of the vegetative apices of the plants grown under low light intensity at Day 60 after seed germination was significantly ($P<0.001$) lower than those of plants grown under high light intensity at all sampling times (Figure 6.1). In high light, the

C:N ratio of the shoot apices increased gradually during the early transition stage (Day 40), followed by a slight decrease at Day 45 and a subsequent increase at Day 55.

In Figure 6.2, the %C in the low light intensity treatment at Day 60 is significantly lower than %C in all the high light intensity treatment readings, particularly at Day 60 when the paired measurements were taken. In the high light treatment, there appears to be a significant decrease from Day 35 to Day 40 and then a gradual increase from Day 40 to Day 60.

In Figure 6.3, %N, unlike carbon, is significantly higher in the low light treatment at Day 60 than in all the readings for the high light treatment. Again this is particularly so at Day 60 when the paired measurements were taken. In high light, there appears to be a significant gradual decrease from Day 30 to Day 40 (transition), then a gradual increase from Day 40 to Day 50, followed by another significant decrease after that.

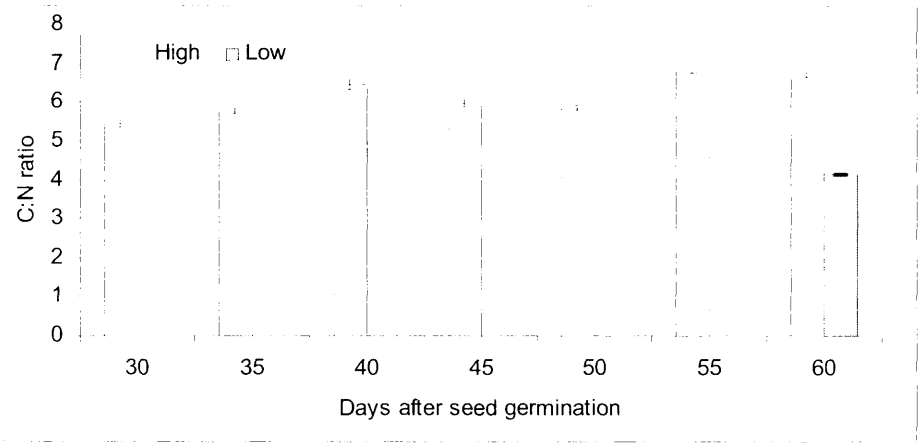

Figure 6.1 Relative concentrations of carbon:nitrogen (C:N) ratio in shoot apices of *Swainsona formosa* on a dry weight basis. For the treatments, High = high light intensity (800 μmol $m^{-2}s^{-1}$) and Low = low light intensity (150 μmol $m^{-2}s^{-1}$). Results are the means ± standard error of four repetitions.

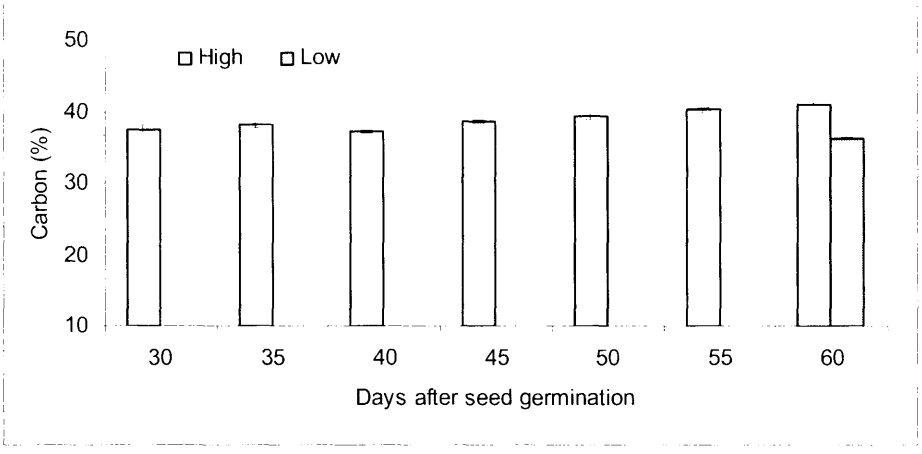

Figure 6.2 Percentage of carbon in shoot apices of *Swainsona formosa* on a dry weight basis. For the treatments, High = high light intensity (800 μmol $m^{-2}s^{-1}$) and Low = low light intensity (150 μmol $m^{-2}s^{-1}$). Results are the means ± standard error of four repetitions.

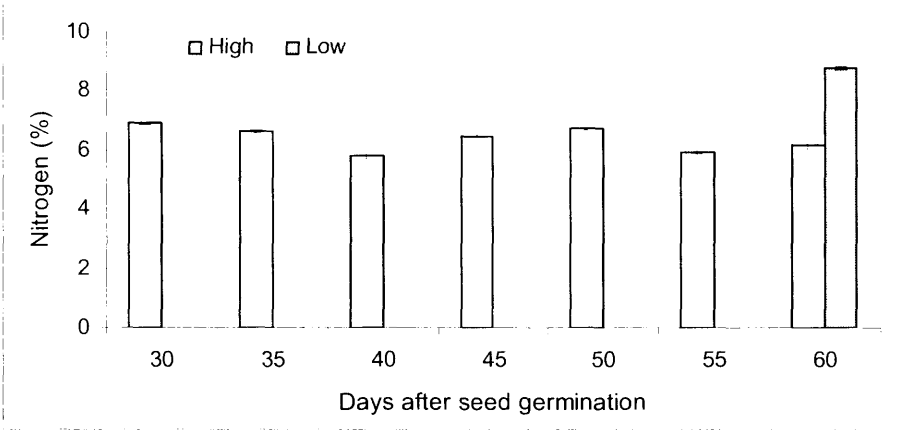

Figure 6.3 Percentage of nitrogen in shoot apices of *Swainsona formosa* on a dry weight basis. For the treatments, High = high light intensity (800 μmol $m^{-2}s^{-1}$) and Low = low light intensity (150 μmol $m^{-2}s^{-1}$). Results are the means ± standard error of four repetitions.

6.4 Discussion

In *S. formosa*, the C:N ratio of the vegetative shoot apices was approximately 30% lower in plants grown under low light conditions compared with those grown under high light conditions. Similar results were observed by Gary *et al.* (2003) who reported the concentration of $[CH_2O]_n$ in tomato (*Lycopersicon esculentum*) vegetative organs was 30 – 50% lower in plants that were exposed to low C assimilation conditions compared with those exposed to high C assimilation conditions. Paul and Foyer (2001) suggested that $[CH_2O]_n$ repression of photosynthesis may be viewed as a mechanism that optimises the whole plant C and N balance, and sugar signalling is dependent on the nitrogen status, as well as the C status, of the plant. It can be suggested that a low supply of substrate (C) caused by unfavourable conditions (inadequate light) can limit overall metabolism. Light intensity can affect the metabolism and distribution of endogenous $[CH_2O]_n$ and in this way may influence flowering. Under low light conditions, there may be insufficient C from the sum of photosynthesis and reserve carbon to satisfy plant requirements for the induction of flowering. In *S. formosa*, under low light conditions, the C:N ratio and %C may not have been sufficient to induce phase transition from the vegetative to the reproductive stage. An appropriate balance between C, N and sugars (Chapter 5) is needed for the induction of flowering in *S. formosa*.

Assuming that differential changes in the supply of $[CH_2O]_n$ and N relative to one another are the floral stimulus or are the prerequisites for the floral stimulus to be effective (Raper *et al.*, 1988), parallel increases or decreases in $[CH_2O]_n$ and N would not be expected to result in floral transition, while unequal changes in the relative concentration in the labile pools would be expected to result in floral transition (Rideout *et al.*, 1992). The results showed reduced %N and %C at early transition stage but an increased C:N ratio in the shoot apices. The present study supports the C:N ratio hypothesis and demonstrates that an increased ratio is associated with floral transition.

Coruzzi and Zhou (2001) reported that the ability of plants to regulate N uptake and metabolism involves sensing both inorganic (i.e. nitrate) and reduced or organic N (e.g. ammonium, glutamine). They also suggest sugars regulate gene expression and this may have an adaptive value, resulting in altered C allocation among different parts of the plant, thereby optimising the investment in C and N that could result in changes to

the whole-plant morphology. In this experiment the status of C and N were determined from the shoot apices only, and not the whole plant. If C and N were measured from other parts of the plant, such as the leaves and stems, the results might have been different to those reported here.

The analysis of %C and %N in the *S. formosa* shoot apices showed an inequality in the relative availability of %C and %N in the shoot apical meristem, the increase in %C being higher than the increase in %N after the conversion from the vegetative to the reproductive stage occurred. Similarly, Raper *et al.* (1988) found that the increase in $[CH_2O]_n$ was higher than the increase in N prior to and during floral transition in the shoot apical meristem of day-neutral tobacco (*Nicotiana tabacum*). No genetic evidence for this model exists to date, but analysis of $[CH_2O]_n$ to N ratios, prior to and during floral transition, shows that under vegetative conditions, the ratio is consistent but at the time of flowering nitrogen transport to the apex becomes proportionally greater (Rideout *et al.*, 1992). Interaction between the C and N metabolisms in plants is important to ensure efficient assimilation of these two major nutrients and this may allow flowering to occur. Carbon skeletons are essential for the incorporation of inorganic N into biologically relevant molecules, such as amino acids, proteins, nucleic acids and a large number of secondary metabolites (Krapp *et al.*, 2005). For the results to be more conclusive, a larger number of proteins (N compounds) were analysed in the experiment reported in Chapter 8.

The translocation of nutrients to the shoot apical meristem during floral transition is associated with flowering in many species (Tremblay and Colasanti, 2006). Under high light conditions, the increase in the C:N ratio at 40 days after seed germination, the beginning of the transition stage, may indicate active cellular action at the meristematic region. As a result of cellular differentiation, the apical meristem becomes a stronger sink. Under low light intensity, $[CH_2O]_n$ may be stored in leaves and other organs, thus inhibiting the production of new assimilates (Gary *et al.*, 2003), or low light intensity may reduce sink strength. The results obtained support the nutrient diversion hypothesis of floral induction, which assumes that changes of relative sink strength favouring assimilate supply cause the shoot apical meristem to undergo floral transition (Sachs, 1977). In some species, such as *Sinapis alba*, it has been suggested that the time at

which these assimilates arrive at the apex is just as important as the quantity received (Sachs, 1987).

Nitrogenous assimilates in the shoot apices may be derived from xylem to phloem transfer and amino acids produced in the leaves (Krapp *et al.*, 2005). The efficiency of C and N assimilation pathways is a main factor in determining plant growth. For example, under *in vitro* conditions, high N concentrations in the medium usually stimulate vegetative growth; the vegetative growth then retards reproductive development (Dielen *et al.*, 2001). In *S. formosa*, %C in low light plants is always significantly lower than in high light plants, while %N is always significantly higher in low light than high light plants, particularly at Day 60 when the paired measurement was taken. Carbohydrate distribution within a plant may be affected by the N supply, which strongly influences the process of C assimilation, allocation and partitioning (Kaiser, 1997). In many species, reduced N nutrition can retard growth, resulting in increased $[CH_2O]_n$ concentration in the shoot and thus accelerating flowering (Davenport *et al.*, 2006). An examination of *Arabidopsis* suggests that N deficiency initiates transcriptional changes that can be integrated in a pathway directing the accumulation of sugars and starch in the shoots and the increased translocation of sucrose to the roots (Hermans *et al.*, 2006). Carbon metabolites and C:N ratio both regulate the expression of several genes involved in N acquisition and metabolism (Domoney *et al.*, 2006), and nitrate regulates many genes assigned to sugar metabolism (Rolland *et al.*, 2006). The mode of N acquisition (fixation versus assimilation) seems to influence %N accumulation in the shoot apices of low light grown *S. formosa* plants. To reach a more precise conclusion on the effect of the C:N ratio on flowering of *S. formosa*, it is necessary to understand the relative contributions of assimilate supply ($[CH_2O]_n$ and N fertilisation) to flower initiation. The effects of exogenous sugar application on the flowering of *S. formosa* was tested to determine whether increased assimilate supply (%C) promotes flowering; this is reported in Chapter 7.

6.5 Conclusion

This experiment supports the idea that flowering of *S. formosa* is influenced by C and N status of the shoot apex. A possible activator of the transition of the shoot apex from the vegetative to the reproductive phase is a quantitative change in the C:N ratio; an increased ratio is important to trigger floral transition. Reduced %N and %C at the early

transition stage, and increased C:N ratio in the shoot apices, could be used as indicators for stage changes. To flower, plants need to maintain an appropriate C:N ratio.

Chapter 7 Effect of Exogenous Sugar Application on the Flowering of *Swainsona formosa*

7.1 Introduction

In *S. formosa*, an increase in endogenous glucose (Chapter 5) and an increase in the C:N ratio (Chapter 6) were found prior to flower differentiation in the shoot apices, suggesting that flowering in *S. formosa* was linked to changes in carbohydrates levels and/or proportions of %C and %N. Previous work in Chapter 5 showed that plants grown under low light intensity conditions produced no flowers and no glucose was detected in their shoot apices.

Carbohydrates are needed for growth, organised development, and as an energy source for biosynthetic processes and for structure of carbon skeletons. Sugars regulate the expression of a large number of genes in various plant species; for example, the genes involved in photosynthesis are repressed by sugars (Jang and Sheen, 1994). It is also thought that sugar may act directly as a signalling molecule that triggers changes in gene expression in plants (Price *et al.*, 2004).

The coordination of floral development with the availability of nutrients, such as soluble sugars, may help ensure an adequate supply of building materials and energy with which to complete specific developmental programmes. Sucrose has been shown to promote flowering in many species (reviewed in Gibson, 2005).

In this chapter, the possibility of using exogenous sugar applications as a replacement for high light intensity conditions is considered with the objective of evaluating the effect of exogenous sugar applications on the flowering of *S. formosa*. Several experiments using exogenous sugar applications were conducted, to try to identify the correct methodology, type, concentration and frequency of application to induce flowering in *S. formosa*. The objective of this chapter was to evaluate the effect of exogenous sugar application on flowering of *S. formosa*.

7.2 Materials and methods

7.2.1 *In vivo* (Experiment 1, 2, 3 and 4)

Seeds were germinated as described in Chapter 3. After transplanting to plastic pots, plants were raised in growth cabinets. The growth cabinets were set with a 16-hour photoperiod and a light intensity of 150 ± 10 µmol $m^{-2}s^{-1}$. Day/night temperatures were set at 30 °C (day) and 18 °C (night).

From earlier experiments, the most suitable time for the application of the chemical treatments was determined to be 40 days after seed germination, when the plants had produced up to three true pinnately compound leaves (adult leaves); the results in Chapter 4 also indicated that the transition stage occurred at this time in plants grown under high light intensity. At 40-days-old, the plants were treated with the appropriate sugar solutions as described in Experiments 1, 2, 3 and 4 (Figure 6.1*A-D*). All the required sugar concentrations were prepared with deionised distilled water. A wetting agent (Triton®, octyl phenoxy polyethoxy) was used at 0.1% of the spray volume to facilitate the adherence of the sugar to the plant tissue. The control plants were treated with deionised distilled water plus the wetting agent. The analytical grade D-Glucose was obtained from Ajax chemicals (Australia), the microbiology grade D(-)-Fructose from Fluka BioChemika (Switzerland) and the plant tissue culture grade II quality sucrose from Sigma-Aldrich, Inc. (USA).

7.2.1.1 Experiment 1: Effect on flowering of types and concentrations and frequency of application of sugar spray applied to S. formosa *plants grown under low light intensity conditions.*

The treatments were control (water), sucrose, fructose and glucose at concentrations of 1, 10 and 100 g L^{-1}. The selected range of sugar concentrations was based on the findings of the experiment discussed in Chapter 5. The treatments were applied at 40 days after seed germination (single spray), or at 40 days after seed germination with a repeat spray at 50 days (double spray) (see Figure 6.1*A*). The application time, 40 days after seed germination, was chosen because earlier results (Chapter 4) showed the transition stage occurred at this time in plants grown under high light intensity conditions. The treatments were applied as a whole-plant spray until run off

(approximately 10 mL per plant) using manual pump dispensers. The treatments were applied in the afternoon and irrigation was avoided for about 10 – 12 hours before and after the treatment. Foliar sprays were used because they can be applied relatively quickly and are less costly than other methods (Gent and McAvoy, 2000).

7.2.1.2 Experiment 2: Effect on flowering of types and concentrations and frequency of application of sugar applied with a brush to the apical bud of S. formosa *plants grown under low light intensity conditions.*

The treatments and the time of application were similar to those in Experiment 1, with the treatment solutions (1 mL) being applied to the apical bud of the vegetative plants with small soft brush (see Figure 6.1*B*). This method is more labour intensive but allows for precise application at the target site.

7.2.1.3 Experiment 3: Effect on flowering of types and concentrations of sugar applied by placing a piece of absorbent cotton onto the apex of S. formosa *plants grown under low light intensity conditions.*

The treatments and the initial time of application were similar to those in Experiment 1, with the treatment solutions being applied by placing a piece of absorbent cotton soaked in the given solution onto the apex (1 mL) for 24 hours (see Figure 6.1*C*). The treatments were repeated on three consecutive days. This method is also more labour intensive but it allows for precise application at the target site. The repeat applications prolong the time for the plants to absorb the sugar solution compared with the application method used in experiment 2.

7.2.1.4 Experiment 4: Effect on flowering of types and concentrations of sugar injected into apices of S. formosa *plants grown under low light intensity conditions.*

The treatments and the time of application were similar to those in Experiment 1. The treatment solutions (0.1 mL per shoot) were injected into shoot apices 5 cm below the apex using a 1 mL hypodermic syringe with a 0.45 x 13 mm needle (Terumo corporation) (see Figure 6.1*D*). Shoot injection is also time consuming, but allows the precise application of a defined amount of sugar. This technique is particularly suited to

scientific experimentation, where it is important to know the precise quantity of the applied sugar.

7.2.2 *In vitro* (Experiment 5)

Seeds were germinated as described previously in Chapter 3. After transplanting to plastic pots, the plants were raised in growth cabinets. The growth cabinets were set with a 16-hour photoperiod. Day/night temperatures were set at 30 °C (day) and 18 °C (night). There were two treatments: a high light intensity (800 ± 50 μmol $m^{-2}s^{-1}$) and a low light intensity (150 ± 10 μmol $m^{-2}s^{-1}$).

The shoot apices (1.5 cm) of 35-days-old plants (plants still in the vegetative stage, and just before transition occurred in plants grown under high light intensity, as reported in Chapter 4) were excised from the axillary branches and surface-sterilised by soaking in 1.0% w/v sodium hypochlorite for 15 minutes. The shoot apices were then rinsed three times in sterile water, and then trimmed and cultured in one of the induction media for six weeks.

The basal medium was MS medium (Murashige and Skoog, 1962). Table 7.1 lists the constituents and the amounts for the preparation of 1 L of MS basal medium from the stock solutions. The preparation of the stock solution is described in Appendix 1.

Table 7.1 Chemical constituents for the preparation of 1 L of MS basal medium (for more information refer to Appendix 1).

Chemical Constituent	Amount
Nitrate stock	10 mL
Sulphate stock	10 mL
Halide stock	10 mL
PBMo stock	10 mL
NaFeEDTA stock	10 mL
Vitamin stock	10 mL
Agar	10 g

To 600 mL of distilled water, 10 mL of each stock solution was added, with constant stirring. Sucrose and glucose were added directly to the media before autoclaving and

the volume made to 1 L with distilled water. The pH of the medium was adjusted to pH 7 for sucrose and pH 7.5 for glucose using 1N NaOH or 0.5 N HCl with an Activon Model 210 pH meter before the addition of the Agar (MBI, Marine BioProducts Inc., Canada). This two mixtures were dispensed (30 mL) into 250 mL polycarbonate culture vessels and capped with polyethylene screw caps. The media were then autoclaved at 1.1 kg cm^{-2} (103 kPa) at 121°C for 20 minutes. After autoclaving the pH of both media was around 5.5. The fructose was filter-sterilised (Millipore™ 0.22 µM) and added to partially-cooled autoclaved media; autoclaving can cause toxic degradation products of fructose (Druart and De Wulf, 1993). After autoclaving, the pH was adjusted aseptically to pH 5.5. To improve air exchange, the caps of the culture vessels were replaced with caps with a ventilation hole plugged with sterile non-absorbent cotton wool.

The culture vessels were placed on a shelf in the culture growth room situated in the Tissue Culture Laboratory of the School of Rural Science and Agriculture (Figure 6.1*E*). The temperature of the culture room was maintained at 25 ± 2 °C with a 16-hour photoperiod provided by cool fluorescent white tubes. Light intensity at the surface of the vessels was 50 ± 10 µmol m^{-2} s^{-1}.

7.2.2.1 Experiment 5: Influence of carbohydrate source and concentration on the in vitro *flowering of shoot culture of* S. formosa.

For the induction of floral buds, eight induction media were prepared. Three types of sugar, sucrose, fructose and glucose, media and four different concentrations of exogenous sugar: 1.5, 3.0, 4.5 or 6.0% w/v were used (see Figure 6.1*E*). The advantage of the *in vitro* technique is that it allows for precise control of the environmental conditions in the experiment and ensures that the sugar is readily available to the explants.

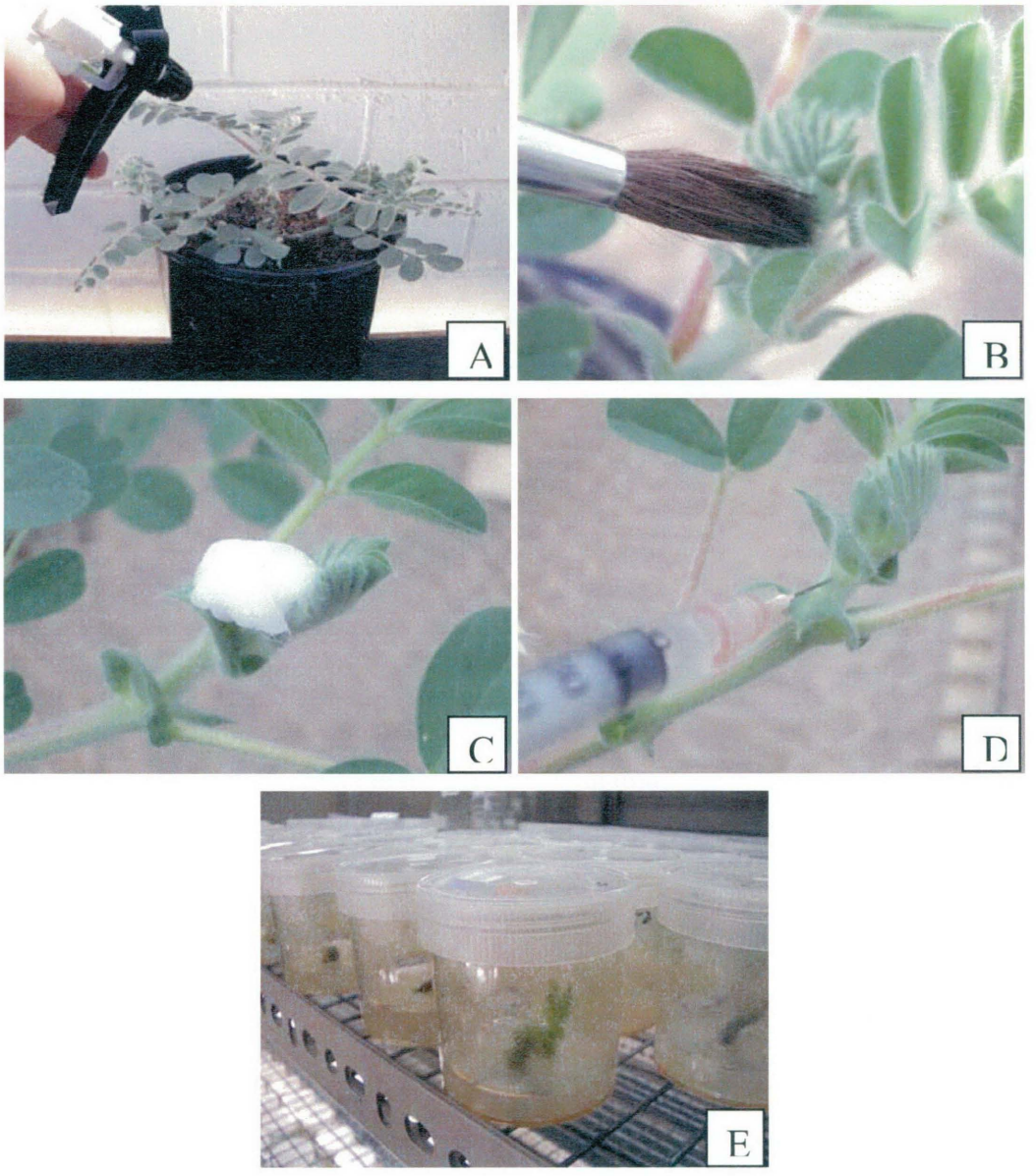

Figure 7.1 Application methods for applying the exogenous sugar to the *Swainsona formosa* plants (*A*) spraying method (*B*) brush painting method (*C*) placing a piece of absorbent cotton onto the apex method (*D*) injection method (*E*) *in vitro* method.

7.3.2 Data collection and statistical analysis

7.2.3.1 In vivo (*Experiment 1, 2, 3 and 4*)

Experiments 1, 2, 3 and 4 were laid out separately in a fully randomised factorial design with three replications (4 plants per replicate). The plants were assessed every week after the sugar applications (unless otherwise mentioned) until week four (equivalent to 68 days after seed germination). The percentage of plant shoot apices converted to floral

apices was recorded. A dissection microscope was used to observe the morphogenetic responses.

7.2.3.2 In vitro (*Experiment 5*)

In experiment 5, the plants were assessed every two weeks after the sugar applications (unless otherwise mentioned) until week 6 *in vitro* (equivalent to 77 days after seed germination). The percentage of shoot apices giving rise to floral organs was recorded. A dissection microscope was used to observe the morphogenetic responses. The experiment was arranged in a completely randomised design. There were 12 plants per treatment, and the experiment was repeated three times. However, the results were not statistically analysed owing to the low flowering response *in vitro*.

7.3 Results

All treatments used in experiments 1, 2, 3 and 4 failed to induce flowering in *S. formosa*. The sugar caused diseases and thrips infestation problems (Figure 7.2), especially when applied as a spray at high concentration (100 g L^{-1}). Injection method resulted in 100% shoot mortality.

Figure 7.2 Leaves of plants showing thrips infestation at high sugar concentration (100 g L^{-1}) applied as foliar spray. Scale bar = 1 cm.

In experiment 5, when the shoot apices from the low light grown stock plants were cultured on the induction media, they started to elongate after a week. Shoots arising from the axillary buds appeared at the nodes of explant stems, and other shoots (presumably adventitious) formed from the base of the stems. No flowers were observed in any cultures from low light grown stock plants after 6 weeks, whereas, some of the

explants from the high light grown stock plants cultured on medium containing 3.0% and 4.5% sucrose developed flowers within 4 to 6 weeks (Figure 7.3). The rate of flowering was 25% after 4 weeks and 50% within 6 weeks of culture initiation (the end of the experimental period).

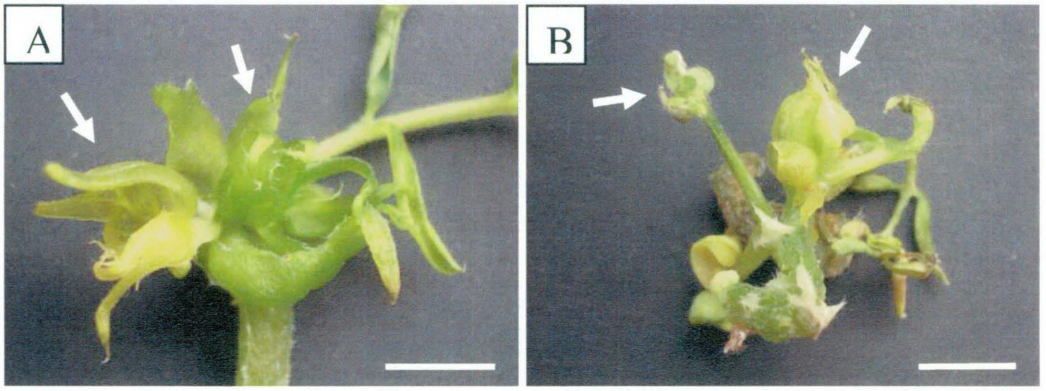

Figure 7.3 *In vitro* flowering of *Swainsona formosa* (A) *In vitro* flowering on MS medium supplemented with 4.5% sucrose. (B) Inflorescence just after emergence from shoot. Scale bar = 1 cm.

7.4 Discussion

Physiological and genetic analyses of flowering have shown that multiple environmental and endogenous inputs influence the timing of the switch for the vegetative to the reproductive stage (Boss *et al.*, 2004). The multiple pathways quantitatively regulate an overlapping set of common targets, the floral pathway integrators, whose activities commit the shoot apical meristem to a reproductive stage. An emerging idea is that changing the predominance of these input pathways could account for much of the plasticity and diversity of flowering control within and between plant species (Simpson and Dean, 2002). A possible activator of the transition of the shoot apex from the vegetative to the reproductive phase is a quantitative change in soluble sugars. In this sense, there is evidence which suggests that the supply of more assimilates than otherwise required for vegetative growth is part of the signal which activates flower initiation (Chapter 5 and 6).

The results presented in this study indicate that exogenously applied sugars under *in vivo* conditions failed to induce the transition from a vegetative to a reproductive apex in *S. formosa*. Failure to flower depends on many factors, such as the use of ineffective sugar types and concentrations along with inappropriate application methods.

Incorrect times of application, including prevailing environmental conditions, are important factors in the uptake and incorporation of sugar. Mengel (2002) suggested that temperature did not affect rates of penetration and the application of treatments should be done in the evening to take advantage of the high humidity during the night; such conditions should prolong the absorption time available to the plants.

The spray application of sugars to plants is known to be involved in a series of complex interdependent events. The main components consist of the formulation of the sugars, atomisation of the spray solution, transport of the spray droplets to the intended plant surface, impaction leading to spreading and retention, spray droplet residue formation, penetration, and transport of the sugar to a reaction site (Bukovac *et al.*, 2002). The nature and distribution of spray droplet residues are important factors in improved penetration of compounds from droplets and also the remaining residue. It appears that in spite of the use of a wetting agent, uptake/penetration of the sugar solutions may not have occurred because of the fine hairs that cover the leaves and shoots of *S. formosa*. Furthermore, exogenous application of sugar can lead to diseases and insect infestations. Therefore, the spray method cannot be recommended.

The failure to induce flowering of *S. formosa* may also reflect an inability to uptake the sugar provided. Sugar droplets when applied exogenously (e.g. spraying, brushing) can dry quickly on the axillary surface before being taken up by the plant (Schonherr *et al.*, 2000). However, the cotton absorption method, which prolonged the absorption time, also failed to induce flowers. One would not expect leaves to take up large amounts of foliar sugars. Foliar uptake of solutes requires either penetration of the leaf cuticle or passage through stomata (Eichert *et al.*, 2002). Although uptake through the stomatal pore could provide a pathway into the leaf, the architecture of the pore largely prevents liquid penetration (Ziegler, 1987). The permeability of cuticles to water, ions and polar solutes has been found to be extremely small (Mengel, 2002). However, penetration of the solution to the plant cells could depend on the molecular size of the solution; the monosaccharides (glucose and fructose) tested have smaller molecule sizes and are therefore likely to be taken up by plant cells more readily than the disaccharide (sucrose). Moreover, the flowering of *S. formosa* may be limited by several internal and external factors, such as metabolic activity, nutritional status, cuticle thickness, leaf age,

solute mobility and resistance inside the leaf, droplet dimension and shape, light level, relative humidity and temperature (Mengel, 2002).

Furthermore, exogenously applied sugars might not have been taken up by the plants, or, if taken up, then not in sufficient quantities to induce flowering in *S. formosa* grown under low light condition; this, however, was not measured. Specific interactions between the wetting agent and the active ingredients may also affect rates of foliar penetration (Schonherr *et al.*, 2000).

To avoid the problems associated with the plant surface morphology (hairs), in Experiment 4 plants were injected with sugar solutions and in Experiment 5 plants were grown under *in vitro* conditions. The injection method was too damaging, with physical damage causing the death of the injected shoots. A smaller-sized micro-needle is recommended in future trials. There are some reports on the successful use of the injection method. For example, Su *et al.* (2001) reported that the need for low temperatures to induce flowering in *Phalaenopsis hybrida* can be overcome under high temperatures by injecting gibberellic acid at 0.5 μg shoot^{-1} into the cavity of the second visible bud scale below the flowering shoot apex. Kadowaki *et al.* (2001) indicated that the injection of sugar solutions (sucrose, glucose and fructose) into the stems of sweet potato (*Ipomoea batatas*) plants have a dual effect on root production. First, increased sugar concentrations in the plant increase the activity of adenosine 5'-diphosphate glucose pyrophosphorylase and sink activities, and second, the increased C supply quantitatively promotes starch synthesis and accumulation in the roots. In future trials, the correct site of application, as well as the size of the needle, needs to be considered.

Excised *S. formosa* shoots grown *in vitro* produced flowers. The percentage of plantlets flowering depends upon environmental conditions under which the stock plants were grown and the carbon source of the culture medium. The explants from the high light grown stock plants cultured on medium containing 3.0% and 4.5% sucrose developed flowered. The type of carbon source and its concentration affects *in vitro* flowering in many plant species (Ziv and Naor, 2006). In this study, the percentage of plantlets flowering was influenced by both the concentration and type of sugar added to the media.

Autoclaving causes sucrose breakdown (Khuri and Moorby, 1995), with sucrose hydrolysis normally reaching 10% during autoclaving (Druart and De Wulf, 1993). Slesak *et al.* (2004) found that, after autoclaving, the sucrose content of the medium decreased slightly (to 93% of the initial level) and this decrease was accompanied by the appearance of fructose (3%) and glucose (4%). Mezzetti *et al.* (1991) also reported around 5% of sucrose is hydrolysed into glucose and fructose during autoclaving. It is possible that the balance of these sugars in a 3.0 to 4.5% sucrose medium becomes suitable for inducing flowering of *S. formosa*. In addition, different plant species require different types of sugars for *in vitro* induction of flowering (Taji *et al.*, 2002).

Sucrose proved to be more effective in inducing flowering of *S. formosa* than either glucose or fructose. Sucrose concentrations of 3.0 and 4.5% were optimal for floral induction, while high sucrose levels at 6.0% failed to induce flowering. Sucrose can be rapidly and completely hydrolysed at the beginning of the culture period (Nikolova *et al.*, 1991), or it can gradually diminish until the end of the culture period (De Riek *et al.*, 1997). In both the cases cited above, the glucose and fructose concentration in the medium increased in proportion to the decrease in sucrose, as shown by Slesak *et al.* (2004) in *Brassica napus*. Slesak *et al.* (2004) reported that, during culturing, the sucrose content decreased continuously, by a total of 73% by the end of culture period (Day 30). Simultaneously, the amounts of glucose and fructose in the medium increased to 41% and 30%, respectively. This result is not surprising since it is known that sucrose is hydrolysed by cell wall-bound invertase to glucose and fructose and the sugars obtained are incorporated into the cells. The work by Nikolova *et al.* (1991), De Riek *et al.* (1997), and Slesak *et al.* (2004) leads me to suggest that, for *S. formosa*, *in vitro* flowering required the presence of both glucose and fructose in the culture medium; the glucose and fructose were provided by the hydrolysis of the sucrose in the medium, partly as a result of autoclaving and partly as a result of the plant's interaction with the medium (Slesak *et al.*, 2004). All concentrations of the glucose only and fructose only treatments failed to induce flowering.

The rate of flowering of *S. formosa in vitro* increased with the increasing age of the cultures. Similar results was obtained by Tisserat *et al.* (1990), with the rate of flowering 5 – 10% after 16 weeks and 90% within 32 weeks of culture initiation in *Citrus limon*, when culturing vegetative lateral buds from 6-year-old trees. In *Pisum*

sativum, the percentage of flowering increased with the increasing age of the cultures until the second sub-culture; the ability to produce flowers was lost beyond the third sub-culture (Franklin *et al.*, 2000). This may result from loss of vigour in the cultures or an increase in somatic mutation (Taji *et al.*, 1997).

High exogenous sugar concentrations in media have also been reported to suppress floral induction by extending the late vegetative growth phase (Ohto *et al.*, 2001). Sugar applied to the roots of the plants may also stimulate cell divisions in this competing sink tissue, thus lowering assimilate supply to the apical meristem. In an experiment with tomato, *Lycopersicon esculentum*, Dielen *et al.* (2001) found that explants produced flowers in a medium containing 3% w/v sucrose, whereas higher sucrose levels (4.5, 6 and 9% w/v) were found to impair growth and inhibit flowering. This partially supports the results in Experiment 5 (Chapter 7) where the 3% w/v sucrose level promoted flowering and level at 6% w/v inhibited flowering of *S. formosa*. The 4.5% w/v sucrose level, however, did promote flowering in *S. formosa*. All these results indicate that, for *in vitro* flowering to be maximised, sucrose is required at an optimal concentration which is different for different species.

Apart from its metabolic role, sugar represents the major osmotic component of most media. Hydrolysis of one sucrose molecule into two hexose molecules (glucose and fructose) can substantially decrease the osmotic potential of the cell and increase the driving forces for water uptake (Lambrechts and Kolloffel, 1993). High concentrations of sugar might decrease osmotic potential and cause osmotic stress (Do and Cormier, 1990), although the role of osmotic pressure on the metabolites affected by high sugar concentration is not clear. Moreover, sugar concentrations may affect gene expression that induces flowering (Rolland *et al.*, 2006).

The positive results for some treatments confirm that sugar can be taken up by explants. However, the sugar content in the culture media at the end of the experiment ideally should be measured, so that the quantity and the type of sugar taken up by plants can be determined. Changes in endogenous soluble sugar levels in the plantlets at the end of the experiment should also be measured, and the interaction between sugars and other components of the media should be considered. There is a need for further work to establish a reliable method to investigate sugar uptake, sugar metabolism and the

interaction between various components in the media to determine the factors that affect *in vitro* flowering.

Plantlets coming from low light grown stock plants did not flower. That is possibly due to other factors, such as level of endogenous hormones and other cofactors (differences in carbohydrate transport and accumulation within tissues, and plant metabolism), which may play a role in the conversion of vegetative buds to flowering buds. Bernier (1988) suggested that flowering can only occur when the limiting factors are present at the apex in the appropriate concentrations and at the right times.

In vitro flowering can be induced by supplementing plant growth regulators in the culture medium. In *Pisum sativum*, it is essential to add NAA or IBA to the medium for flower induction (Franklin *et al.*, 2000). By enriching the culture medium with BA, Goh and Yu (2000) succeeded in inducing *in vitro* flowering of *Dendrobium* hybrids. The use of plant growth regulators in the culture medium may substitute for the need for high light intensity conditions for flowering of *S. formosa* and this should be considered in future research.

Exogenously applied sugars were unable to induce flowering in plants grown under low light intensity in both *in vivo* and *in vitro* conditions. It appears that the transition to flowering of *S. formosa* depends primarily on the environmental conditions under which the explant source was grown (e.g. light intensity) and the result of an inter-play of biochemical factors including assimilates (e.g. carbohydrates, C:N ratio and protein) and phytohormones, which are likely to participate in floral induction. To determine if the treatments used in the *in vivo* experiments themselves prevented flowering, the experiments should be repeated with plants grown under high light conditions (positive control).

7.5 Conclusion

Results from this chapter and previous chapters confirm that *S. formosa* requires adequate light intensity for flower induction and development. Exogenous applications of sugars were not effective in inducing flowering. All treatments failed to induce the transition to flowering in *S. formosa* plants grown under low light conditions. The failure to flower depends on many factors, such as the use of ineffective concentrations

of sugars along with inappropriate application methods. The Spraying method can cause disease and infection, while the injecting method caused physiological and physical damage to the plants. In all *in vivo* treatments, the applied sugars might have not been taken up by the plants or were taken up at insufficient concentrations to induce flowering in *S. formosa*. The success of i*n vitro* flowering of *S. formosa* is the result of an inter-play of factors, including environmental conditions under which the explant source was grown, and types and concentrations of exogenous sugars used in the culture medium.

Chapter 8 Comparison of Protein Expression in the Shoot Apices at the Vegetative and Flowering Stages in *Swainsona formosa*

8.1 Introduction

The transition of a shoot meristem from the vegetative to the flowering stage requires the expression of new genetic information (Suarez-Lopez, 2005), with some genes that are inactive in the earlier vegetative period being expressed during flowering. The expression of these genes should be accompanied by the occurrence of proteins specific to flowering and not found in vegetative plants (Buchanan *et al.*, 2000). Flowering induction and flower development are highly complex processes that require the coordinated expression of numerous genes throughout the different stages of development (Bernier and Perilleux, 2005). When protein changes occur during development both, the morphogenesis and the biochemistry will reflect the extent to which gene action is expressed. Environmental factors, such as light irradiance and temperature, affect also the pattern of gene expression (Stevens *et al.*, 2000).

Plant development is accompanied by a differential modification of proteins reflecting the developmental stage and also a pattern of molecular differentiation (Thompson-Coffe *et al.*, 1992). Changes in the patterns of proteins associated with floral developmental stages have been obtained in many species (see Chapter 2 section 2.7.1.3). Studies by Steward *et al.* (1971) on floral induction of *Tulipa* bulbs revealed that certain basic proteins (histones rich in lysine) and several esterases developed in the shoot apex prior to the development of floral organs. Iwahori *et al.* (1990) found changes in protein content and protein electrophoretic patterns (particularly those of ribulose bisphosphate carboxylase/oxygenase) in the buds of satsuma mandarin cv. Owari trees responding to girdling (girdling accelerates flower initiation). Furthermore, Cremer *et al.* (1992) found qualitative and quantitative changes in protein composition in *Sinapis alba* shoot meristems during floral transition induced by photoperiod.

One-dimensional gel electrophoresis is a relatively simple technique that can provide basic qualitative information about changes in the patterns of proteins associated with

plant developmental stages. However, the main limitations are that the process is unable to detect very small and low levels proteins, and it is unable to further separate complex mixtures of proteins into smaller components (Wong *et al.*, 1994).

One-dimensional gel electrophoresis was used to determine changes in proteins in the shoot apices of *S. formosa* during shoot growth and development. Such information will be an aid in better understanding the flowering process and has the potential for specific proteins to be used as molecular markers in the identification of developmental stages. The objective of the present study was to investigate protein concentrations and patterns in the shoot apices collected from different developmental stages around the time of floral transition, using plants grown under high and low light intensity conditions.

8.2 Materials and methods

8.2.1 Plant material and growth conditions

Seeds were germinated as described in Chapter 3. After transplanting to plastic pots, the plants were raised in growth cabinets at either 800 ± 50 μmol $m^{-2}s^{-1}$ (high light) or 150 ± 10 μmol $m^{-2}s^{-1}$ (low light). The growth cabinets was set with 16-hour photoperiod. Day/night temperatures were set at 30 °C (day) and 18 °C (night). Samples were taken at different developmental stages (see Chapter 4) to represent vegetative, floral transition, initiation and differentiation, and flower (flower buds can been seen with naked eye) stages, as indicated in Table 8.1. As Yusuf *et al.* (2002) found that at low light intensity the apices remained vegetative until Day 121 after seed germination, the plants grown under low light intensity were only sampled at Day 60. This helped to avoid high numbers of analyses and also the limitation of the space in the growth cabinets. The plants grown under the high light intensity treatment were sampled at Days 30, 35, 40, 45, 50, 55 and 60. For both light intensity treatments and all sampling times the samples were taken from the two axillary branches.

Table 8.1 Sampling development stages (Days after seed germination) for protein profiling of the shoot apices of *Swainsona formosa* grown under high light intensity and low light intensity.

Light intensity	Days after seed germination	Apex developmental stages
High light (800 μmol $m^{-2}s^{-1}$)	30 – 35	Vegetative
	40 – 45	Floral transition
	45 – 55	Floral initiation and differentiation
	60	Flower
Low light (150 μmol $m^{-2}s^{-1}$)	60	Vegetative

8.2.2 Protein extraction

The experiment was conducted using facilities in The School of Biological. Biomedical and Molecular Sciences, UNE. Five hundred micrograms of shoot apices (1 cm in length) were sampled at different dates and immediately stored at -80 °C until used. The shoot apices were ground to a fine powder in a liquid nitrogen-cooled mortar. The ground samples were homogenised with 1 mL of sodium dodecylsulphate (SDS) sample buffer (0.5 M Tris-HCl pH 6.8, glycerol, 10% SDS, 2-β mercaptoethanol, 0.05% bromophenol blue and deionised distilled water). Homogenates were shaken for a minute and then boiled at 95 °C for 5 minutes. The homogenates were centrifuged for 12 minutes at 12000 g. The supernatant containing the proteins was recovered and centrifuged again at 12000 g for 5 minutes. The resulting supernatants were stored at -20 °C until used.

8.2.3 Protein quantification

Protein concentrations were determined based on precipitation of protein using a 2-D Quant kit from Amersham Biosciences (Castle Hill, NSW, Australia). Bovine serum albumin (BSA) was used as the standard protein. The absorbance of the solution was measured with a 480 nm UV probe (Shimadzu, Japan) against a protein reagent/deionised distilled water blank. The protein concentrations were calculated employing a BSA standard curve, which was constructed with concentrations ranging from 0 – 40 μg mL^{-1} (Coumans-Moens, J., 2006 pers comm., 19/06/2006). Each sample was replicated three times.

8.2.4 One-dimensional gel electrophoresis

One-dimensional protein electrophoresis was carried out in 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) with modification. Samples were centrifuged at 12000 g for 5 minutes in a microcentrifuge. The amount of extracted protein used for the protein gel electrophoresis varied for each sample; each sample was to represent an equivalent amount of 100 µg total extractable protein. Molecular-weight standards (Precision Plus Protein™ Standards Kaleidoscope, Bio-Rad) were included as markers.

The following materials were used for preparing gels :

Stacking gel : 0.125 M Tris-HCl pH 6.8, 0.1% SDS T = 4%, C = 3.33%.

Separating gel : 0.375 M Tris-HCl pH 8.8, 0.1% SDS T = 12%, C = 3.33%.

Electrophoresis was performed at 12 °C and 8 mA per gel using the continuous buffer system (Hoefer Scientific Instruments, 1994). The running buffer contained 3.03 g Tris base, 14.4 g glycine and 1 g SDS per litre. The duration of electrophoresis was around 18 hours (until the tracking dye had reached the bottom of the gel).

8.2.5 Protein staining

After electrophoresis, gels were stained according to the Candiano *et al.* (2004) method with 0.1% w/v Coomassie® Brilliant Blue G-250 for a day, followed by destaining several times in deionised distilled water.

8.2.6 Gel analysis

Gels were recorded with a charge coupled device (CCD) camera (Infinity). The proteins were identified according to their molecular weights, by comparing their migration with those of standards of known molecular weight.

Qualitative analyses of the gels were performed by a band to band comparison. Bands were scored on a presence/absence basis. The appearance and/or disappearance of protein bands were considered to be qualitative changes, whereas, the changes in the intensities of protein bands were considered quantitative. The protein bands were analysed using the BIO-PROFIL Bio-1D Version 11.9 (Viber-Lourmat, France).

8.3 Results

Under high light conditions, floral induction proceeded normally, while under the low light conditions the apices remained vegetative throughout the experimental period of 60 days (confirmed by inspection under a stereomicroscope). The protein concentrations in the *S. formosa* shoot apices changed during development (Figure 8.1). Under high light conditions, a slow decrease in the concentrations of proteins was observed up to Day 40, with a large increase at Day 45 coinciding with the floral transition stage (see Chapter 4, Figure 4.2). After Day 45, the protein concentrations remained almost constant, decreasing slightly up to Day 55, but increasing again at Day 60. The highest protein concentrations were recorded at Day 60 in plants grown under high light intensity conditions.

Comparing shoot apices of similar age (60 days), plants grown under the high light conditions had significantly higher protein concentrations than plants grown under the low light conditions ($P<0.001$). Protein concentrations at Day 60 for plants grown under the low light intensity conditions were similar to the vegetative plants grown under high light intensity (Days 30 – 40).

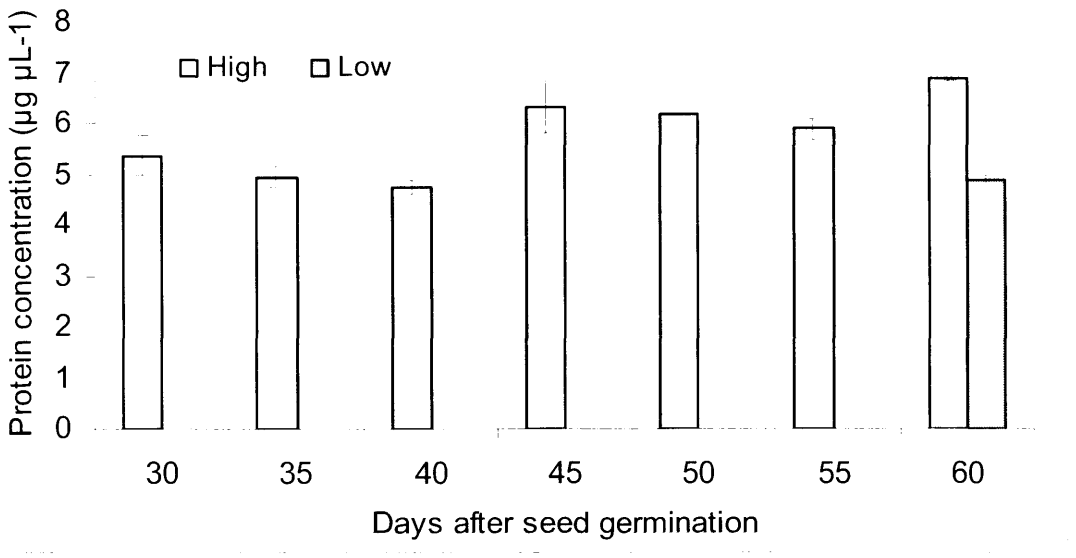

Figure 8.1 The changes in protein concentrations in *Swinsona formosa* shoot apices for different sampling days (Days after seed germination). Vertical bars represent standard error (n = 3). If no bar is visible, standard error is smaller than symbol size. For the treatments, High = high light intensity and Low = low light intensity.

One-dimensional gel electrophoresis of the high light shoot apices, extracted at different developmental stages, showed a number of protein bands common to all stages (Figure 8.2). At Day 30 and Day 35 (vegetative stage) only faint bands were visible but the banding patterns were similar to each other.

The protein patterns of the shoot apices aged between 40 and 55 days (corresponding to floral transition, initiation and differentiation stages) of the high light intensity conditions and 60 days under low light intensity were common to all of the extracts examined during these sampling times, but varied in band intensity between sampling times. Changes in band intensity reflect changes in the concentrations of these proteins per unit weight of the extracted tissue. A unique band at approximately 89 kDa was found in plants grown under high light conditions at Day 45 and plants grown under low light conditions at Day 60 (Figure 8.3). The 51 kDa band was much more intense in plants grown under low light intensity than plants grown under high light intensity (Figure 8.2). A protein band at approximately 66 kDa showed a marked increase in band intensity and a major band at 34 kDa was only found in the flower (60 days). The 79 kDa band was present in the vegetative apices and disappeared from the apices after the flowers could be seen with the naked eye (Figure 8.3).

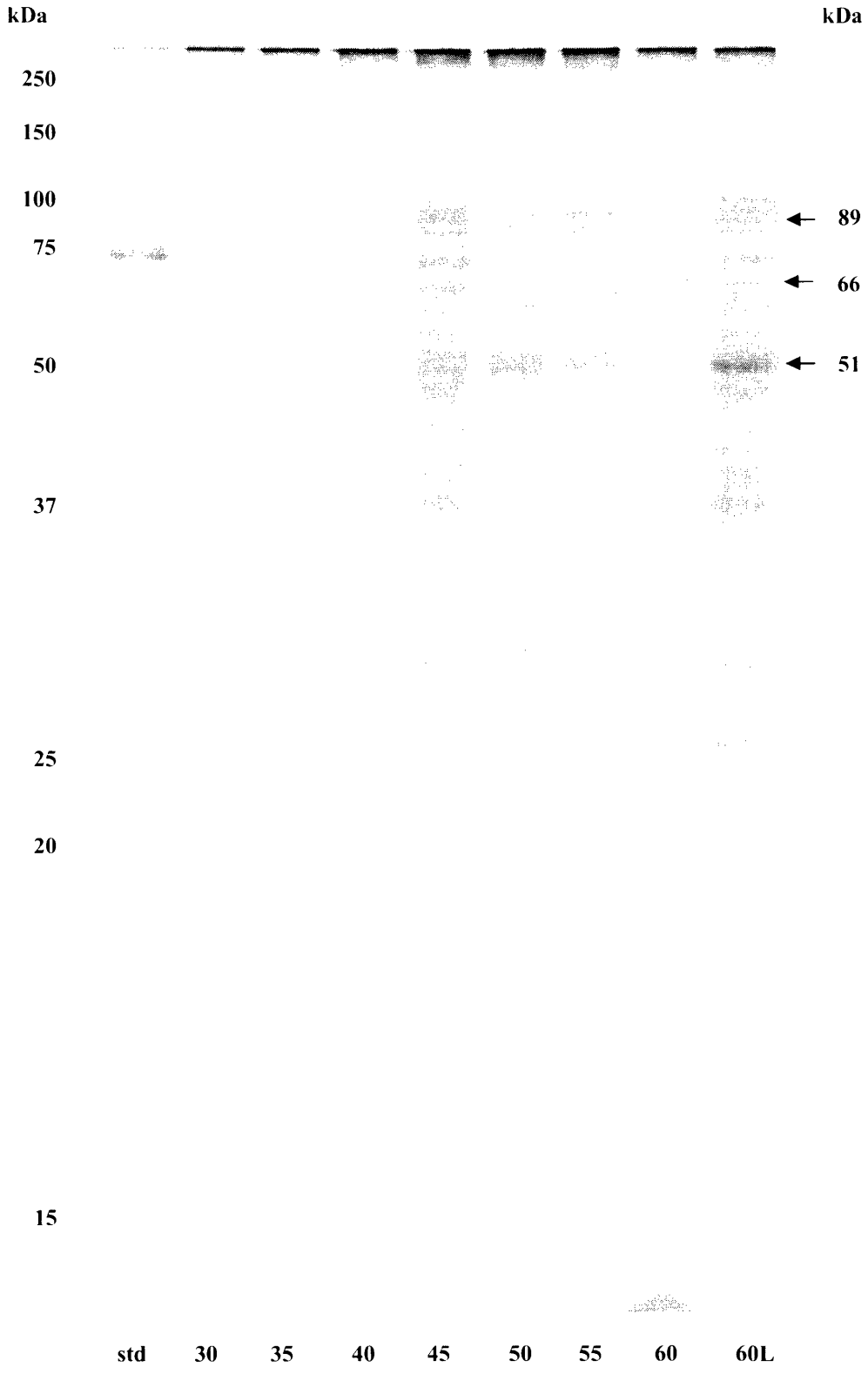

Figure 8.2 The electrophoretic separation on acrylamide gels of the soluble proteins of *Swainsona formosa* shoot apices: Changes with time in the protein patterns. std = molecular weight of marker given in kiloDaltons (kDa). 60L = apices grown in low light intensity for 60 days. Gel lanes were loaded with equal amounts of protein (100 μg).

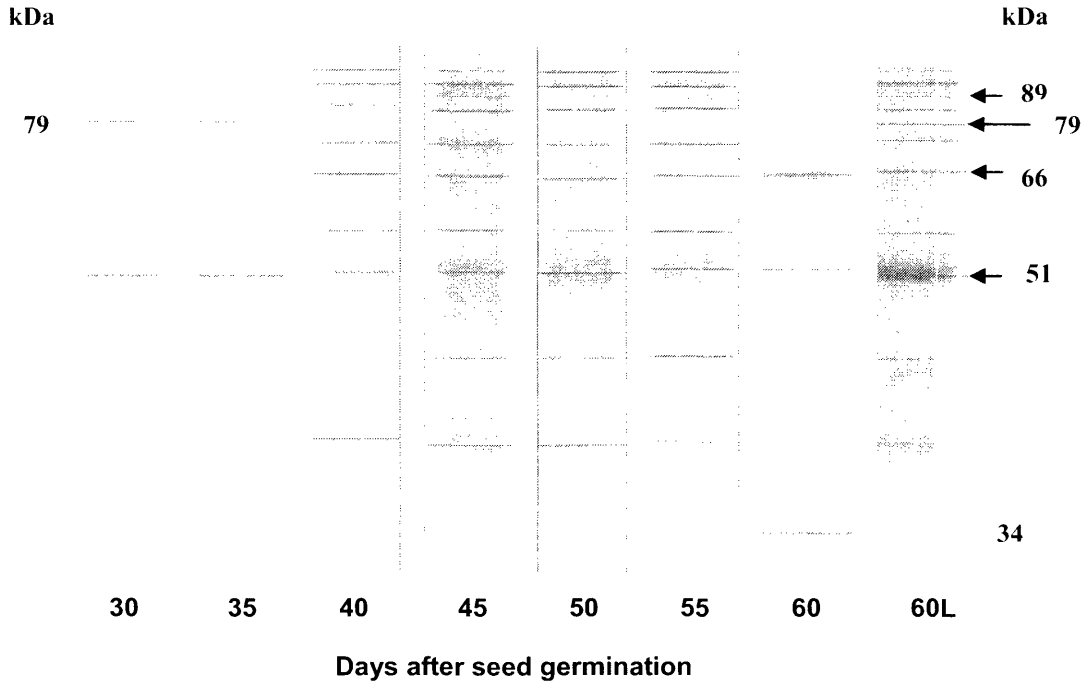

Figure 8.3 The presence and/or absence of the 34, 51, 66, 79 and 89 kDa bands over time in *Swainsona formosa* shoot apices. 60L = apices grown in low light intensity for 60 days. Gel lanes were loaded with equal amounts of protein (100 μg).

8.4 Discussion

The investigation of protein concentrations and the electrophoretic patterns in shoot apices of *S. formosa* revealed a close relationship between the proteins and the developmental stages. An increase in protein concentrations and electrophoretic bands coincided with the transition from the vegetative stage to the early reproductive stage demonstrated in Chapter 4. The protein concentrations of plants grown under high light intensity increased significantly at the transition stage, whereas the protein concentrations of the plants grown under low light intensity at 60 days were similar to the vegetative plants grown under the high light intensity conditions. These observations indicated that both an increase in protein concentrations and a change of proteins present constitutes one of the early biochemical processes in the floral development of *S. formosa*.

8.4.1 Protein concentrations and composition: differences in vegetative and reproductive apices

Protein concentrations were higher in the floral apices than in the vegetative apices. This result is in accordance with the results obtained for the evoked meristems of *Sinapis alba* (Pierard *et al.*, 1980) and *Silene coeli-rosa* (Taylor *et al.*, 1990). In *Chrysanthemum segetum*, a greater number of polypeptides were recorded in the prefloral apex than in the vegetative shoot apex (Rembur and Nougarede, 1989). Furthermore, Anderson and Beardall (1991) noted that vegetative plants contain low levels of protein due to the high amounts of structural polysaccharides.

The differences between vegetative and reproductive apices made it possible to identify protein markers related to particular morphogenetic phases. Analysis of the bands indicated that the 79 kDa band was distinctive in that it could be detected only in the vegetative stage. This may reflect the physiological stage and may be related to specific physiological functions in the shoot apices. Pierard *et al.* (1977) also indicated that one antigenic protein was present in the vegetative buds of *S. alba* and disappeared from the buds of induced plants after the start of the inductive treatment. Therefore, the 79 kDa protein can be considered as a marker of the vegetative stage of *S. formosa*.

An increase in light intensity may induce the synthesis of some new proteins that are not involved directly in the flowering process. It is also noteworthy that the band intensity of the 51 kDa protein was much greater in plants grown under low light than plants grown under high light. Degradation of the 51 kDa protein in the shoot apices under high light conditions could be one of the reasons for this observation. It seems that as long as the 51 kDa protein is there the plant remains vegetative.

Plants grown under low light intensity may have insufficient amounts of proteins to facilitate the conversion of the vegetative apices to floral apices. Moreover, changes in light intensity alter the protein composition in the shoot apices of *S. formosa*. When exposed to high or low light intensity during growth, plants react with a variety of adaptations to meet appropriate growth and maintenance. For example, the soluble proteins of Norway spruce (*Picea abies*) was markedly lower in shade needles, possibly due to the lower synthesis of RuBPC (ribulose-1,5-bisphosphate carboxylase), the major soluble protein of assimilation tissue (Bertamini *et al.*, 2006). The carboxylating

enzyme RuBPC is not fully activated at low irradiance and thus the degree of activation may regulate the flux of carbon through the photosynthetic pathway (Usuda *et al.*, 1985). In a similar report, the leaves of *Cenchrus ciliaris* plants grown under shade had a relatively low content of soluble proteins (Baig *et al.*, 2005). Peroxidase activity in *C. ciliaris* increased with a decrease in irradiance and its isozyme patterns were different among treatments. Some changes in peroxidase activity and isozyme patterns have been shown to take place during the transition of *Crocus sativus* to flowering (Ebrahimzadeh and Abrishamchi, 2001). In contrast to the above, the crude protein content of *Codonopsis lanceolata* was higher under shade conditions (55% shade) (Lee *et al.*, 1996). Vyas *et al.* (1996) reported that 25% as well as 50 % shading increased the plant water status, transpiration rate, and chlorophyll, protein, and amino acid contents in cluster bean (*Cyamopsis tetragonoloba*).

8.4.2 Protein concentrations and composition: variation in reproductive apices over time

Changes in protein expression by plants grown under high light intensity were also dependent on plant age. In this experiment, the protein patterns changed before the first signs of visible morphogenetic changes (see Chapter 4, Figure 4.1). The results agree with the finding of Barber and Steward (1968) for tulip (*Tulipa* sp.) and Pierard *et al.* (1980) for *S. alba*. Different groups of cells in the apex are differentially sensitive to proteins, and raising or lowering the concentrations of proteins might affect the relative rates of division in the different groups of cells so as to alter morphogenetic patterns (Applewhite *et al.*, 2000).

In *S. formosa*, the appearance of the 89 kDa protein occurred at both Day 45 high light and Day 60 low light, indicating that it has no causal significance in floral induction, since it occurred in both high light plants before flowering and in low light plants which do not flower. The extract from 60-day-old floral apices had the highest protein concentrations and showed a marked increase in band intensity at the 66 kDa band. A 34 kDa protein, which may be needed for the development of advanced floral structures, can only be detected at flower stage. These results suggest that different proteins are characteristic not only of different stages of development, but also of different organs (e.g. shoot apex, flower). All plant parts differ in their protein complements (Libal-Weksler *et al.*, 1997; Kehr *et al.*, 1999). In the tulip, the protein complements of sepals,

petals, anthers and pistils are different from each other and from those of the leaves (Barber and Steward, 1968). In addition, plant organ development is accompanied by a differential modification of proteins that reflects a pattern of molecular differentiation (Thompson-Coffe *et al.*, 1992).

The qualitative changes at early transition stage (Day 40) indicated that distinct groups of proteins are involved in different steps of the transition to flowering. It is coincident with findings in Chapters 5 and 6 that changes in carbohydrates and C:N ratios occurred at the early transition stage. Many interactions may possibly be involved between C, N and protein metabolisms, as C skeletons are essential for the incorporation of inorganic N into biologically relevant molecules, such as amino acids, proteins, nucleic acids and a large number of secondary metabolites (Krapp *et al.*, 2005).

This study was specifically limited to one-dimensional gels to gain a preliminary view of gross protein changes during meristem development under high and low light intensity conditions. It is suggested that two-dimensional gels will provide more information on possible changes in gene expression (Abrishamchi *et al.*, 2004). Future technical improvements in protein separation, detection, and identification will further help to enhance the resolution of protein analysis. In addition, future work should consider more sampling days to investigate more fully the effect of both age and developmental stage on protein expression. The identification and assessment of protein modifications, which finely tune the cellular function of each protein, will further help to interpret developmental processes (Hochholdinger *et al.*, 2006).

8.5 Conclusion

It may be concluded that the morphological transition of *S. formosa* plants to flowering is correlated with protein concentrations and patterns. High light intensity conditions caused changes in protein concentrations and types, and the changes facilitated flower initiation and differentiation. These protein changes were absent from the vegetative apices and other apices grown under low light intensity conditions. The timing of the changes coincided with those observed in morphology (Chapter 4), carbohydrates (Chapter 5), and C:N ratios (Chapter 6). Further studies of protein morphogenetic markers, as well as their genes and conditions for expression, are promising approaches to understanding the regulation of morphogenetic processes.

Chapter 9 General Discussion and Future Directions

9.1 Introduction

Sturt's desert pea (*Swainsona formosa*) is an Australia native legume, which has potential as an ornamental pot plant and is also suitable for hanging baskets and as a cut flower. The successful use of *S. formosa* as a commercial crop depends on increasing our knowledge of the regulation of flowering, which may, in turn, provide commercial growers with practical strategies to control flowering in this uniquely spectacular plant. The purpose of this research was to investigate the changes in morphology, sugar levels, C:N ratio and protein expression in the shoot apices of *S. formosa* plants during the transition period from the vegetative to the floral stage, to better understand the mechanisms of flowering in this plant.

This thesis has presented both a comprehensive literature review and several experiments focusing on the changes in the morphology and physiology associated with the early floral development of *S. formosa*. This chapter aims to integrate the work of this thesis and assess the degree to which the research objectives presented in Chapter 1 have been fulfilled. Some directions for future research are also suggested.

9.2 Summary and consolidation of the literature review

The first part of the literature review considers the commercial importance of, and provides general information on *Swainsona formosa*, the plant examined in the present study. A knowledge of the growth stages of floricultural crops is critically important in making management decisions, but the information on the growth stages of *S. formosa* is insufficient to make these decisions. The stereomicroscopy and scanning electron microscopy work presented in Chapter 4 helps to resolve questions about the correct flowering time and stages of floral development of *S. formosa*. This is the first complete description of the floral ontogeny in a member of tribe Galegeae in papilionoid legumes.

The second part of the literature review, presents a general framework to understanding the process of flowering. It covers a range of research areas in plant physiology, for

both *in vivo* and *in vitro* conditions. Flowering processes are complex and encompasses a variety of anatomical, morphological, physiological, and biochemical processes.

The flowering process involves major changes in the pattern of morphogenesis and cell differentiation at the shoot apical meristem, ultimately leading to the production of the floral organs. Flower development occurs in a temporal sequence and is regulated by various genes. Floral induction mechanisms determine flowering time and precede floral evocation, which consists of the events occurring in the shoot apical meristem that commit it to form flowers. The developmental signals that bring about floral evocation include: internal factors, such as plant maturity, nutrition status, hormones and other compounds, and external (environmental) factors, such as light, temperature and stress conditions. The interactions of these internal and external factors enable plants to synchronise their reproductive development with the environment.

Multiple lines of evidence indicate that flowering is regulated by the availability of biochemical assimilates, such as nutrients (e.g. carbohydrate, carbon and nitrogen), proteins and plant growth regulators (Gibson, 2004). Generalisations about flowering, however, are limited. The research findings on the roles and effects of these assimilates on flowering are not consistent. This is because the responses of most plants are species specific creating a large number of exceptions to each rule.

9.3 Floral ontogeny of *Swainsona formosa*

Under the growing conditions described in Chapter 4, the conversion of the apices of *S. formosa* from the vegetative to the floral stage started to occur 40 – 46 days after seed germination for axillary branches and 46 – 52 days for central stems.

Scanning electron micrographs of shoot apices dissected during the transition, define eight morphologically distinct stages of shoot apex development. The flower ontogeny of *S. formosa* conforms with the common model of flower development in legumes: the order of floral organ initiation within each whorl is unidirectional, except for the petal whorl, which is simultaneous; and the flower is organised into five whorls and shows a pentamerous arrangement of sepals and petals, ten stamens in two whorls and a central carpel (Tapingkae *et al.*, in press). It is expected that this study will provide a

morphological framework for the interpretation of future molecular, physiological and developmental studies in the flowering of *S. formosa*.

9.4 Changes in sugar levels, Carbon:Nitrogen ratio and protein expression in the shoot apices during the transition period from the vegetative to the floral stage

One of the impediments to the commercialisation of *S. formosa* as a pot plant is its inability to produce flower under low light conditions. This thesis investigated changes in sugar content (Chapter 5), C:N ratio (Chapter 6) and protein expression (Chapter 8) during flower initiation and development of *S. formosa*. These changes were closely correlated with morphogenetic events during early flower development, indicating that these changes are an integral part of the flowering process in *S. formosa*.

It is difficult to distinguish between signalling and metabolic effects in plants (Francis and Halford, 2006). In this study, there are two possible scenarios for the transition to flowering. First, an external change of environment (light intensity) is sensed by the plant, generating a remote signal leading to gene expression which in turn drives metabolic flux to induce flowering. Second, the change in environment directly affects biochemical flux (e.g. sugars, proteins), which may lead to altered gene expression, which drives flux to induce flowering.

Coordination between sugar and other nutrient metabolic pathways is essential to optimise the use of energy resources. For example, a number of genes involved in nitrogen-assimilation are co-regulated by sugars, and nitrogen availability extensively regulates carbon-metabolic-gene expression (Rolland *et al.*, 2006). Signalling networks that control flowering may need an adequate balance of assimilates for switching from the vegetative to the reproductive phase. The mechanisms that govern this metabolic balancing act involve feedback of information on metabolite concentrations and a responsive alteration of enzyme activity (Finkemeier and Sweetlove, 2006).

The results from this thesis confirm that phase change in *S. formosa* is governed by light intensity, and by the genetic and biochemical competence of the shoot apex to respond to that factor. Reproductive development in plants is influenced by the availability of

substrates that are used in the shoot meristems as they develop. The changes in sugar content, C:N ratio and protein expression in the shoot apices indicates a higher demand by apical meristematic tissues (sink). The timing of changes in sucrose quantity (Chapter 5) matched the timing of morphological changes (Chapter 4), indicating a key role for sucrose in flower development. The changes in the sugar composition of the shoot apices are associated with quantitative changes of sugar translocation and changes of enzyme activities related to floral transition. The balance between glucose, fructose and sucrose are important for flowering to occur (Chapters 5 and 7). Adequate glucose supplies are required for floral initiation and development in *S. formosa*. One possible mechanism through which glucose can be sensed is via its interaction with hexokinase (glucose sensors) (Rolland *et al.*, 2006). However, the role of sugars in day-neutral plants is unclear, since in these species flowering results from endogenous and autonomous signals which are genetically controlled (McDaniel, 1996).

Reduced %N and %C at the early transition stage, and an increased C:N ratio in the shoot apices, could be used as indicators for stage changes. It can be suggested that the time at which these assimilates arrive at the apex is just as important as the quantity that is received. To flower, plants need to maintain an appropriate C:N ratio. The mode of N acquisition (fixation versus assimilation) seems to influence %N accumulation in the shoot apices of low light grown *S. formosa* plants.

The protein concentrations and the electrophoretic patterns in the shoot apices of *S. formosa* (Chapter 8) revealed a close relationship with the floral developmental stages (Chapter 4). Changes in the protein patterns over time also matched important changes in sucrose concentrations (Chapter 5). The protein concentrations of plants grown under high light intensity increased significantly at the transition stage, whereas plants grown under low light intensity at 60 days was similar to vegetative plants grown under high light intensity conditions (e.g. Day 30 and Day 35). These observations indicate that both an increase in protein concentrations and a change of proteins present constitutes one of the early biochemical processes in the floral development of *S. formosa*.

9.5 Effect of exogenous sugar applications on flowering

Results obtained from experiments in Chapter 7 showed that exogenously applied sugars (spraying, brushing, absorbent cotton on apex, injecting) failed to induce

flowering of *S. formosa* plants grown under low light conditions. Although failure to flower depends on many factors, the likely cause in these trials may be the use of ineffective application methods.

In vitro flowering can be achieved within 4 to 6 weeks by placing shoot apices from high light grown stock plants on Murashige and Skoog medium supplemented with 3.0 to 4.5% sucrose. *In vitro* flowering of *S. formosa* not only depends on environmental conditions under which the stock plants were grown but also the carbon source and concentration of sucrose in the culture medium. This study using plant tissue culture techniques supports the role of carbohydrates in providing the energy and carbon skeletons needed for flower initiation. Sucrose was effective in *in vitro* flowering because it was available in its own right and was also able to be hydrolysed into glucose and fructose, both needed for floral development (Chapter 5).

The results of this work provide the basic information on several factors required for flowering in *S. formosa*. Although refinements of these findings are required for commercial application, the results from this research may underpin future commercial protocol development for this species. Furthermore, for the first time successful production of *in vitro* flowering in *S. formosa* has been reported.

9.6 Directions for future research

Apart from improving our understanding of the flowering mechanism in *S. formosa*, the knowledge gained through this research could be used to manipulate and control flowering in the future. Studies in Chapter 4 have linked the time to flowering of *S. formosa* to photothermal time. The time of flowering, therefore, could possibly be predicted by generating a model to simulate the time of floral initiation. This model will be of value for growers to indicate how to change the environment to advance or delay the rate of growth in accordance with market demand and to predict optimal environmental regimes for plant growth, while minimising fuel consumption.

The major challenges now facing plant biochemists are to understand how changes in nutrient availability and environment are sensed and signalled, and to understand how different signalling pathways are integrated and how multiple signals are interpreted. Factors controlling key metabolic processes, such as photosynthesis, starch synthesis

and enzymatic activities, should be investigated at precise developmental stages in response to external factors, to determine the efficiency of a sugar-metabolic pathway. Despite the findings in this thesis, many questions concerning the interaction of C and N metabolisms, and especially the regulation of this interaction, are still open. To enhance the ability of protein analysis to separate complex mixtures of proteins into further components, examining other protein analysis methods will help achieve further understanding of the developmental processes. In addition, future work should consider more sampling days to further investigate the effects of both age and developmental stage on protein expression and the C:N ratio. Further repeat experiments to validate the conclusions by using a full set of controls should also be considered.

In vivo application of sugars to induce flowering is not practical and should not be pursued. *In vitro* system should be adapted to study carbohydrate, as well as a preliminary study *in vitro* of growth regulators' involvement in flowering. A commercial protocol is needed for the *in vitro* method, so that further work to improve the quality of the flowering plant can be undertaken.

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Appendix 1

Culture media

MS Stock Solutions

Chemical	concentration ($g\ L^{-1}$ stock)
MS Nitrate Stock	
Ammonium nitrate, NH_4NO_3	165.0
Potassium nitrate KNO_3	190.0
Sulfate Stock	
Magnesium sulphate, $MgSO_4.7H_2O$	37.0
Manganous sulphate, $MnSo_4.H_2O$	1.69
Zinc sulphate, $ZnSO_4.7H_2O$	0.86
Cupric sulphate, $CuSO_4.5H_2O$	0.0025
Halide Stock	
Calcium chloride, $CaCl_2.2H_2O$	44.0
Potassium iodide, KI	0.083
Cobalt chloride, $CoCl_2.6H_2O$	0.0025
PBMo Stock	
Potassium phosphate, KH_2PO_4	17.0
Boric acid, H_3BO_3	0.62
Sodium molybdate, $Na_2MoO_4.2H_2O$	0.025
NaFeEDTA Stock	
Ferrous sulphate, $FeSo_4.7H_2O$	2.784
Ethylenediaminetetraacetic acid, disodium salt, Na_2EDTA	3.724
Vitamin Stock	
Glycine	0.20
Nicotinic acid	0.05
Pyridoxine HCl	0.05
Thiamine	0.01
Myo-Inositol	10

All solutions were made up at 100x in 1 L of deionised water.
These solutions were stored in the refrigerator at 5°C. The same batch of stock solutions was used through for all MS basal media preparations.