Genetic diversity and flowering in *Clianthus* and New Zealand *Sophora* (Fabaceae)

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Abstract

Clianthus and New Zealand *Sophora* species are woody legumes endemic to New Zealand, with high ornamental value and biodiversity significance. Research was conducted to address the fact that little is known about the details of their developmental characteristics, genetic structure and relatedness of the wild populations, and their molecular mechanism of flowering.

Genetic diversity and relatedness of all remaining wild populations of *Clianthus* and samples of all New Zealand *Sophora* species were investigated using ISSR and AFLP markers. Genetic relationships were established for *Sophora* species, *Clianthus* wild populations and cultivars, and most individuals in each of the wild *Clianthus* populations. The molecular evidence did not support the recent separation on morphological grounds of the two *Clianthus* species, *C. maximus* and *C. puniceus.*

Postharvest treatments were tested to extend vase life of the short-lived cut *Clianthus maximus* and *Sophora tetraptera* flowers. Appropriately treated *Clianthus* cut flowers lasted 10-12 days in the vase, with over 80% of flowers opening. Similar postharvest treatments did not improve the vase performance of cut *Sophora* flowers.

Detailed calendars of vegetative and reproductive growth, and of floral ontogeny were developed for *Clianthus* and *Sophora.* Contrasting behaviours of both vegetative and reproductive growth were observed between these two legumes. A long period of summer-autumn dormancy of vegetative and reproductive growth in *Sophora*, and mass abortion of initiated *Clianthus* inflorescences during most of the year were observed. Unusual floral ontogeny processes, with precocious carpel initiation and delayed petal development, were observed in both species.

An efficient two-step quantitative real-time RT-PCR protocol for detailed gene expression analysis of large numbers of samples was developed using SYBR Green DNA dye and a LightCycler instrument. The consistency of this protocol was optimised with regards to sample and template preparation, primer design, and determination of appropriate internal controls for gene expression quantification. Differences of gene expression in the range of 5-7 orders were effectively detected.

Putative partial homologues of *LEAFY, APETALA1, PISTILLATA,* and *AGAMOUS* were isolated from both *Clianthus* and *Sophora.* Detailed temporal and spatial expression of each floral identity gene was investigated using quantitative real-time RT-PCR. The expression patterns, together with the sequence similarity, showed that these new isolated gene fragments were most probably *LEAFY, APETALA1, PISTILLATA,* and *AGAMOUS* homologues in *Clianthus* and *Sophora,* and that the ABC model of floral development is generally applicable to both species. However, there were important variations in temporal expression patterns compared to those of herbaceous species. A bimodal expression pattern of *LEAFY* and *APETALA1* homologues was observed in *Sophora*, but not in *Clianthus*, coincident with their contrasting patterns of floral initiation and development.

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Abbreviations

AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BLAST	basic local alignment search tool
c.	approximately
cDNA	complementary DNA
cm	centimeter
cpDNA	chloroplast DNA
DTT	dithiothreitol
g	gram
h	hour
ISSR	inter-simple sequence repeats
ITS	internal transcribed spacer
km	kilometer
l	litre
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger RNA
ng	nanogram
nmol	nanomole
nr DNA	nuclear DNA
P	probability
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
RT	reverse transcription
s	second
SE	standard error
STS	silver thiosulfate
v/v	volume/volume
w/v	weight/volume
µg	microgram
µl	microlitre
^{o}C	degrees Celsius

Chapter 1 Introduction

1.1 Overview

The research reported in this thesis focuses on two native woody legumes, *Clianthus* and *Sophora*, both of which were included in the Public Good Science Fund Native Ornamental Plants Programme. The research was carried out mainly at Massey University under subcontract to the New Zealand Institute for Crop and Food Research Limited.

There were a number of objectives of this research, which was undertaken in order to assist the development of the wholesale nursery and floriculture export industries. An increased understanding of genetic diversity within *Clianthus* and *Sophora* was required. It was also required that knowledge be gained of cut flower postharvest performance within these genera. In addition, the flowering process within these genera needed to be understood in terms of describing floral phenologies and the expression patterns of key genes associated with the flowering process.

1.1.1 Basic aspects and conservation status of *Clianthus*

Clianthus (kowhai ngutukaka or kakabeak) is a leguminous shrub (Fabaceae: Papilionoideae: Galegeae), endemic to New Zealand. With its showy flowers, it is also an outstanding horticultural subject. It was one of the first endemic plants to be grown in cultivation and is still widely cultivated as a common garden plant in New Zealand (DOC New Zealand, 1994). Taxonomically, it is most closely related to the genera *Swainsona*, *Montigena* and *Carmichaelia* (Heenan, 1998a, 1998c; Wagstaff et al., 1999).

Banks and Solander collected the first botanical specimens of *Clianthus* in 1769. Although originally named *Donia punicea*, the name *Donia* had already been used, and the new genus *Clianthus* was erected to accommodate *C. puniceus* (Heenan, 2000). In addition, a "southern form" of *Clianthus* occurring on the eastern coast of the North Island was described and named *C. maximus* by Colenso (1885). *Clianthus maximus* was later reduced to the variety *C. puniceus* var. *maximus* by Kirk (1899), a name it retained for the next 100 years. However, the two species *C. puniceus* (G. Don) Sol. ex Lindl. and *C. maximus* Colenso are once again recognised on the basis of vegetative and floral morphology by Heenan (2000).

Clianthus maximus is currently found almost exclusively within 50 km of the eastern coast of the North Island of New Zealand. The only recent occurrence of *C. puniceus* in the wild was reported to be on Moturemu Island in Kaipara Harbour, north of Auckland (Heenan, 2000). Seeds were collected from this site in 1996 at the time when the last parent *C. puniceus* plant died, and the resulting seedlings grown under supervision in well documented collections (S. Benham, pers. comm.).

Clianthus puniceus was a "mystery" to Colenso (1885), who described how he had never seen it growing in the wild except on 2-3 smaller islands off northern New Zealand. He believed the species had been "long and assiduously cultivated" by Māori (along with important food and fibre crops). The significance of the plant to Māori and the transport of its seed had also been observed by Taylor (1855).

Unfortunately, details of the cultivation and trade of this ornamental plant were never documented. The only recent occurrence of *C. puniceus* in the wild was that described above, on Moturemu Island in Kaipara Harbour, north of Auckland (Heenan, 2000). However, Cameron and Wright (1993) were unsure of the original provenance of this population, believing that it may have been a remnant of Māori introduction and cultivation.

In the most recent assessment of New Zealand threatened plants, *C. puniceus* is considered to be critically endangered (de Lange et al., 1999). *C. maximus* is considered to be a vulnerable species that requires some conservation priority as populations are becoming depleted and its security is not assured (de Lange et al., 1999). Cultivated populations of *Clianthus* are not under threat, but appear also to contain very limited genetic diversity (Shaw, 1993). Heenan (2000) suggested that the genetic diversity of *C. puniceus* could perhaps be enhanced by examing the gene pool of cultivated plants of unknown origin. Appropriate molecular (e.g., RAPD or AFLP) studies should be made of plants from Moturemu Island and cultivated plants of unknown origin to establish the amount of genetic diversity in *C. puniceus*.

Some population and morphological studies have been conducted for *Clianthus* (Heenan, 1995; Shaw and Burns, 1997; Heenan, 2000), but limited genetic information was revealed using biochemical and molecular markers. Preliminary investigations utilizing comparison of SDS-PAGE profiles of *Clianthus* seed proteins (Gardiner, 1991) indicated that genetically distinct populations existed in the wild. In addition, working with a small

data set, the RAPD technique had shown promise for distinguishing variation among *Clianthus* populations (Gardiner et al., 1993).

However, the genetic status at the species level involving all populations still remains unknown. None of these studies tracked the whole cycle of vegetative and reproductive development of these species. Neither had the detailed flowering process, from floral initiation to organ differentiation and development, been studied, nor was there any information regarding the molecular mechanisms involved in flowering of this genus.

1.1.2 Basic aspects of *Sophora*

Sophora (kowhai) (Fabaceae: Papilionoideae: Sophoreae) is one of the four major New Zealand native woody legume genera, together with *Clianthus*, *Montigena* and *Carmichaelia* of the tribe Galegeae. It is considered one of the New Zealand's most beautiful trees and was adopted as the national flower (Salmon, 1996). Until recently, it was thought that three *Sophora* species, *S. microphylla*, *S. tetraptera*, and *S. prostrata*, occurred in New Zealand (Allan, 1961; Yakovlev, 1967; Heenan, 1998a).

Sophora tetraptera is considered the earliest in ancestry of the New Zealand taxa, giving rise to the other two species (Hurr et al., 1999). It is also the most popular in cultivation with the highest horticultural value. *S. tetraptera* is the North Island kowhai, found originally growing wild along streamsides and lowland forest margins from East Cape to the Ruahine Mountains. The endemic *Sophora prostrata* occurs on the eastern side of the Main Divide, particularly around Banks Peninsula and the Canterbury foothills of the South Island. *Sophora microphylla* is the most varied and most widespread species. Several of its forms were recently recognized at the species level by Heenan et al. (2001) and named as *S. chathamica*, *S. godleyi*, *S. fulvida*, *S. longicarinata* and *S. molloyi*, based on morphological and growth habit characters.

All of the now eight *Sophora* species occurring in New Zealand are endemic, and belong to the section *Edwardsia*, which is one of the largest groups in *Sophora*, including about 19 species whose distribution is centred on islands in the southern Pacific Ocean (Peña et al., 2000; Heenan et al., 2001; Mitchell and Heenan, 2002).

Although some species of New Zealand *Sophora* are considered regionally endangered or vulnerable and might be potentially endangered in the near future (Shaw, 1993), most

species are not yet considered to be nationally threatened (Heenan et al., 2001). The most worrying aspect of the conservation of the New Zealand *Sophora* species has been considered to be not the individual species requirements but the serious damage being inflicted on the wild gene pools through planting for revegetation, horticultural and Māori amenity purposes (Godley, 1972). These mixed plantings increase the risk of hybridism with natural populations of the different *Sophora* species, and make it difficult to determine the natural distributions of the species (Heenan et al., 2001). In this respect, knowledge about the genetic status of the natural populations of *Sophora* species, as well as understanding their vegetative and reproductive characters and flowering process, would be beneficial to the conservation the species.

Indeed, the interspecific relationships have proven difficult to establish within the eight New Zealand *Sophora* species. Several studies were conducted to elucidate relationships among New Zealand *Sophora* using molecular data. Chloroplast DNA sequences (Hurr et al., 1999) and nuclear DNA sequences (Mitchell and Heenan, 2002) failed to discriminate differences at the species level. The previously considered "ancestor" of New Zealand *Sophora* species, *S. tetraptera*, was even placed in the "ingroup" relative to other species (Hurr et al., 1999). Currently, the phylogenetic relationships of the New Zealand *Sophora* species have yet to be resolved. Such resolution might occur if molecular markers such as AFLPs and ISSRs were to be used.

Furthermore, there are no studies tracking either the whole cycle of vegetative and reproductive development of these species, or the detailed flowering process, from floral initiation to organ differentiation and development. There is no information regarding the molecular mechanisms involved in flowering of this genus.

1.2 Molecular markers and their application in the determination of genetic diversity and genetic relatedness in higher plants

1.2.1 Genetic diversity and species conservation

One of the major factors responsible for the endangering and subsequent extinction of plants is the small numbers of populations and low numbers of individuals within populations (Gemmill et al., 1998). Although detailed demographic data are known to be very important in designing conservation management programme for the short-term survival of endangered taxa (Lande, 1988), poorly planned reintroduction programmes based on demographic data can sometimes introduce population bottlenecks, with further reduced effective population size compared to the founding population. Such populations may be misleading for conservation purposes and potentially increase the threat of extinction by inbreeding (Friar, et al., 2000).

For these reasons, genetic variation within a taxon is thought to be critical for the long-term survival and continued evolution of a population or species (Huenneke, 1991). Holsinger et al. (1999) stated that loss of genetic diversity threatens the ability of populations to respond adaptively to future environmental change, and it may even threaten the short-term persistence of populations if the expressed effects of inbreeding depression are sufficiently severe. Low genetic diversity has often been taken as evidence that a population has experienced a bottleneck or is vulnerable to future extinction (Bouzat et al., 1998; Spencer et al., 2000). Therefore, an understanding of genetic structure, especially genetic diversity, is very important for endangered species, by providing useful data for planning action, such as reinforcement of populations on the verge of extinction, re-introduction, or *ex situ* culture or seed collections (Given, 1994; Gaudeul et al., 2000).

An estimation of genetic diversity at the phenotypic level may not be reliable because of the influence of environment on gene expression, and requires relatively large-scale field experiments in the area studied (Henry, 1997; Pham and Hintum, 2000). Neutral genetic markers, such as allozyme and DNA markers, can avoid complicating environmental effects and provide an ideal tool for assessing genetic diversity (Bataillon et al., 1996; O'Hanlon et al., 1999). Allozyme markers have proven to be closely associated with various life history traits, and can therefore produce important information for

evolutionary and conservation biology (Hamrick and Godt, 1989, 1996). However, practical constraints and low levels of polymorphism have limited the application of allozyme markers, and have brought about an increasing interest in DNA-marker based methods (Nybom, 2004).

A number of different types of DNA markers, including restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites (SSR), and inter-simple sequence repeats (ISSR), have recently become available (Morran et al., 2000; Patzak, 2001; Marsh and Ayres, 2002; Uptmoor et al., 2003; Vijayan et al., 2004; Souframanien and Gopalakrishna, 2004), and have made it possible to estimate intra- and inter-specific genetic diversity from nuclear genomes of natural plant populations with relative ease (McRoberts et al., 1999; McBreen et al., 2003; Vijayan et al., 2004).

Although co-dominant markers such as RFLP and SSR provide higher levels of polymorphism than dominant markers, such as RAPD, AFLP, and ISSR (O'Hanlon et al., 1999; Huang and Sun, 2000; Patzak, 2003), they may still be less useful for assessing genetic relationships, because co-dominant markers require large quantities of pure DNA or pre-developed primers specific to the species in question (Paul et al., 1997; Lanham and Brennan, 1999; Garcia-Mas et al., 2000). These are often unavailable or limited for endangered species (Sun et al., 1999; McRoberts et al., 1999; Maguire et al., 2002; Turpeinen et al., 2003). Large numbers of PCR-based molecular markers, such as AFLP, ISSR, and RAPD can easily be generated in a short time, using very low amounts of DNA, and no previous knowledge of genomic information (Paul et al., 1997; Wolfe et al., 1998; Sun et al., 1999; Palacios et al., 1999; Garcia-Mas et al., 2000), making these the molecular markers of choice for many projects.

1.2.2 RAPD markers

Randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990) or arbitarily primed polymerase chain reaction (AP-PCR) (Welsh and McClelland, 1990) is a PCR based molecular marker technique with short oligonucleotide primers of arbitary (random) sequence (Gillies et al., 1999). RAPD markers are recognized as a powerful and very useful DNA-based method for initial assessment of genetic variation, especially the assessment of genetic diversity of plant species. The procedure only requires PCR

technology, which is faster, less labour intensive, and less technically demanding than other methods, such as RFLP (Sun et al., 1999; McRoberts et al., 1999; Souframanien and Gopalakrishna, 2004). RAPD data can be accumulated with nanogrammes of low quality DNA from the target organisms without the need for previous information of DNA sequences or marker development, which are often unavailable in wild and less genetically studied plants (Sun et al., 1999; Nybom, 2004).

RAPD markers can reveal higher polymorphic levels and identify higher genetic diversity than allozyme markers. Using RAPD markers, Esselman et al. (2000) could distinguish 100% and 60% of the individuals of two populations of *Dendroseris berteroana* and *D. pruinata*, respectively, in which no genetic diversity had been detected using allozyme data. In the study of an extremely rare species in Australia, *Zieria prostrata*, Hogbin and Peakall (1999) identified a relatively high level of genetic diversity within and among the few extant populations using RAPD markers, and provided crucial information for the conservation management of the species. Using the same technique, Lowe et al. (2000) detected significant genetic diversity between and within the two morphologically similar species, *Irvingia gabonensis* and *I. wombolu*, which were undergoing domestication at that time. Application of these results to domestication programmes and conservation approaches had been proposed. Similar results have been obtained for other rare, endemic or endangered species such as *Agrostis adamsonii* (James and Brown, 2000), *Rosmarinus tomentosus* (Martin and Bermejo, 2000), *Martes americana* (McGowan et al., 1999) and *Caesalpinia echinata* (Cardoso et al., 1998).

However, despite these practical advantages, the value of RAPD markers in genetic diversity studies has been questioned because of a number of drawbacks to the technique. One frequently cited limitation of RAPD in a variety of applications in molecular genetics, is the lack of reproducibility in the fingerprints that they produce (McRoberts et al., 1999; Uptmoor et al., 2003). The presence of co-migrating markers is also possible. These putatively similar bands originating from RAPD in different individuals are not homologous although they may share the same size in base pairs. This situation may lead to misleading results when calculating genetic relationships (Garcia-Mas et al., 2000).

1.2.3 AFLP markers

The amplified fragment length polymorphism (AFLP) technique is a recently developed

molecular technique through which selected restriction fragments from the digestion of total DNA are amplified by the polymerase chain reaction (Vos et al., 1995). This technique includes three major steps: (1) restriction digestion of DNA to be analysed with a pair of restriction enzymes; (2) ligation of short oligonucleotides to the ends of all digested fragments; and (3) high-stringency selective amplification using a subset of PCR primers complementary to the added oligonucleotide, with a few additional nucleotides added at the 3' end. Each added base can reduce the number of fragments amplified by a factor of 16 (Henry, 1997; O'Hanlon and Peakall, 2000).

The AFLP technique combines the reliability of the RFLP technique with the power and ease of the PCR technique (Zhang et al., 1999). The major advantage of AFLP markers, compared with other marker systems such as RFLPs and SSRs, is its ability to generate large numbers of polymorphic markers. Since the whole genome can be sampled, it can, in general, generate up to hundreds of informative genetic markers in a single primer pair, with a higher number of polymorphic loci (Palacios et al., 1999; Lubberstedt et al., 2000), which is often up to several dozen-fold higher than that that obtained using RFLP and RAPD techniques (Sharma et al., 1996; Ajmone Marsan et al., 1998; Mueller and Wolfenbarger, 1999; Talhinhas et al., 2004). For instance, Zhang et al. (1999) regenerated 48-74 AFLP bands per primer pair in *Cynodon* spp. Similarly, an average of 77 bands per primer pair were obtained in *Limonium cavanillesii* (Palacios et al., 1999).

In comparing the efficiency of different marker systems in cultivated barley accessions, Russell et al. (1997) found that there was wide variation in the average number of genotypes revealed by each of them. With AFLPs, an average of 17.2 genotypic classes for each primer pair could be distinguished. Nearly all primer combinations were able to discriminate between the 18 accessions used. However, this figure decreased to 5.38, 3.41 and 2.37 with SSRs, RAPDs and RFLPs, respectively. In detecting genetic diversity in maize inbreds from different sources, Ajmone Marsan et al. (1998) obtained an average of 34.8 polymorphic bands per AFLP primer pair, compared with 4.14 polymorphic bands per probe-enzyme combination for RFLP marker. Similary, Purba et al. (2000) could find polymorphism between four populations of *Elaeis quineensis* for all of the five tested AFLP primer pairs. However only 45% of RAPD primers and 2.5% of RFLP enzyme/probe combinations showed polymorphism within the same species.

The Marker Index (MI) provides an overall measure of marker or diversity efficiency (Powell et al., 1996). In investigating the genetic diversity among wheat cultivars using

AFLP, SSR and RFLP markers, the average MI of 3.41, 0.24 and 0.18 were obtained for these three markers, respectively (Bohn et al., 1999). Similarly, Lubberstedt et al. (2000) observed an average MI of 16.4 for AFLP markers, but below 4.0 for both RAPD and RFLP markers in maize (*Zea mays*).

AFLPs are generally considered reliable molecular markers. In a comparison of the reproducibility of AFLPs, RAPDs and SSRs between different laboratories using standardized PCR conditions, O'Hanlon and Peakall (2000) concluded that AFLPs were as reproducible as SSRs and far more reproducible than RAPDs. Of the total of 172 AFLP fragments, only one was inconsistently amplified in one out of six laboratories, which was subsequently shown to be the result of DNA template variation. In contrast, only 10 of 42 RAPD fragments were amplified across all nine laboratories. The high reproducibility of the AFLP markers were also stated and confirmed by the results of the majority of authors (Jones et al., 1997; Ajmone Marsan et al., 1998; Garcia-Mas et al., 2000; Lubberstedt et al., 2000; Campbell et al., 2003).

The AFLP technique is even more useful in detecting genetic diversity in genetically depauperate natural populations, rare species and endangered species (Krauss, 1999). In using this technique, the current diversity status was also demonstrated for a number of tree and shrub species, such as *Salix* spp. (Barker et al., 1999), *Cirsium dissectum* (Smulders et al., 2000), *Elaeis guineensis* (Purba et al., 2000), *Camellia sinesis* (Paul et al., 1997), *Ribes grossularia* (Lanham and Brennan, 1999), *Manihot esculenta* (Roa et al., 1997), and some endangered species such as *Astragalus cremnophylax* (Travis et al., 1996), *Moringa oleifera* (Muluvi et al., 1999), *Sicherus flabellatus* (Keiper and McConchie, 2000), and *Phormium* spp. (McBreen et al., 2003).

A major drawback of AFLP markers is that they are usually assumed to be dominant as for RAPD markers. It is also more technically sophisticated and more expensive to develop than RAPD markers. However, these difficulties need to be balanced against the significantly larger number of polymorphic markers generated per assay unit (Garcia-Mas et al., 2000; Gaudeul et al., 2000). Therefore, the overall efficiency of the AFLP method is higher than that of RFLP, RAPD and SSR methods, and is highly recommended for the investigation of genetic diversity in rare and endangered species with limited diversity and lack of previous knowledge of genomic information (Lanham and Brennan, 1999; Garcia-Mas et al., 2000; Patzak, 2003).

1.2.4 ISSR markers

The inter-simple sequence repeat (ISSR) technique is another powerful molecular marker system, first developed by Zietkiewicz et al. (1994). ISSR relies on the ubiquity of microsatellites in eukaryotic genomes. A single primer composed of a microsatellite sequence anchored by two to four arbitrary nucleotides is used to amplify the DNA between two opposite microsatellites of the same type placed whithin a short distance of one another (less than a few kb) in the genome. Any changes in these two sites, e.g. lack of the repeated sequence, a deletion or insertion that modifies the distance between repeats, or differences in the length of the microsatellite, will cause the absence or variation of an amplified fragment and generate polymorphisms between individuals. Since microsatellites usually occur in considerable numbers of loci dispersed throughout the genome and certain simple repeat sequences such as $(GT)_n$ and $(CAC)_n$ are common to all eukaryotic genomes, universal primers can be generated to match these microsatellites. The sequences of repeats and anchored nucleotides are selected randomly (Zietkiewicz et al., 1994; Wolfe et al., 1998).

The ISSR technique combines the high reliability and variability of SSR markers and the speed and ease of RAPD markers, and has several advantages over other marker systems (Marsh and Ayres, 2002; Souframanien and Gopalakrishna, 2004). Since ISSR markers target divergence in regions containing dispersed repetitive DNA and can rapidly differentiate closely related individuals, it is especially useful in detecting clonal variation and fingerprinting closely related individuals. It has great potential in the study of natural populations for addressing questions ranging from conservation biology to molecular ecology and systematics. The utility of the technique has been demonstrated in a wide range of applications and plant families (Wolfe et al., 1998; McBreen et al., 2003; Budak et al., 2004; Sudupak, 2004; Vijayan et al., 2004). It is especially attractive because it avoids the need to carry out costly cloning and sequencing inherent in the original microsatellite-based approach and the requirement of previous knowledge of genomic information (Squirrel et al., 2003). In this respect, this is especially important to endangered, rare and less commercially important species, which are generally little studied for their genetic structure (Nybom, 2004).

Since the ISSR technique can produce a much larger number of fragments per assay unit, it is a more powerful fingerprint tool than SSR, RFLP and RAPD marker systems (Qian et

al., 2001; Marsh and Ayres, 2002). To some extent, its efficiency might be comparable to that of AFLP markers since a higher percentage of polymorphic bands can be generated compared with the AFLP method (Blair et al., 1999; Vijayan et al., 2004). Huang and Sun (2000) obtained an average of 52 bands per PCR reaction and 62.2% polymorphic bands among *Ipomoea* intraspecific accessions. Pasqualone et al. (2000) were able to distinguish a set of 30 Italian durum wheat cultivars and 22 breeding lines using only two ISSR primers. In estimating the genetic diversity among 42 Indian elite rice varieties, Esselman et al. (1999) demonstrated that ISSR markers detected more diversity than RAPD markers and allozymes. In one population, eight different genotypes were found among the 10 plants with ISSR markers whereas no diversity was found with RAPD markers. Fang et al. (1997) showed that ISSR markers generated by 11 primers were able to identify 17 unique phenotypes among 48 vegetatively propagated trifoliate orange accessions, while few polymorphisms could be detected among accessions with seven isozyme loci and 38 probe-enzyme combinations of RFLP markers. In an attempt to detect the genetic diversity and phylogenetic relationship among nine wild populations of *Brassica oleracea*, Panda et al. (2003) found no difference among these populations using chloroplast DNA PCR-RFLP markers generated from 12 primer pairs and three restriction enzymes. However, both within and among population variations were detected using seven selected ISSR primers.

ISSR markers are also more reliable and reproducible than RAPDs because of the higher annealing temperature contributed by longer primers (Zietkiewicz et al., 1994; Palacios et al., 1999; Qian et al., 2001). Kantety et al. (1995) found that 98% of the ISSR bands were repeatable across maize DNA extractions and separate PCR runs. Estimates of genetic distances using ISSR markers usually correlated with those using SSR (Patzak, 2001; Uptmoor et al., 2003), and DNA sequence related markers such as nrDNA-ITS region and rbcL gene (McBreen et al., 2003; Talhinhas et al., 2004). ISSR markers were also shown to be closely associated with morphological traits, especially life history traits (Nybom, 2004).

Compared to results obtained with RAPD and AFLP, ISSR tends to produce higher estimates of within-population variation, and may also attribute more of the variation to lower levels (Wolfe et al., 1998; Qian, et al., 2001; Marsh and Ayres, 2002). Therefore, this technique is especially useful to endangered, rare and less commercially important species, which are generally little studied for their genetic structure. Indeed, ISSR

markers have been widely used for detecting genetic diversity of rare and endangered plant species, such as *Senecio layneae* (Marsh and Ayres, 2002), *Changium smyrnioides* (Qiu et al., 2004), *Cycas guizhouensis* (Xiao et al., 2004), and *Alnus maritime* (Schrader and Graves, 2004).

The only major drawback of using ISSR markers seems to be their dominant nature, a drawback in common with AFLP and RAPD markers (Gaudeul et al., 2000; Krauss, 2000).

In summary, each marker has its advantages and drawbacks. Different types of genetic markers may also yield inconsistent information about genetic diversity (Thomas et al., 1999). It is clear that genetic diversity identified from more than one technique can provide more appropriate information for designing subsequent conservation strategies. Since both *Clianthus* and *Sophora* to be investigated in the current study may have limited genetic diversity as revealed by preliminary studies, and there is a lack of molecular information (e.g. genomic DNA sequences, RFLP probes and microsatellite primers), a combination of several powerful techniques such as ISSR and/or AFLP and RAPD might be necessary for appropriate interpretation of genetic diversity in these species.

1.3 Functions and expression of floral identity genes

Flowering is influenced by interactions of genetic, environmental and physiological factors. In recent years, a significant understanding has been gained regarding the molecular mechanisms of flowering. In general, flower development can be divided into four steps that occur in a temporal sequence. Firstly, in response to either or both environmental and endogenous signals, the shoot apical meristem switches from vegetative growth to reproductive growth, a process controlled by a large group of flowering time genes. Secondly, 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. Thirdly, the meristem identity genes activate the floral organ identity genes in discrete regions of the flower. Fourthly, the floral organ identity genes activate downstream "organ building" genes that specify the various cell types and tissues that constitute the four floral organs (Jack, 2004). This section of the review will mainly focus on genes involved in the last three steps, especially the floral meristem identity genes and the floral organ identity genes.

1.3.1 Floral meristem identity genes

There are two overriding patterns of reproductive development in angiosperms. In the first case, vegetative meristems are directly transformed into determinate floral meristems. In the second case, the vegetative meristems are first transformed into inflorescence meristems, which then generate the floral meristems (Meeks-Wagner, 1993). In both of the two herbaceous model species, *Arabidopsis thaliana* and *Antirrhinum majus*, flowering follows the second pattern. The floral meristems are produced in the bract axils of the inflorescence (Fosket, 1994).

Genes involved in the identity of the floral meristem were first identified from mutants in which flowers were replaced by shoots with inflorescence-like properties. The *FLORICAULA* (*FLO*) gene of *Antirrhinum* (Coen et al., 1990) and *LEAFY* (*LFY*), its orthologue from *Arabidopsis* (Weigel et al., 1992), encode transcription factors that impose a floral identity on primordia that arise from the flank of shoot apical meristems after their floral induction. Ectopic expression of the *LFY* transcription factor in non-reproductive meristems can cause a conversion to a floral meristematic fate in a variety of species, demonstrating that it is a master regulator of floral development

(Weigel et al., 1992; Weigel and Nilsson, 1995; Peña et al., 2001). Floral meristem identity is also promoted by *SQUAMOSA* and *APETALA1* in *Antirrhinum* and *Arabidopsis*, respectively. Similar ectopic expression studies have shown that *AP1* is also sufficient for specification of floral meristem identity (Mandel et al., 1992; Mandel and Yanofsky, 1995). Both *LFY* and *AP1* encode sequence-specific DNA binding transcription factors. *AP1* is a member of the MADS (derived from MCM1, AGAMOUS, DEFICIENS, and SRF box family (Huijser et al., 1992; Mandel et al., 1992), whereas *LFY* encodes a plant-specific protein that exhibits no strong similarity to other genes in *Arabidopsis* (Coen et al., 1990; Weigel et al., 1992).

LFY homologues are present in a wide range of flowering and non-flowering plant species (Frohlich and Parker, 2000; Gocal et al., 2001). In many developmental contexts, *LFY* is necessary and sufficient to specify a meristem as floral (Weigel and Nilsson, 1995). *LFY* homologues have been isolated and studied from many dicotyledonous species including cauliflower, tobacco, pea, pine, eucalyptus, poplar and violet cress (Anthony et al., 1993; Kelly et al., 1995; Hofer et al., 1997; Pouteau et al., 1997; Mouradov et al., 1998; Southerton et al., 1998a; Rottmann et al., 2000; Shu et al., 2000). More recently, Bomblies et al. (2003) demonstrated that the *Zea mays LFY* homologues, *ZeaFLO/LFY1* (*zfl1*) and *zfl2*, are crucial in promoting the transition to flowering and the activation of floral organ identity genes, similar to what is observed for *LFY* homologues in core eudicots. These studies showed that the *LFY* gene lineage is highly conserved across dicots and monocots (Chuck and Hake, 2005).

In *Arabidopsis*, the greatest expression of *LFY* was found in regions of the inflorescence meristem that formed floral meristems and in newly formed floral meristems. However, *LFY* was also expressed at low levels during vegetative development (Weigel et al., 1992). The *LFY* homologues in tobacco were found to be active not only in floral meristems, but also in indeterminate vegetative meristems (Kelly et al., 1995). Blasquez (1997) observed that *LFY* combines properties of flowering time and floral meristem identity genes. He analysed the expression of *LFY* during the *Arabidopsis* life cycle and found that *LFY* was extensively expressed during the vegetative phase. However, increasing the copy number of endogenous *LFY* reduced the number of leaves produced before the first flower was formed.

Mutations in *AP1* also have a stronger effect on flowers that develop at early positions on the shoot than on those that develop later. However, *ap1* mutant structures are less

affected than shoots formed in *lfy* mutants. *SQUA* and *AP1* are perhaps not as critical as *FLO* and *LFY* in controlling floral meristem identity as evidenced by the phenotypic characteristics of their null mutants. The phenotypes of *lfy* and *ap1* single and double mutants indicate that these genes have partially redundant functions. *lfyl ap1* double mutants show a more severe phenotype than either single mutant, with flower-like structures seen only very rarely (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993).

Although *AP1* and *LFY* are the major floral meristem identity genes, other genes such as *CAULIFLOWER* (Bowman et al., 1993; Kempin et al., 1995), *FRUITFULL* (Gu et al., 1998; Ferrándiz et al., 2000), and *AP2* (Jofuku et al., 1994; Okamuro et al., 1996) play secondary roles in specifying floral meristem identity.

Apart from determing the meristem fate, the other role of floral meristem identity genes is to activate the expression of organ identity genes that act later in flower development (Weigel and Meyerowitz, 1993). Analysis of a *LFY* responsive enhancer in the homeotic gene *AGAMOUS* indicated that direct interaction of *LFY* with the enhancer was required for its activity in plants (Busch et al., 1999). Expression of *APETALA3* (*AP3*) and *PISTILATA* (*PI*) is lost in *lfy ap1* double mutants, and expression of *AGAMOUS* is directly activated by *LFY*. Thus *LFY* is a direct upstream regulator of floral homeotic genes (Lohmann and Weigel, 2002; Jack, 2004).

1.3.2 Floral organ identity genes and the ABC model of flowering

After having been activated by floral meristem identity genes, several groups of floral organ identity genes interact, either directly or indirectly, within the events that monitor the development of flowers (Cseke and Podila, 2004). Among the models for the control of floral organ identity (Haughn and Somerville, 1988; Schwarz-Sommer et al., 1990; Bowman et al., 1991; Coen and Meyerowitz, 1991), the ABC model is the most widely known and has formed the foundation of our understanding of floral development. It was based on work done with homeotic mutants of *Arabidopsis* and *Antirrhinum*, and suggests three functional classes of genes (A, B, and C) working in combination to specify the four concentric whorls of floral organs (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Yanofsky, 1995) (Fig. 1.1 section I).

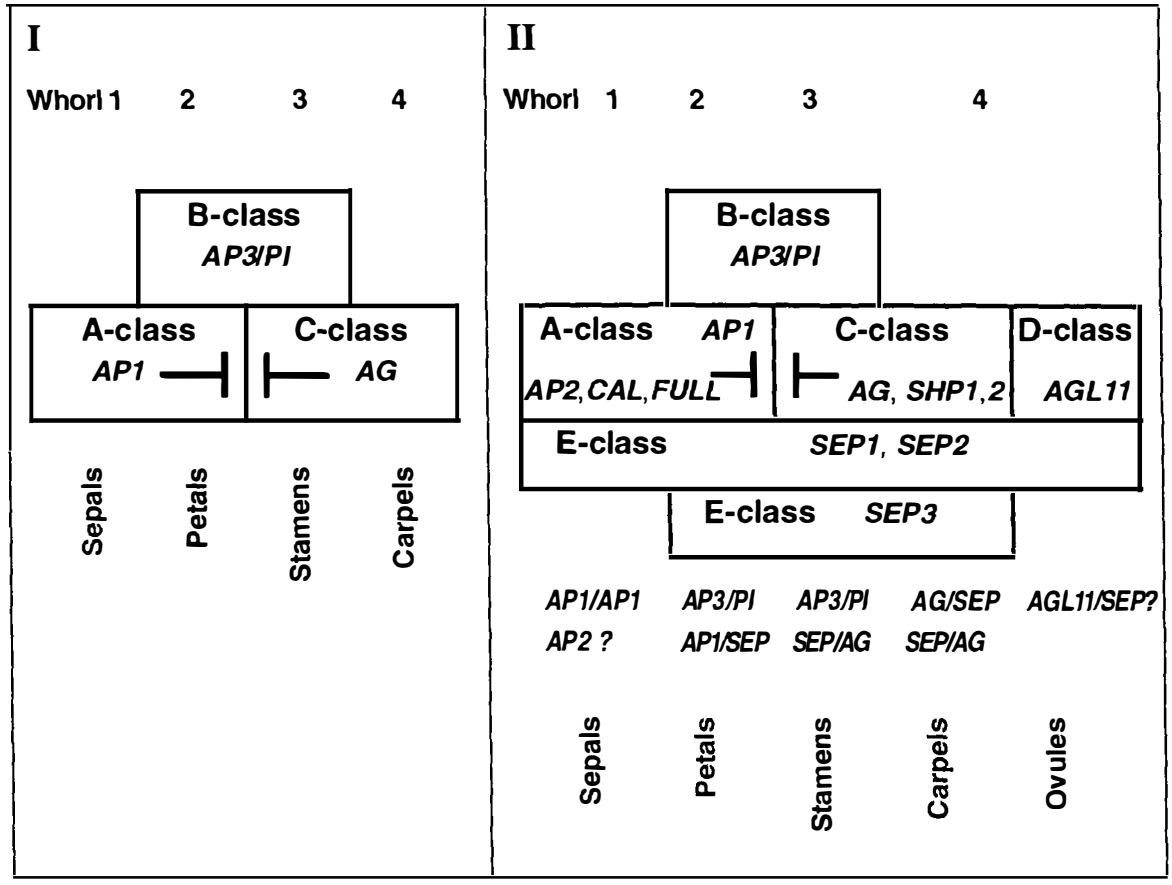

Figure 1.1 The traditional (I) and expanded (II) ABC model of floral development

Adapted from Cseke and Podila (2004). The expanded ABC model includes functional domains of D- and E-class genes and possible MADS-box protein "quartets" active in the development of each floral whorl.

In *Arabidopsis*, activity of A-function genes, such as *AP1* and *APETALA2* (*AP2*), alone specify the development of sepals in whorl 1. The combined activity of A- and B-function genes, such as *PISTILATA* (*PI*) and *APETALA3* (*AP3*), leads to the development of petals in whorl 2. B- and C-function genes, such *AGAMOUS* (*AG*), work together to produce stamens in whorl 3. And the C-function gene alone specifies carpel formation in whorl 4. The A- and C-function genes appear to be mutually exclusive in their activity. For example, *AP1* inhibits the expression of *AG* in whorls 1 and 2 while *AG* inhibits *AP1* in whorls 3 and 4 (Coen and Meyerowitz, 1991; Yanofsky, 1995).

It is suggested that the overall purpose of C-function genes is to distinguish between reproductive and non-reproductive organs, while that of B-function genes is to discriminate between male and female reproductive organs (Saedler et al., 2001). Over the years the ABC model has been shown to be useful in explaining the development of

the reproductive organs of many plant species including angiosperms and even gymnosperms (Ng and Yanofsky, 2001b; Theissen, 2001; Becker and Theissen, 2003; Komeda, 2004).

The spatially restricted patterns of ABC gene expression depend on the action of the floral meristem identity genes. *LFY* plays a pivotal role in this process, regulating the initiation of expression of A, B, and C class genes (Weigel and Meyerowitz, 1993; Parcy et al., 1998). Although *LFY* acts to regulate the expression of *AG* and *AP1* via direct transcriptional activation (Busch et al., 1999; Wagner et al., 1999), it acts both directly and indirectly, via *AP1*, to regulate *AP3* (Ng and Yanofsky, 2001a; Lamb et al., 2002). *LFY*, however, is expressed throughout the young flower primordium and therefore cannot provide the spatial specificity for activation of the ABC genes (Weigel et al., 1992). Since *AP1* is initially expressed throughout the young flower primordium and only later becomes restricted to the first and second whorls, negative regulation by *AG* is sufficient to explain the spatial pattern of *AP1* expression. *LFY*, in combination with spatially restricted *WUSCHEL* (*WUS*) activity, can limit *AG* expression to the centre of the floral meristem. *LFY* interacts with another factor, *UNUSUAL FLORAL ORGANS* (*UFO*), to limit *AP3* activation to the second and third whorls (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Lee et al., 1997). *UFO* is expressed in the presumptive second and third whorls early in floral development (Ingram et al., 1995; Lee et al., 1997). Thus the action of *LFY*, combined with spatially restricted cofactors, can explain the overlapping domains of ABC gene expression patterns (Parcy et al., 1998).

B-class genes such as *PI* and *AP3* are expressed in the petal and stamen whorls of flowers (Bowman et al., 1989; Jack et al., 1992; Goto and Meyerowitz, 1994). Mutations in either of these genes trigger the conversion of petals to sepals in the second whorl and stamens to carpels in the third whorl (Parenicova et al., 2003). *PI* and *AP3* act as a heterodimer *in vitro* (Goto and Meyerowitz, 1994; Riechmann et al., 1996a, b), and the functionality of *AP3* appears to be contained in the C-terminal domain of MADS box sequence (Lamb and Irish, 2003).

C-class genes, such as *AG*, interacting with B-class genes such as *AP3* and *PI*, define the formation of stamens while *AG* genes define carpels (Honma and Goto, 2001; Theissen, 2001). *AG* expression in whorls 3 and 4 is also known to inhibit *AP1* expression thus setting up the mutually exclusive zones predicted in the ABC model of floral development. *Arabidopsis ag* mutants show conversion of stamens to petals and carpels to

a secondary flower in which the pattern of sepals, petals, petals repeats more than five times (Bowman et al., 1989). This is due in part to the expression of *AP1* in whorls 3 and 4, but it also indicates that *AG* is required for the determinacy of the floral meristem. Therefore, it is likely that the purpose of C-function genes is to distinguish between reproductive and non-reproductive organs (Saedler et al., 2001).

Recently, reverse genetic studies of other MADS-box genes have led to the proposal of an additional group of floral organ identity genes, the D-function genes, which are thought to have a specific role in ovule identity (Colombo et al., 1995). Similarly, recent studies have shown that another group of closely related genes, *SEPALLATA1, 2, 3* (*SEP1, 2, 3*), are required for petal, stamen and carpel identity, and are thus necessary for the function of B and C class genes (Pelaz et al., 2000). The true function of these genes may be found through the formation of quartets (Pelaz et al., 2000; Theissen, 2001) (Fig. 1.1 section II). This has recently shed light on the diversification of function among paralogues of the *SEP* genes in grasses (Malcomber and Kellogg, 2004). The failure of floral organs to develop with the correct identity in A, B, C, and E class mutants demonstrates that the ABCE genes are necessary to specify floral organ identity. When expressed ectopically, the ABCE genes are sufficient to direct organ identity in flowers but not in vegetative leaves (Jack et al., 2004).

Within the genomes of many plant species, there is a high degree of genetic redundancy within the floral identity genes. This often inhibits the interpretation of gene suppression studies and mutant phenotypes. There are several examples of genetic redundancy of these genes in *Arabidopsis* alone, such as *AP1/CAL/FUL* (Ferrándiz et al., 2000) and the three *SEP* genes (Pelaz et al., 2000). Many aspects of ABCDE gene functions appear to be conserved across the angiosperms (Becker and Theissen, 2003), but comparisons among divergent taxa are complicated by the complex evolutionary history of these genes. Furthermore, many of these genes are not merely expressed in floral tissues and are suspected to function in floral and/or vegetative development (Alvarez-Buylla et al., 2000; Johansen et al., 2002; Cseke et al., 2003b).

1.3.3 Expression of floral identity genes in woody perennials

1.3.3.1 Expression of *LFY/FLO* homologues

In woody species, a similar situation to that in model species seems to be in effect for

LFY/FLO homologues. *ELF1* and *ELF2*, the *Eucalyptus globulus LFY* homologues, have been identified by Southerton et al. (1998a). Similar to *LFY*, *ELF1* is expressed early in the transition to flowering as well as in vegetative meristems. Over-expression of *ELF1* in transgenic *Arabidopsis* results in the conversion of shoot meristems into floral meristems, the phenotypes being very similar to the over-expression phenotypes of 35S::LFY within *Arabidopsis* itself (Southerton et al., 1998a).

The *LFY* homologue in *Populus trichocarpa*, *PTLF*, shares high levels of sequence identity with *LFY*. *In situ* hybridisation studies showed that the gene was expressed most strongly in developing inflorescences. Expression was also seen in leaf primordia and very young leaves, most notably in apical vegetative buds near inflorescences, but also in seedlings. Although ectopic expression of the *PTLF* cDNA in *Arabidopsis* accelerated flowering, only one of the many tested transgenic lines of *Populus* flowered precociously. The majority of trees within a population of 3-year-old transgenic hybrid *Populus* lines with *PTLF* constitutively expressed showed few differences when compared to controls. This suggests the presence of differing interactions that may be active during floral transition and development in *Populus* (Rottmann et al., 2000).

A partial *LFY* homologue from *Metrosideros excelsa*, *MEL*, was isolated by Sreekantan et al. (2004). Temporal expression of *MEL* was characterised by a bimodal pattern. *MEL* was expressed at low levels in autumn, increased during cymule primordia initiation in late autumn, decreased during winter, and increased again during organogenesis in spring. *In situ* hybridization showed that *MEL* was expressed, sequentially, in the apex of the inflorescence, on the cymule primordia, on floral primordia, in sepals and petals, in stamens and the inner regions of the gynoecium, and in anthers and ovules at a late developmental stage. *MEL* expression was also detected in the inner walls of cup-like structures formed by the developing primordia and on the subtending bracteoles where it was mainly seen in the axils. No expression of *MEL* was detected on the stalk-like structures supporting the developing floral primordia (Sreekantan et al., 2004).

A very similar situation to *MEL* was described by Walton et al. (2001) in *ALF*, a partial *LFY* homologue from *Actinidia deliciosa*. *ALF* also had bimodal patterns of expression in developing first-order axillary buds and their subsequent shoots, consistent with the 2-year cycle of axillary bud, flower and fruit development observed in this species. The first expression peak was observed in the initiated second-order meristems with the first-order buds (late spring of the first growing season), while the second peak occurred

approximately 10 months later, when those meristems differentiated flowers (late spring of the second growing season). *In situ* hybridisation studies showed that *ALF* expressed throughout the developing first-order buds during late spring of the first growing season, and in differentiating flowers during the spring of the second growing season.

Two *LFY* homologues, *AFL1* and *AFL2* were isolated from *Malus domestica* (Kotoda et al., 2000; Wada et al., 2002). *AFL1* was expressed only in the floral bud during the transition from vegetative to reproductive growth, whereas *AFL2* was expressed in the vegetative shoot apex, floral buds, floral organs and roots. Transgenic *Arabidopsis* with over-expressed *AFL2* showed accelerated flowering and gave rise to several solitary flowers directly from rosette axils. *AFL1* had similar effects, but the phenotypes of the transgenic *Arabidopsis* with *AFL1* were weaker than those with *AFL2*. These results suggest that both genes have similar functions in flower differentiation to that of *LFY* (Kotoda et al., 2000; Wada et al., 2002).

The *Vitis vinifera LFY* homologue, *VFL,* was expressed in the vegetative shoot apical meristem, leaf primordia and the growing leaf until later stages of development, suggesting a role for *VFL* not only in flower meristem specification, but also in the maintenance of indeterminacy before the differentiation of derivatives of the apical meristem: flowers, leaves, or tendrils (Carmona et al., 2002).

In gymnosperms, a *LFY* homologue in *Pinus radiata, NEEDLY* (*NLY*) was isolated by Mouradov et al. (1998) and was found to be expressed both in reproductive and vegetative meristems. *NLY* expression was detected during vegetative development at least five years before the transition to the reproductive phase. However, expression of *NLY* in transgenic *Arabidopsis* promoted floral fate, demonstrating that *NLY* is indeed a functional homologue of the *FLO/LFY* genes of angiosperms.

Despite some variation in expression, especially the bimodal temporal expression patterns in some species such as *Metrosideros* and *Actinidia*, the basic role of *LFY* homologues in most woody species is similar to those of the model species *Arabidopsis.*

1.3.3.2 Expression of A-class genes

In *Eucalyptus globulus*, the *AP1* homologues, *EAP1* and *EAP2,* were expressed in the operculum and in the receptacle of developing flowers (Kyozuka et al., 1997).

Over-expression of *EAP1* and *EAP2* in *Arabidopsis* causes similar phenotypes to *AP1* overexpression in *Arabidopsis*, strongly suggesting a similar function. This suggests that *Eucalyptus* may have a similar mechanism behind the transition to flowering as well as aspects of floral development.

MdAP1 (or *MdMADS5*), an *AP1* homologue in *Malus domestica*, was exclusively expressed in sepals (Kotoda et al., 2000) and in the flesh cortex during fruit development (Yao et al., 1999). Unlike the characteristic expression of *AP1*, no *MdAP1* expression was found in petals or petal primordia, and *MdAP1* did not seem to express at earlier stages. However, over-expression of *MdAP1* in *Arabidopsis* resulted in phenotypes similar to *Arabidopsis* over-expressing *AP1* (Kotoda et al., 2002). Another *Malus AP1* homologue, *MdMADS2*, expressed in all stages of floral development. However, immunolocalization showed that *MdMADS2* protein was excluded from stamen and carpel primordia during later development (Sung et al., 1999). Interestingly, ectopic expression of *MdMADS2* in transgenic tobacco showed early flowering and shorter bolts, but did not produce any changes in the floral organs (Sung et al., 1999). Another *Malus AP1* homologue, *MdMADS12*, expressed in leaves, vegetative shoots, and floral tissues (van der Linden et al., 2002). Therefore, some aspects of *Malus* floral development probably differ from those found in herbaceous species.

MESAP1 is the partial *AP1* homologue from *Metrosideros excelsa* isolated by Sreekantan et al. (2004). Temporal expression of *MESAP1*, like *MESLFY*, were also characterised by a bimodal pattern, expressing at low levels in autumn, increasing during cymule primordia initiation in late autumn, decreasing during winter, and increasing again during organogenesis in spring. *In situ* hybridization showed that *MESAP1* was first expressed on the cymule primordia in early developmental stages, and in the developing sepals, petals and the perianth in later stages. Expression was also detected on the subtending bracteoles, mainly concentrated in their axils.

AAP1 is a partial homologue of *AP1* from *Actinidia deliciosa* isolated by Walton et al. (2001). Similar to *MESAP1*, *AAP1* also had a bimodal expression pattern in developing first-order axillary buds and their subsequent shoots, consistent with the 2-year cycle of axillary bud, flower and fruit development observed in this species. The first expression peak was observed in the initiated second-order meristems with the first-order buds (late spring of the first growing season), while the second peak occurred approximately 10 months later, when those meristems differentiated flowers (late spring of the second

growing season). *In situ* hybridisation studies showed that *AAP1* expression was limited to developing second-order axillary meristems within the first-order buds during late spring of the first growing season, and in sepals and petals in differentiating flowers during the spring of the second growing season. This suggested that important developmental events were occurring very early in kiwifruit first-order axillary bud development and it was likely that this included floral commitment (Walton et al., 2001).

Populus tremuloides AP1 homologues, *PTM1* and *PTM2*, were isolated from male and female catkins. Sequence based phylogenetic analysis indicates that *PTM1* and *PTM2* belong to the *AP1* family of genes with *PTM1* being most closely related to *AP1* out of the then known *AP1* homologues in woody species (Cseke et al., 2003a). Consistent with the floral meristem identity function of *AP1*, *PTM1* and *PTM2* expressed exclusively in floral tissues at very early stages of reproductive development when visible sex-specific organs had not yet been formed. Expression declined as the flower buds entered winter dormancy; after winter, the expression levels rose again, then declined as the flowers reached maturity (Cseke and Podila, 2004).

A similar floral structure to *Populus* was found in *Betula pendula.* The *AP1* homologues, *BpMADS3*, *BpMADS4*, and *BpMADS5*, resulted in an extreme early flowering phenotype when over-expressed in transgenic tobacco (Cseke and Podila, 2004). All three genes act early during the transition to the reproductive phase and might be involved in the determination of the identity of inflorescence and/or flower meristem (Elo et al., 2001).

VAP1 is an *AP1* homologue from *Vitis vinifera* (Calonje et al., 2004). *VAP1* is expressed in lateral meristems, inflorescence and flower meristems. During flower organ development, its expression is detected in petals, stamens and carpels, but not in sepals. This does not support its role as an A-function gene in grapevine. Furthermore, *VAP1* is also expressed throughout tendril development, even in very young plants that have not undergone flowering transition, indicating that this expression is independent of the flowering process and that this could represent a novel role that would have been recruited for the development of tendrils in *Vitis* (Calonje et al., 2004).

Homologues of A-class genes in many woody species have very similar functions to those of model species in determining the floral meristem and the outer whorl floral organ identity. However, homologues of A-class genes in some woody species seem to have varied functions compared to their *Arabidopsis* counterparts. These variations suggest that

the A-class gene is less conserved across different species.

1.3.3.3 Expression of B-class genes

In woody species, B-class genes are also expressed similarly to *PI* in *Arabidopsis*. *EGM2*, from *Eucalyptus globulus*, is a single copy gene expressed in the petals and stamens (Southerton et al., 1998b). However, weaker expression was also found in vegetative shoots, leaves, and seedlings. In *Malus*, the *PI* homologue *MdPI* is expressed in the petals and stamens of flower buds (Yao et al., 2001). The phenotypes of *MdPI* mutants are strikingly similar to those of the *Arabidopsis pi* mutant in that they show conversion of petals to sepals and stamens to carpels (Yao et al., 2001). This, as well as the identical expression pattern to *PI* in the flowers, suggests that the *MdPI* gene has similar activity to *PI*. In addition, *MdMADS13* may be the *Malus AP3* homologue. Like *MdPI*, its expression is found only in floral tissue with the greatest abundance in the petals and stamens (van der Linden et al., 2002). Thus, it is likely that *Malus* possesses B-class genes that may function almost identically to those found in *Arabidopsis*.

PTD, a B-class gene homologue from *Populus trichocarpa*, has sequence and expression similarity to that of *AP3* (Sheppard et al., 2000). While *PTD* expression is observed in the inner whorl of both male and female flowers of these two whorled species, the expression pattern becomes specific to stamen primordia and development as the flowers mature. No transcripts were found in female trees at these later stages, suggesting that, despite the lack of petals in these flowers, the *PTD* gene functions along similar lines to those of *AP3* and *PI* by simply reducing its area of influence. While *PTD* is more divergent than other B-class genes, it may be one of the important factors that helps to specify the sex of *Populus* species (Sheppard et al., 2000).

B-class genes are also present in gnetophytes and gymnosperms which do not produce petals and whose reproductive organs come in the form of cones. *GGM2* and *GGM15* are *AP3/PI* homologues from *Gnetum gnemon* (Winter et al., 1999). *GGM2* expression is specific to the antherophores of male cones, and no expression was found in female tissues. This again suggests some conservation of the B-class function with restriction of the floral activity to stamens.

In situ hybridization of *DAL11*, *DAL12* and *DAL13*, *PI* homologues from *Picea abies*, indicates that each of these genes is expressed only in developing pollen cones, although

their expression patterns vary somewhat within the cone (Sundstroem and Engstrom, 2002). Expression of *DAL11* or *DAL12* in transgenic *Arabidopsis* causes phenotypes similar to the over-expression of *PI*, while the overexpression of *DAL13* triggers the formation of anthers in all four whorls. This strongly suggests a conserved B-class functional role in woody species. *DAL11*, *12*, and *13* were also shown to form homo- and heterodimers similar to the proposed interaction between *AP3* and *PI*.

Similarly, expression of *DGL* from *Pinus radiata* is restricted to the pollen strobili (male cones) and is not detected in female cones (Mouradov et al., 1999). *DGL* expression was first detected in male cone primordia and persisted through the early stages of pollen cone bud differentiation. Thus, it may play a role in distinguishing between male and female reproductive organs, and there may be an ancestral role of B-class genes in specifying male reproductive organs.

Both sequences and functions of B-class genes are highly conserved between woody species and herbaceous model species. This conservation also seems to extend to woody gymnosperms.

1.3.3.4 Expression of C-class genes

C-class gene homologues have also been isolated and studied in a number of woody species. The *Malus AG* homologue, *MdMADS15*, expressed in stamens and carpels. However, expression was also found in sepals and at very low levels in leaves (van der Linden et al., 2002). In *Populus trichocarpa*, both *PTAG1* and *PTAG2* expressed at very early to late stages of floral development in the inner (stamen) whorl of flowers from male trees or the inner (carpel) whorl of flowers from female trees. However, unlike *AG*, the *PTAG* genes also expressed in vegetative tissues such as leaves and stems, although Brunner et al. (2000) proposed that these MADS-box genes do not achieve their real function in these tissues.

In gymnosperm species, the *AG* homologue from *Ginkgo biloba*, *GBM5*, expressed in reproductive organs such as stamens, ovules, and the female gametophyte, but it was also detected in young leaves from both male and female trees (Jager et al., 2003). *SAG1* from *Picea mariana* (Rutledge et al., 1998) also expressed in the reproductive cones with reducing levels of expression in maturing male cones and relatively constant expression levels in female cones within the developing ovuliferous scale. However, RT-PCR also

detected *SAG1* transcript in the leaves and vegetative buds. Overexpression of *SAG1* in transgenic *Arabidopsis* resulted in the same phenotype as overexpression of *AG* in *Arabidopsis* (Rutledge et al., 1998). *DAL2* from *Picea abies* (Tandre et al., 1995) and *PMADS2* from *Pinus resinosa* (Liu et al., 2003) also expressed in very similar patterns to those of *GBM5* and *SAG1*, strongly suggesting that C-function genes are conserved across angiosperms and gymnosperms.

In conclusion, the activities of most floral meristem and organ identity genes in woody species are somewhat similar to those of their *Arabidopsis* homologues, especially in species with four-whorl flowers such as *Malus* and *Eucalyptus*. In woody species having flowers with only two whorls, such as *Populus*, some genes appear to have alternative or greatly narrowed functions, although they may have a very high level of sequence similarity to herbaceous model species such as *Arabidopsis*. In gymnosperm species, the major classes of genes have similarities in the processes of reproductive development. Interestingly, no A-class genes have been reported in gymnosperm species to date. However, the majority of studies regarding floral meristem and floral organ identity genes in woody species are limited to the characterization of expression patterns. Because of the long juvenile period for most woody species, functional studies have been limited to heterologous systems where the expression of foreign genes might be questionable (Csecke and Podila, 2004). The use of phenotypic mutants to analyse gene function is also very limited, with few such studies having been reported (Yao et al., 2001). Furthermore, despite the fact that protein interactions are key to gene function, few studies regarding protein interactions have been reported in woody species (Sundstroem and Engstrom, 2002).

Many aspects of the control of flowering in woody perennial species, such as competence for flowering, timing of initiation and long-term temporal and complex spatial dimensions, are different from that in annual model species, and are still very poorly understood (Brunner and Nilsson, 2004). The recent public release of the complete genome sequence of *Populus trichocarpa* (Strauss and Martin, 2004; DiFazio, 2005) (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), raises the possibility of whole genome comparative studies among woody (*Populus*), herbaceous dicot (*Arabidopsis*) and monocot species (*Oryza*). This would enable direct comparisons of the regulatory networks controlling flowering between these distantly related angiosperms.

1.4 Summary

Both *Clianthus* and New Zealand *Sophora* are of great biodiversity, biological and potentially commercial significance. *Clianthus* is critically endangered and needs urgent action toward species conservation and management. However, little is known about their genetic structure and relatedness, detailed vegetative and reproductive growth characters, or the molecular mechanisms underlying flowering.

Molecular markers such as AFLP and ISSR are powerful and reliable tools to reveal the genetic structure, especially genetic diversity and relatedness at or below the species level. These markers have been widely used and are thought to be particular useful in rare and endangered species, in which little or no previous molecular information is available.

The ABC model of floral development seems applicable to a wide range of angiosperm species and many gymnosperm species, including woody perennials, but with variation in expression patterns and/or functions in many cases. Floral organ identity genes, especially B- and C-class genes, are much less studied in woody species. No study of floral organ identity gene expression in woody legumes has been reported, and few such studies in the whole leguminous family have been reported. No study, including the herbaceous model species, has tracked the expression patterns across all the ABC class genes together with their up-stream regulator, *LFY*, in one species and the same experiment.

Given that partial gene redundancy and complicated interactions among redundant genes exists in each group of floral organ identity genes, and that gene function study is hampered in woody perennials by their long juvenile period, in this thesis the applicability of the ABC model was assessed by tracking the temporal and spatial expression patterns of one gene from each class. However, it shoud be noticed that for New Zealand native species, gene cloning and transformation is strictly regulated.

1.5 Aims of the study

The aims of the research reported in this thesis were to obtain comprehensive information on vegetative and reproductive development with an emphasis on the molecular mechanisms of flowering, genetic status, and genetic relationship within populations and among species of *Clianthus* and native *Sophora* species. The main objectives were to:

- Reveal the genetic diversity of all the remaining wild populations of *Clianthus puniceus* and *C. maximus*, and some commercial cultivars using ISSR and AFLP markers, and to provide a molecular test of the recent separation of *Clianthus* into two species, *C. puniceus* and *C. maximus*, based on morphological grounds.

- Reveal the genetic variation among New Zealand *Sophora* species using ISSR and/or AFLP markers in order to resolve the phylogenetic relationships of New Zealand *Sophora* species, and to provide a molecular test of the recent separation of *Sophora chathamica*, *S. Godleyi*, *S. fulvida*, *S. longicarinata* and *S. molloyi* from *Sophora microphylla* based on morphological and growth habit characters.

- Investigate the developmental characters of *Clianthus* and *Sophora* by monitoring their vegetative and reproductive growth cycles, and track their floral development process through microscopic studies.

- Evaluate the potential to be new cut flower species and attempt to extend the longevity of the short-lived cut flowers of *Clianthus* and *Sophora* by investigating the role of sucrose, and other commonly used postharvest chemicals, and to eventually develop a practical postharvest treatment protocol.

- Isolate, using non-cloning strategies, and characterize homologues of *LFY* and ABC class genes *AP1*, *PI*, and *AG* from both *Clianthus maximus* and *Sophora tetraptera*.

- Quantify the detailed temporal and spatial (developmental) expression characteristics of each of the newly isolated floral genes using the state-of-the-art real-time RT-PCR technique in order to test the hypothesis that the ABC model of floral development in herbaceous species is applicable to both woody legumes.

Chapter 2 Analysis of genetic diversity and genetic relatedness of *Clianthus* and *Sophora* populations using molecular markers

2.1 Introduction

A high proportion of species in the New Zealand flora, including those in the genus *Clianthus* and *Sophora* of the family Fabaceae, are categorised as critically endangered, endangered or vulnerable (de Lange et al., 1999), and are threatened by the steady loss of native forested cover that has occurred over the last 150 years (Ogden et al., 1998).

Clianthus had been considered a monospecific genus for most of the last 100 years, with *Clianthus maximus* reduced to *C. puniceus* var. *maximus* by Kirk (1899). However, the two species *C. puniceus* (G Don) Sol. Ex Lindl. and *C. maximus* Colenso were again recognised recently on the basis of vegetative and floral morphology (Heenan, 2000). *Clianthus maximus* is currently found almost exclusively within 50 km of the eastern coast of the North Island of New Zealand (Fig. 2.1). The only recent occurrence of *C. puniceus* in the wild was reported to be on Moturemu Island in Kaipara Harbour, north of Auckland (Heenan, 2000) (Fig. 2.1). Seeds were collected from this site in 1996 at the time when the last parent *C. puniceus* plant died, and the resulting seedlings grown under supervision in well documented collections (S. Benham, pers. comm.).

All *Sophora* taxa found in New Zealand are endemic species, and belong to section *Edwardsia* of the genus *Sophora* (Hurr, 1996; Heenan et al., 2001). The taxonomy of New Zealand *Sophora* has had a complex history despite the general consistency of the three species, *Sophora tetraptera*, *S. prostrata* and *S. microphylla*. *Sophora microphylla* contained several varieties including *chathamica*, *fulvida*, *godleyi*, *longicarinata* and *molloyi* (Allan, 1961; Yakovlev, 1967 cited by Heenan et al., 2001). However, all these varieties previously under *S. microphylla* were recently recognized at the species level, based on morphological and growth habit characters (Heenan et al., 2001).

Knowledge of genetic diversity in the remaining wild populations and the genetic relatedness of these populations may be beneficial to the conservation of these species in a number of aspects (see Section 1.2.1). Molecular markers such as random amplified

polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and amplified fragment length polymorphisms (AFLP), have proved to be the most effective markers for revealing genetic diversity and genetic relatedness, and have been widely used for the study of plant genetic diversity, particularly for rare and endangered species, such as *Senecio layneae* (Marsh and Ayres, 2002), *Changium smyrnioides* (Qiu et al., 2004), and *Alnus maritime* (Schrader and Graves, 2004). The great advantage of molecular markers is that it simultaneously assays variation at multiple, independent (but anonymous) loci. Not only does this allow resource-efficient detection of genetic variation at very low taxonomic levels (i.e. good resolution), but it can provide data from multiple, independent characters, allowing an assessment of genomic-level relationships between individuals (Vos et al., 1995; Lanham and Brennan, 1999; Lowe et al., 2000; Garcia-Mas et al., 2000; Squirrel et al., 2003; Nybom, 2004).

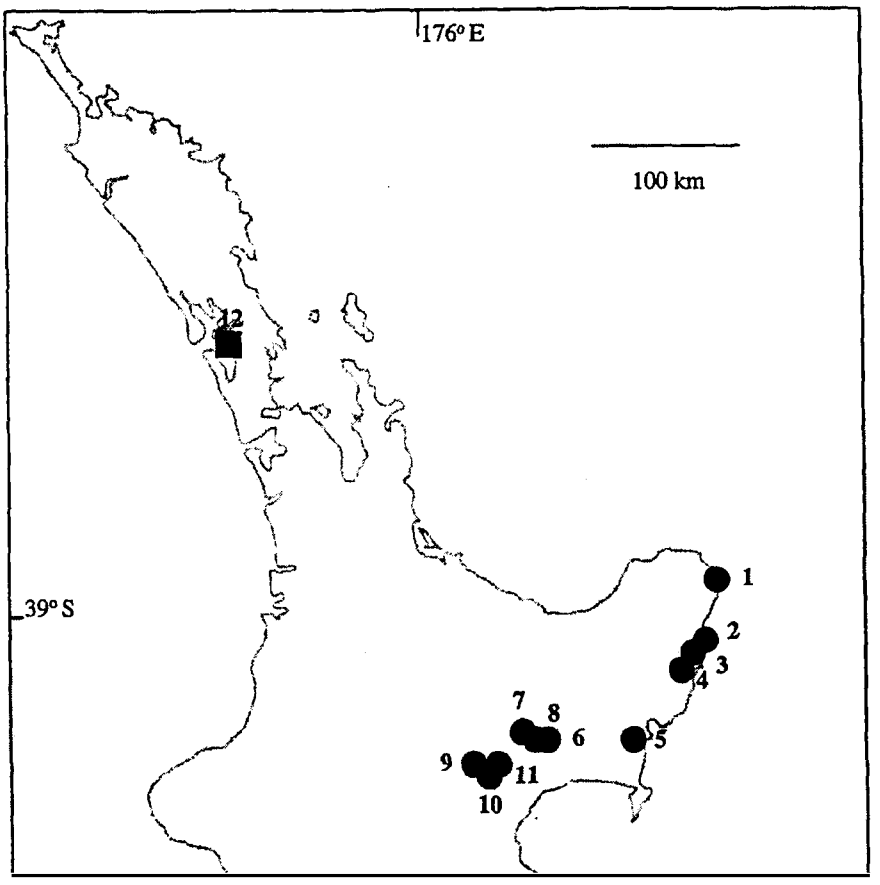

Figure 2.1 Distribution of *Clianthus* used for DNA extraction and analysis: *C. maximus* (●), and the single population of *C. puniceus* (■) derived from seed collected from Moturemu Island, Kaipara Harbour

Population names and localities shown in Table 2.3.

Several studies have been carried out to reveal the phylogenetic relationship of the New Zealand *Sophora* species using nuclear and chloroplast DNA sequence difference. Results of these studies indicated that these strategies have only a very limited ability to discriminate differences at or below the species level (Hurr et al., 1999; Mitchell and Heenan, 2002; Heenan et al., 2004). Apart from a preliminary RAPD study with a small data set (Gardiner et al., 1993), no other information at the molecular level was available regarding the genetic diversity of *Clianthus* populations.

The objectives of the work described in this chapter were to test the hypothesis that genetic diversity within *C. maximus* would not exist in the wild because of the longstanding cultivation and transport of propagules of this species by Māori and European colonists (Colenso, 1885; Shaw and Burns, 1997), and to test if molecular evidence would support the recent separation of the two species based on morphological grounds (Heenan, 2000). In addition, molecular evidence was sought to test for support for the recent separation of *S. microphylla* into the five new *Sophora* species (Heenan et al., 2001).

In order to achieve these objectives, RAPD, ISSR and AFLP markers were used to reveal the genetic diversity and relatedness for almost all plants of the remaining *C. maximus* populations, some plants originally raised from seed of the last *C. puniceus* plants in the wild, and some commercial cultivars. ISSR markers were also used for genetic relatedness analysis of all the eight New Zealand *Sophora* species, with a closely related species, *S. cassioides*, from Chile.

2.2 Materials and methods

2.2.1 Plant material

Young, expanding leaves were harvested in April (autumn) 2000 from 11 known wild populations of *Clianthus maximus* Colenso in the eastern North Island of New Zealand (Fig. 2.1). In addition, leaf samples were provided from *C. puniceus* (G Don) Sol. ex Lindl. plants, which were seedlings raised from seed collected in Kaipara Harbour (Fig. 2.1) at the time when the remaining population of parent plants died out. Up to 28 leaves (one from each plant) were sampled from each *C. maximus* population, a sample being taken from every plant for populations containing fewer than this number of plants. Plants

< c.1 km apart were treated as comprising the same population. Approximately 0.5 g of leaf material was collected per plant and leaf samples were sealed in a plastic bag and held on dry ice for transportation from remote locations. Full location and collection details and voucher specimens of all wild populations were lodged at the Ella Campbell Herbarium, Massey University.

Leaves were also sampled from plants of four named cultivars of *Clianthus* ('Flamingo', 'Maximus', 'Red Cardinal' and 'White Heron'), one *Clianthus* clone released under a trademark (Kaka King® [Naturally Native New Zealand Plants Ltd.), and two *Clianthus* populations of unknown provenance, which were grown and supplied by commercial nurseries.

As for *Clianthus*, young, expanding leaves were sampled in September 2001 from 67 40-year-old adult individual trees of nine *Sophora* species of section *Edwardsia* growing in the Landcare Research experimental nursery at Lincoln. Plants of *Sophora cassioides* were originally collected from Chile and Atlantic Island (Table 2.1). Plants of all other species, which are endemic to New Zealand, were originally collected from a range of New Zealand regions (Table 2.1). These were the same set of plants sampled by Heenan et al. (2001) for phylogenetic studies based on leaf and growth habit characters. Approximately 1 g of leaf material was collected per plant, and leaf samples were sealed in a plastic bag containing silica gel until used for DNA isolation.

Table 2.1 Samples of *Sophora* Section *Edwardsia* used for ISSR analysis

Code	Species	Collection locality
r1-r2	*Sophora cassioides*	Frutillar, Chile
r3		Gough Island, Atlantic Ocean
r4-r6		Chepu River, Chile
r7-r8		Ocean Beach, Chepu, Chile
e1	*S. chathamica*	Bay of Islands, Northland
e2		Whangarei, Northland
e3		Chatham Islands
e4		Manakau Heads, Auckland
e5		Te Whanga Lagoon, Chatham Islands
e6		Paremata, Wellington
e7		Te Whanga Lagoon, Chatham Islands
f1-f5	*S. fulvida*	Whatipu, Auckland
f6		Piha, Auckland
a1	*S. godleyi*	Taumarunui, King Country
a2-a3		Pohangina, Wellington
a4		Taumarunui, King Country
a5		Pipiriki, Wanganui
a6-a7		Waitotara, Wanganui
a8		Taihape, Wellington
a9		Ohingaiti, Wellington
l1-l5	*S. longicarinata*	Takaka Hill, Nelson
m1	*S. microphylla*	Taihape, Wellington
m2		Rakaia Gorge, Canterbury
m3		Hundalee Hills, Canterbury
m4		Rakaia Gorge, Canterbury
m5		Haast, Westland
m6		Roxburgh, Otago
m7		Haast, Westland
m8		Heaphy River, Nelson

(Table 2.1 continued)

Code	Species	Collection locality
m9	*S. microphylla*	Pareora, Canterbury
m10		Heaphy River, Nelson
m11		Banks Peninsula, Canterbury
m12		Dunedin, Otago
m13		Woodside Creek, Marlborough
m14		Wairau River, Marlborough
m16		Waiouru, Wellington
m17		Warkworth, Auckland
m18		Wairarapa, Wellington
m19		Murchison, Nelson
m20		Hapuku, Marlborough
m21		Maruia, Canterbury
m22		Seed ex beach, Chatham Islands
c1	*S. molloyi*	Stephens Island, Cook Strait
c2		Turakirae Head, Wellington
c3		Stephens Island, Cook Strait
p1-p4	*S. prostrata*	Waitohi River, Canterbury
t1-t4	*S. tetraptera*	Frasertown, Hawke's Bay

2.2.2 DNA Extraction and quantification

For RAPD, ISSR and AFLP analysis of *Clianthus* populations, the following method was used for genomic DNA isolation. Each leaf sample of c. 0.5 g was crushed in a sealed plastic bag with 2 ml of extraction buffer containing 140 mM sorbitol, 220 mM Tris-HCl (pH 7.5), 20 mM Na_2EDTA (Disodium ethylenediaminetetra-acetic acid), 0.8 M NaCl, 0.8% w/v cetyl trimethylammonium bromide, 1% w/v N-lauroyl sarcosine, and 1% w/v polyvinylpyrrolidone. A 1.6 ml sample of the resulting pulp was extracted with 0.4 ml of a chloroform and octanol mixture (24:1 v/v) at 65°C for 30 min, and then centrifuged at

12000g for 10 min. DNA was precipitated from the aqueous layer by adding 1 ml ice-cold isopropanol. The DNA pellet was recovered following a 5 min centrifugation at 12000g, washed in ethanol, dried under vacuum, and resuspended in sterile, distilled water.

For ISSR analysis of *Sophora* samples, DNA was extracted from silica-gel dried leaf samples using a protocol modified from Doyle and Doyle (1987). Approximately 50 mg tissue was ripped into small fragments using clean tweezers and placed into a 1.6 ml micro centrifuge tube, and ground into a fine powder using a 'grinding' tool made from a glass pasteur pipette (Volac). These tools were made by heating the pipette at the position where it becomes narrowed in a bunsen flame. As the glass melted, the end of the thicker section was moulded in the flame to create a small bulb that, once cooled, would comprise the grinding surface of the tool. One grinding tool was prepared for each tissue sample to avoid cross contamination between samples.

When the tissue had been ground to a fine powder, 600 µl of CTAB extraction buffer containing 2% w/v CTAB (cetyl-trimethyl-ammonium bromide, Sigma, St Louis, MI, USA), 1% w/v PVP (polyvinyl-pyrrolidone, Sigma), 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, and 20 mM EDTA (BDH chemicals, NZ) was added to the tube. The tube was capped and agitated to thoroughly mix the contents, and placed in a heating block at 65°C for c. 1 h incubation with occasional gentle agitation.

After incubation, 600 µl of chloroform was added to the tube, and the contents thoroughly mixed by vigorous agitation. The tube was then centrifuged at 5000g for 1 min. The upper aqueous phase (containing the DNA) was then transferred to a new 1.6 ml micro centrifuge tube, using a pipette with a wide-bored tip (i.e. a normal 1 ml tip with approximately 5 mm cut from the narrow end with a scalpel blade) to minimise mechanical shearing of the DNA. 600 µl of isopropanol was added to the collected aqueous solution. The tube was then inverted once to gently mix the contents, and left to stand on ice for 5-10 min to allow DNA to be precipitated.

The precipitated DNA (in white spools at about half the depth of the solution) was transferred to a new 1.6 ml micro centrifuge tube containing c. 800 µl 80% ethanol. The tube was gently inverted to wash the DNA precipitate in the ethanol solution. The ethanol was then tipped from the tube (with the DNA pelleted by gentle centrifugation if necessary). The tube was left uncapped (but covered by foil) for c. 10 min to allow the DNA to air dry. 30-100 µl TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) buffer was added

depending on the amount of DNA present. The tube was then left uncapped (but covered by foil) for an additional c.1 h to allow any remaining ethanol to evaporate. The DNA was resuspended by gentle agitation, and by overnight incubation 4-7°C. The extracted DNA was then stored at -20°C until required.

DNA quantitation was by visual comparison of DNA dilutions electrophoresed on 0.9% Ultrapure (Amersham, UK) agarose gels with 50 ng of lambda phage DNA (Life Technologies) as standard. Electrophoresis runs were for 1.5 cm at 3 V/cm, and DNA staining was with 0.75 mg l^{-1} ethidium bromide. Under these conditions *Clianthus* DNA ran as a clear band at the same position as the standard.

2.2.3 RAPD amplification

The PCR reaction mixtures (15 µl) in 96-well reaction plates contained 21 mM tris HCl (pH 8.4), 51 mM KCl, 1.3 mM $MgCl_2$, 0.1 mM of each dNTP, 0.2 µM of each primer, 1.5 ng of *Clianthus* DNA, and 0.44 U of Gibco BRL Platinum Taq polymerase (Life Technologies, USA), and were overlaid with 15 µl paraffin. Amplification was conducted in a thermal cycler (Hybaid PCR Express, UK) programmed for 1 cycle of 2.45 min at 94°C, 40 cycles of 55 s at 94°C, 55 s at 37°C, and 1.39 min at 72°C, followed by a final extension of 10 min at 72°C. The reactions were then held at 4°C.

Amplified products were electrophoresed for 8 cm on gels consisting of 0.22% UltraPure agarose (Amersham), 0.22% NuSieve agarose (FMC, Rockland, Maine), and 0.44% Separide Gel Matrix (Life Technologies) in TAE at 5.5V/cm for 2.5 h. Gels were stained with ethidium bromide at 0.75 mg l^{-1}. They were visualised under ultraviolet light and then photographed (64T Kodak Ektachrome film; Eastman Kodak, Rochester, NY). RAPD product sizes were estimated using a 100-base pair DNA standard ladder (Life Technologies).

A subset of eight DNA samples from different populations was used to screen 120 oligonucleotide primers (Operon Technologies, Alameda, Calif.) for the purpose of selecting primers that produced polymorphic, reliable and scorable markers. These primers were then used to screen all the DNA samples. To ensure reproducibility between PCR runs, four DNA samples were common to every 96-well reaction plate. Selected RAPD primers used for estimation of genetic variation in *Clianthus* were as follows:

OPP05: 5' CCC CGG T AA C 3'	OPAA01: 5' AGA CGG CTC C 3'
OPAC05: 5' GTT AGT GCG G 3'	OPAC10: 5' AGC AGC GAG G 3'
OPAC12: 5' GGC GAG TGT G 3'	OPAD02: 5' CTG AAC CGC T 3'
OPAD16: 5' AAC GGG CGT C 3'	OPAE08: 5' CTG GCT CAG A 3'
OPAF04: 5' TTG CGG CTG A 3'	OPAF16: 5' TCC CGG TGA G 3'
OPAJ06: 5' GTC GGA GTG G 3'	OPAJ08: 5' GTG CTC CCT C 3'
OPAJ12: 5' CAG TTC CCG T 3'	OPAJ13: 5' CAG CCG TTC C 3'

2.2.4 ISSR analysis

2.2.4.1 Primer selection and PCR amplification

Fifty universal ISSR primers 15-22 nucleotides in length (UBC primer set no. 9, Biotechnology Laboratory, University of British Columbia) were used for initial screening with DNA from a few accessions of commercial cultivars. Seven of the primers (811, 818, 828, 841, 844, 864, 866) that generated larger numbers of polymorphic bands were selected and further used for all the accessions in this study.

Each 20 µl amplification reaction consisted of 1 x PCR Buffer (QIAGEN, California, USA), 1.25 mM of $MgCl_2$, 1 x Q solution (QIAGEN), 0.1 mM of dNTPs, 0.2 µM primer, 20 ng sample DNA and 0.75 U Taq DNA polymerase (QIAGEN). PCR reactions were performed in a PCR Express system (Hybaid Limited, UK) under the following conditions: 2 min at 94°C for initial denaturation, 40 cycles each comprised 45 s denaturation at 94°C, 45 s annealing at 50°C and 90 s extension at 72°C, followed by extension of 5 min at 72°C.

2.2.4.2 Separation of PCR product using agarose gel

Separation of ISSR PCR product was initially conducted using 1% w/v agarose gels containing 1% w/v Seakem LE agarose (FMC BioProducts) in 1x TAE buffer. Before loading on the gel, PCR product was combined with c.1 µl of 10x loading buffer (27.5% w/v Ficoll Ty 400 (Pharmacia, Uppsala, Sweden), 0.44% w/v bromophenol blue (Serva) and 0.44% w/v xylene cyanol (Sigma)). A lane containing 400 ng of 1 kb Plus DNA Ladder (Gibco BRL, Auckland, NZ) was run as a size marker. Electrophoresis was conducted for 60-90 min at 5-6 V/cm in 1x TAE buffer. DNA samples were then

visualised by ethidium bromide fluorescence on an UV transilluminator (wavelength 302 nm, UVP Incorporated), with a digital image captured using a video camera (Panasonic) and Image PC (Scion) software.

2.2.4.3 Separation of PCR products using polyacrylamide gels

ISSR profiling was mainly carried out on silver-stained denaturing polyacrylamide (PAA) gels. Prior to each use, the glass plates used to make these gels were thoroughly cleaned with detergent and then repeatedly rinsed with water to remove any soapy residue. They were then dried with paper towels, and the surface that was to be internal was washed twice with c. 2 ml of 80% ethanol. To the cleaned surface of the shorter plate was added 2 ml of bind-silane solution containing 0.5% v/v glacial acetic acid, and 0.05% v/v Bind-Silane (Pharmacia) in 95% ethanol. This was applied thoroughly to the entire cleaned surface using a Kimwipe (Kimberly-Clark), and allowed to dry for 4-5 min. Excess Bind-Silane was then removed from the plate's surface by four vigorous washes of 2 ml 95% ethanol with a Kimwipe. The repellant RAIN-X® (Donna, USA) was applied to the cleaned surface of the longer plate according to the manufacturer's instructions. This was then dried using a Kimwipe. The treated plates were assembled with a pair of 0.4 mm spacers (Gibco BRL) separating their internal surfaces. The assembled plates and spacers were then clamped together using a S2 casting boot (Gibco BRL).

The PAA gel (5%) solution for the gels was prepared as follows: 7 ml Long Ranger gel solution (BMA, Rockland, USA), 8 M urea, 1 x TBE buffer (90 mM Tris-borate and, 1 mM EDTA pH 8.0, in a total volume of 70 ml). The mixture was filtered through two pieces of Whatman No.1 filter paper. Just prior to pouring the gel, 350 µl of 10% w/v ammonium persulphate (Sigma), and 35 µl NNN'N'-tetramethylethylenediamine (TEMED, BDH) were added to the filtered solution, and mixed with gentle agitation.

The gel solution was slowly dispensed in between the glass plates using a 60 ml syringe. A pair of shark-tooth combs (5.7 mm point-to-point spacing, Gibco BRL) were inserted in reverse orientation to form the sample well. These combs were clamped in place with several large bulldog clips. The gel was then left to polymerise for at least 1 h. It was then either used immediately, or stored overnight at 4°C with a plastic wrap seal.

The casting boot and bulldog clips were removed immediately prior to use, and the glass plate-gel assembly placed into an S2 electrophoresis apparatus (Gibco BRL). Both of the

upper and lower buffer tanks were filled with approximately 600 ml of 1 x TBE buffer. The combs were removed, and a 25 ml syringe was used to flush excess urea and any PAA fragments or air bubbles from the sample well. The gel was then pre-run at 55 W for 45 min. The sample well was again flushed to remove urea, and the shark-tooth combs inserted so that their teeth were just embedded within the gel. Each sample (3.5 μl) to be loaded on a PAA gel was mixed with 1.5 μl of formamide loading dye (98% v/v formamide, 10 mM EDTA, 0.05% w/v bromophenol blue, and 0.05% w/v xylene cyanol) in clean 0.2 ml reaction tubes. This mixture was then denatured at 94°C for 4 min in a PCR machine, and then rapidly cooled to 4°C. Samples were subsequently kept on ice until loaded.

Before and during sample loading, the sample wells were flushed again to remove excess urea and/or air bubbles from the sample well. This should be repeated several times for the unloaded wells during sample loading for best results. 4 μl of 100 bp DNA ladder (Gibco BRL) solution (1 μl in 99 μL of formamide loading dye, and denatured as above) was loaded on either side of the sample set. Electrophoresis was then carried out at 50 W for approximately 3 h, until the bromophenol blue dye front reached the bottom of the gel.

The DNA on the PAA gel was then visualised by silver-staining (Promega, 1998). The glass plates were separated by levering a scalpel blade between them. The short glass plate, to which the PAA gel had adhered, was transferred to a developing tray containing 4 l of 10% (v/v) acetic acid, and gently agitated on a shaker for 2 h to removed the urea from the gel matrix. To remove acetic acid from the gel matrix, the gel was transferred to a clean tray and washed two to three times, with gentle agitation, for c. 5 min in 2 l Milli-Q water.

For silver staining, the gel was placed in a clean tray containing 3 l of stain solution containing 6 mM silver nitrate (BDH) and 0.056% (v/v) formaldehyde (BDH), and gently agitated for 30-60 min. After a brief rinse (for less than 10 s) in 2.5 l of chilled Milli-Q water, the gel was transferred to a clean tray containing 2 l of chilled developing solution (280 mM anhydrous sodium carbonate (BDH), 0.056% (v/v) formaldehyde (BDH), and 50 μM sodium thiosulphate (BDH) added immediately prior to use). The gel was initially agitated vigorously by hand to disperse the brown precipitate that would otherwise form, and then gentle mechanical agitation was continued. When banding patterns first became apparent, the gel was quickly transferred to another clean tray containing an additional 2 l

of chilled developing solution. Mechanical agitation was continued until the ISSR profiles were satisfactorily developed. About 2 l of chilled 10% v/v acetic acid, saved from the urea removing step, was added to the tray as a fixative to stop the reaction. The solutions were vigorously mixed, and left for 3-5 min until the bubbling of CO_2 had ceased. The gel plate was then rinsed in water to remove the acetic acid, and stored upright in an operating fume-cupboard to air dry overnight before scoring.

2.2.5 AFLP analysis

Forty-six individual plants representing the 12 remaining wild populations of *Clianthus* were selected for AFLP analysis according to the previously obtained ISSR and RAPD results. The AFLP procedure used in this chapter was based on the method developed by Vos et al. (1995) with modifications. In brief, genomic DNA was used as template for an initial pre-selective PCR amplification, after being restriction digested and ligated to adapter oligonucleotides. The resulting PCR product was in turn used as template for the subsequent selective amplification to produce the selectively amplified PCR products, from which the polymorphism was evaluated.

2.2.5.1 Restriction digestion of genomic DNA

High molecular weight genomic DNA was used as template for AFLP analysis, after digestion with restriction enzymes. For each sample to be analysed, c. 500 ng of genomic DNA was restricted in a total volume of 25 µl containing 4 U *Mse*I (New England Biolabs), 10 U *Eco*RI (Roche), 50 mM potassium acetate (Sigma), 10 mM magnesium acetate (Sigma), and 10 mM Tris-HCl pH 7.5. Restriction reactions were incubated at 37°C for 3 h, with occasional agitation, and then heated to 70°C for 15 min to denature the restriction enzymes. Five µl of the restriction reaction was electrophoresed on a 1% w/v agarose gel as described in Section 2.2.4.2 to confirm complete digestion.

2.2.5.2 Preparation and ligation of oligonucleotide adapters

To form the target primer sites for the subsequent PCR steps, adapter oligonucleotides were ligated on to the 'sticky-ends' of the digested DNA. The sequences of these adapters were as follows:

*Eco*RI adapter I: 5' CTC GTA GAC TGC GTA CC 3'

*Eco*RI adapter II: 5' GAA TTG GTA CGC AGT CTA C 3'

*Mse*I adapter I: 5' GAC GAT GAG TCC TGA G 3'

*Mse*I adapter II: 5' TTA CTC AGG ACT CAT 3'

These adapters were prepared separately as follows (each in a total volume of 100 µl): 500 pmol each of *Eco*RI adapters I and II, with 45% v/v TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0); 5 nmol each of *Mse*I adapters I and II with 45% v/v TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). These two adapter mixtures were then incubated at 94°C for 4 min, before being allowed to slowly cool to room temperature.

*Eco*RI and *Mse*I adapters were ligated on to the digested DNA of each sample in a 10 µl reaction containing 2.5 pmol *Eco*RI adapter, 25 pmol *Mse*I adapter, 0.5 Weiss U T4 ligase (Gibco BRL), 1 x ligation buffer (Gibco BRL), and 5 µl of digested DNA. Ligation mixtures were incubated overnight at 4°C and stored at -80°C before being used as templates for pre-selective PCR amplifications.

2.2.5.3 Pre-selective PCR amplifications

Pre-amplifications were carried out in a total reaction volume of 20 µl, containing 1 x Q solution (Qiagen), 1 x PCR buffer (Qiagen), 250 µmol of each dNTPs (Boehringer Mannheim), 10 pmol of *Eco*RI pre-amplification primer, 10 pmol of *Mse*I pre-amplification primer, 1 U of *Taq* DNA polymerase (Qiagen), and 1 µl of ligation reaction products. Thermocycling was performed in a MJ Research PTC-200 DNA engine with 94°C for 1 min; 30 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min; then 72°C for 7 min. The *Eco*RI pre-selective primer was designed to be complementary to *Eco*RI adapter II sequence plus an "A" at the 3' end. The *Mse*I pre-selective primer was designed to complementary to *Mse*I adapter II sequence plus a "C" at the 3' end. By using pre-selective primers having an additional one base overhang, only approximately 1/16 of the linkered-digestion products could be amplified.

2.2.5.4 Selective amplifications and PCR product profiling

Selective amplifications were carried out in a total volume of 20 µl containing 1 x PCR buffer (Qiagen), 50 mM $MgCl_2$ (Qiagen), 250 µmol of each dNTPs (Boehringer Manntheim), 10 pmol of *Eco*RI selective primer, 10 pmol of MseI selective primer, 1 U of *Taq* DNA polymerase (Qiagen), and 1 µl of 20-fold diluted pre-selective PCR products.

Thermocycling was performed in a MJ Research PTC-200 DNA engine with the following profile: 94°C for 1 min; 6 cycles of 94°C for 30 s, 65°C for 45 s, 72°C for 1 min; 6 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 1 min; 24 cycles of 94°C for 30 s, 56°C for 45 s, 72°C for 1 min; final extension of 72°C for 5 min. Other two or three base pairs were added at the 3' end of the pre-selective primers to further reduce the number of PCR products in order that they were discriminated on the PAA gel. The selective primer sequences used to screen for the best primer combinations for *Clianthus* genetic diversity analysis were as follows:

*Eco*RI selective primers	E-AAA: 5' GAC TGC GTA CCA ATT CAA A 3'
	E-AAT: 5' GAC TGC GTA CCA ATT CAA T 3'
	E-ACC: 5' GAC TGC GTA CCA ATT CAC C 3'
	E-ACG: 5' GAC TGC GTA CCA ATT CAC G 3'
	E-AGC: 5' GAC TGC GTA CCA ATT CAG C 3'
	E-ATA: 5' GAC TGC GTA CCA ATT CAT A 3'
	E-ATT: 5' GAC TGC GTA CCA ATT CAT T 3'
	E-AGG: 5' GAC TGC GTA CCA ATT CAG G 3'
*Mse*I selective primers:	M-CAA: 5' GAT GAG TCC TGA GTA ACA A 3'
	M-CAC: 5' GAT GAG TCC TGA GTA ACA C 3'
	M-CAG: 5' GAT GAG TCC TGA GTA ACA G 3'
	M-CTA: 5' GAT GAG TCC TGA GTA ACT A 3'
	M-CTC: 5' GAT GAG TCC TGA GTA ACT C 3'
	M-CTG: 5' GAT GAG TCC TGA GTA ACT G 3'
	M-CAAG: 5' GAT GAG TCC TGA GTA ACA AG 3'

To test the efficacy of the selective amplification before PAA gel electrophoresis, 5 µl of PCR product was electrophoresed on a 2% w/v agarose gel (see Section 2.2.4.2). Consistent smearing between 100-1000 bp with at least some emergent bands indicated successful amplification in all samples.

AFLP PCR products were profiled by electrophoresis on denaturing PAA gels and visualised with silver staining (see Section 2.2.4.3).

2.2.6 Data analysis

Individual RAPD bands were scored from enlarged colour photographs, and the profiles of ISSR and AFLP were scored over a light-box. A band at a given size position in any sample was assumed to represent an independent character, and was scored as present (1) or absent (0) in all samples. The resultant binary data matrix was further analysed. Faint bands of doubtful reproducibility were ignored, and those that were very faint compared to others in the same position were scored as absent. Qualitative differences in band intensity were otherwise not considered.

Several different measures of genetic variation were estimated for each wild population and cultivated collection. These were: Polymorphism (*P*) the percentage of loci examined that showed variation; Nei's gene diversity (H_e) (Nei, 1987) and Shannon's diversity index (*I*) (Lewontin, 1973). To assess clonal structure within populations the number of unique multilocus genotypes (*G*) was measured as was the number of ramets per genet (*R/G*), which was the total number of samples divided by the number of unique genotypes. Relationships between population size and genetic diversity parameters were examined using linear regression.

Overall genetic divergence among populations was measured using G_{ST}. Nei's unbiased genetic distance (Nei, 1978) was also estimated between all possible pairs of populations and used to perform a hierarchical cluster analysis (unweighted pair-group method with arithmetic averaging-UPGMA). Correspondence between the genetic and geographic distances among wild populations was examined using a Mantel test of matrix correlation using MANTEL Version 2.0 (Liedloff, 1999) with the null distribution of the Z-statistic based on 1000 random permutations. All diversity estimates and distances were calculated using POPGENE Version 1.32 (Yeh and Boyle, 1997).

When analyses of separate RAPD and ISSR data were found to yield essentially the same results, both quantitatively and qualitatively, the analyses were repeated using the combined data sets. A limited number of individuals (6 from 5 populations) and bands (12 from 3 primers), for which complete data sets for both RAPD and ISSR analyses were not available, were omitted.

2.3 Results

2.3.1 Genetic diversity analysis of *Clianthus* populations using RAPD and ISSR markers

Fourteen RAPD primers selected from 120 screened primers consistently generated between one and six clear amplification products, ranging in size from 350 bp to 1700 bp, giving a total of 50 putative genetic loci, all of which were polymorphic. Data for 38 of these loci were combined with those from the ISSR analyses. For the seven selected ISSR primers, 142 PCR products were generated in total, with more than 20 products per PCR reaction. The mean percentage of polymorphism was 37.32% and the length of the products ranged from 150 bp to 1250 bp (Table 2.2). The primer 818 alone was able to discriminate all the commercial cultivars and many of the wild populations (Fig. 2.2).

Table 2.2 Summary of ISSR PCR amplification results for *Clianthus*

Primer	Nucleotide sequences	No. of bands	Polymorphic bands	Polymorphism (%)	Product length (bp)
UBC 811	$(GA)_8C$,	21	9	42.86	200-1140
UBC 818	$(CA)_8G$	23	8	34.78	180-990
UBC 828	$(TG)_8A$	26	9	4.62	570-1150
UBC 841	$(GA)_8YC$	27	14	51.85	150-1250
UBC 844	$(CT)_8RC$	17	4	23.53	460-810
UBC 864	$(ATG)_6$	13	5	38.46	330-1180
UBC 866	$(CTC)_6$	15	4	26.67	270-890
Total	/	142	53	/	150-1250
Mean	/	20.29	7.57	37.32	/

Y=pyrimidine, R=purine

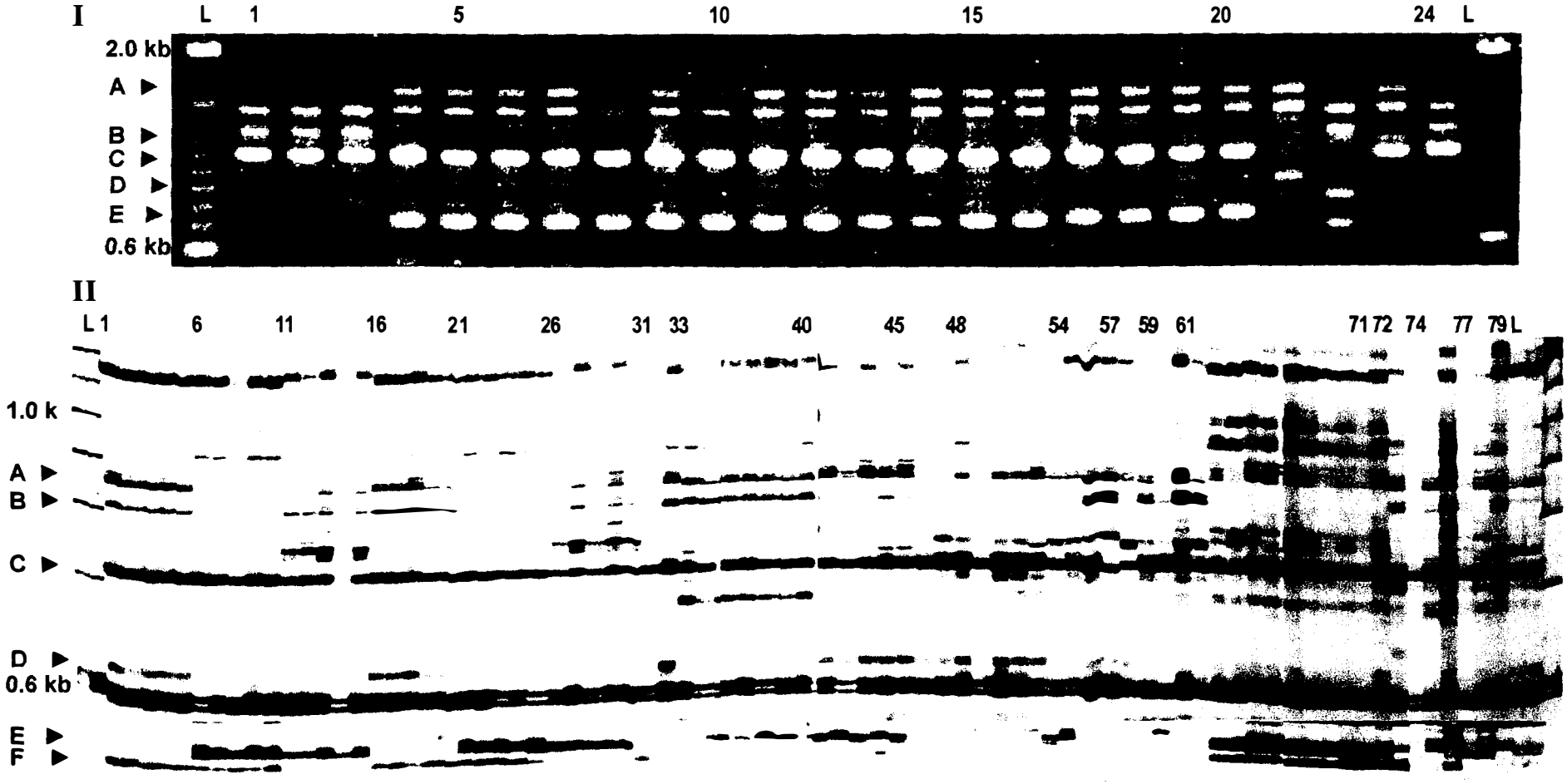

Figure 2.2 Representative RAPD and ISSR profiles for *Clianthus* wild populations and cultivars

I: RAPD profiles generated using 10-bp primer OPAJ13 (Lanes 1-24). 1-4: Mangahauini; 4-20: Koutunui; 21: Moturemu Is; 22: Rosie Bay; 23: Shines Falls; 24: Waipuhatuhatu.

II: ISSR profiles generated using the UBS primer 818 (Lanes 1-79). 1-5: Kaka King; 6-10: White Heron; 11-15: Flamingo; 16-20: Maximus; 21-25: Unknown provenance #1; 26-30: Unknown provenance #2; 31-32: Red Cardinal; 33-39: Koutunui; 40-44: Mangahauini; 45-47: Bartletts; 48-53: Ngamoko; 54-56: Waitio Bay; 57-58: Shines Falls; 59-60: Heidi Track; 61-70: Moturemu Is; 71: Southern Woods; 72-73: Te Heru o Tureia; 74-76: Rosie Bay; 77-79: Waipuhatuhatu.

L: 100-bp DNA ladder. Arrow-heads on the left indicate the fragments that were scored along with fragments from other primers to examine variation within and among populations.

Population genetic variation summary statistics are presented in Table 2.3. Polymorphism (*P*) in the 12 wild populations of *Clianthus* was in the range 0-13.3, and was correlated with population size (R^2=0.61, p<0.05) (Fig. 2.3 A), with the four smallest populations at Southern Woods (N=1), Shines Falls (N=2), Heidi Track (N=2) and Te Heru o Tureia (N=l) (populations 4, 9-11, Fig. 2.1) having the lowest variation, and the larger population having the highest. The number of multilocus genotypes *(G)* was in the range 1-20, and was also positively affected by population size (R^2= 0.90, p<0.0001) (Fig. 2.3 B). There were considerable differences among wild populations in gene diversity (H_e = 0-0.054), the Shannon Index (*I* = 0-0.079) and the number of ramets per genet *(R/G* = 1-1.91). None of these variables was significantly related to population size, although the smallest populations tended to have the lowest H_e and *I*. The single *C. puniceus* population from Kaipara Harbour (population code 1 Fig. 2.1) exhibited moderate variation relative to the 11 *C. maximus* populations.

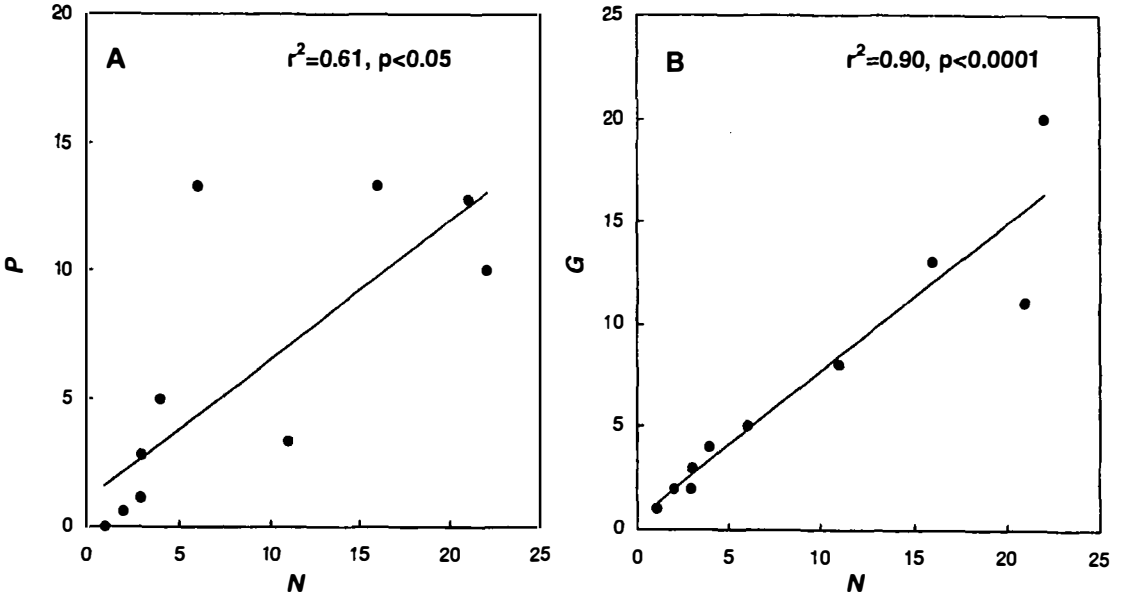

Figure 2.3 Correlation between size of wild *Clianthus* populations and (A) polymorphisms *(P)* and (B) number of multilocus genotypes *(G)*, based on 38 RAPD loci and 142 ISSR loci

Table 2.3 **Genetic variation statistics for 12 wild populations and seven cultivated accessions of *Clianthus* based on 142 ISSR and 38 RAPD loci. Sample size *(N)*; percent polymorphism *(P)*; gene diversity *(H_e)*; Shannon index *(I)*; number of multilocus genotypes *(G)*; number of ramets per genet *(R/G)*. Population codes 1-11: *Clianthus maximus*; population code 12: *Clianthus puniceus* after Heenan (2000)**

Origin	Population	Locality	Code	*N*	*P*	H_e (SD)	*I* (SD)	*G*	*R/G*
Wild	Waipuhatuhatu	Te Araroa	1	22	10.0	0.033 (0.110)	0.049 (0.160)	20	1.10
	Koutunui	Tokomaru Bay	2	16	13.33	0.032 (0.097)	0.052 (0.147)	13	1.23
	Mangahauini	Hikuwai	3	4	5.0	0.020((0.089)	0.030 (0.130)	4	1
	Southern Woods	Tolaga Bay	4	1	0	0	0	1	1
	Bartletts	Wharerata	5	3	1.11	0.005 (0.047)	0.007((0.067)	3	1
	Ngamoko	Waikaremoana	6	6	13.33	0.054 (0.141)	0.079 (0.204)	5	1.20
	Waitio Bay/te Umutiti	Waikaremoana	7	2	2.78	0.014 (0.082)	0.019 (0.114)	2	1
	Rosie Bay	Waikaremoana	8	21	12.78	0.031 (0.094)	0.050 (0.144)	11	1.91
	Heidi Track	Maungaharuru	9	2	0.56	0.003 (0.037)	0.004 (0.052)	2	1
	Shines Falls	Maungaharuru	10	2	0.56	0.003 (0.037)	0.004 (0.052)	2	1
	Te Heru o Tureia	Maungaharuru	11	1	0	0	0	1	1
	Moturemu Is	Kaipara Harbour	12	11	3.33	0.011 (0.060)	0.016 (0.091)	8	1.38
	Mean			7.58(7.90)	5.23(5.53)	0.017 (0.066)	0.026 (0.097)	6 (5.92)	1.15(0.27)
	Overall			91	50.56	0.169 (0.206)	0.251 (0.291)	72	1.26
Cultivated	Kaka King ®			5	0	0	0	1	5
	Flamingo			4	1.67	0.006 (0.048)	0.009 (0.072)	2	2
	White Heron			5	1.11	0.004 (0.034)	0.006 (0.053)	3	1.67
	Maximus			5	2.22	0.008 (0.054)	0.012 (0.081)	4	1.25
	Red Cardinal			2	1.11	0.006 (0.053)	0.008 (0.073)	2	1
	Unknown provenance #1			5	0	0	0	1	5
	Unknown provenance #2			4	1.11	0.004 (0.039)	0.006 (0.059)	2	2
	Mean			4.29 (1.11)	1.03 (0.81)	0.004 (0.033)	0.006 (0.048)	2.14(1.07)	2.56(1.71)
	Overall			30	29.44	0.124 (0.198)	0.179 (0.283)	14	2.14

Inter-population genetic distances for wild populations ranged from D=0.069-0.324 (Table 2.4) with mean population values varying from D=0.155-0.239, suggesting considerable genetic differentiation across the range of *Clianthus* in the wild. Taking all 12 wild populations together, the Mantel test indicated a weak but highly significant correlation between geographic and genetic distance (Fig. 2.4 A, $g = 2.657$, $R^2 = 0.46$, $p<0.001$). Excluding the most geographically isolated of the 12 *Clianthus* populations (that of *C. puniceus* (Heenan, 2000) Kaipara Harbour, population code 12) almost doubled the amount of genetic divergence that could be accounted for by geographic distance, and increased the significance of the relationship (Fig. 2.4 B, $g = 5.101$, R^2 =0.71, $p<0.00001$).

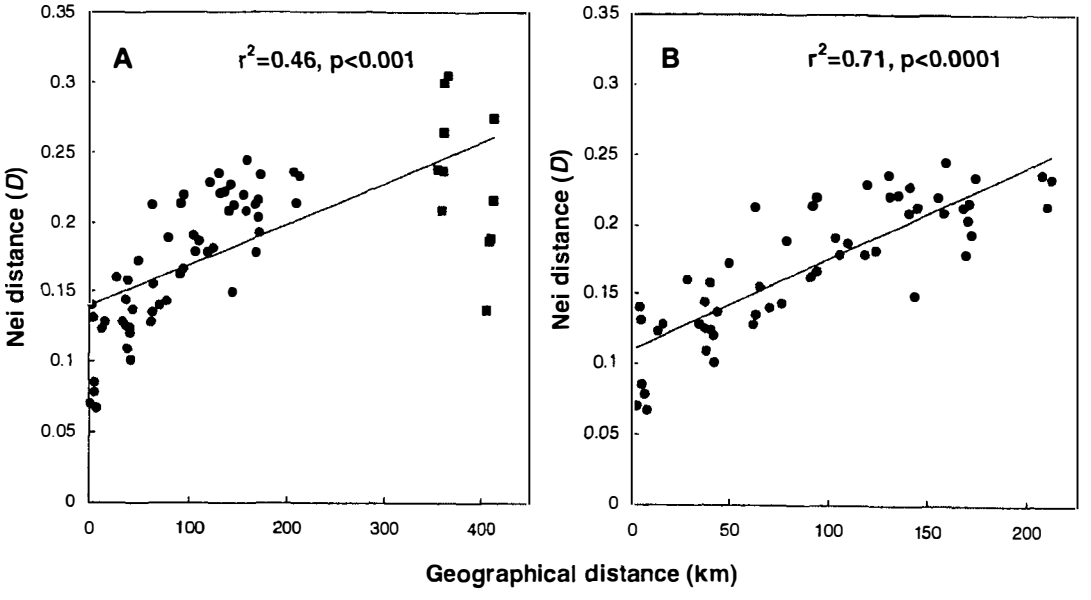

Figure 2.4 Effect of geographical separation of wild *Clianthus* populations on Nei's interpopulation genetic distance for (A) all *C. maximus* populations (●) and that of *C. puniceus* from Moturemu Island, Kaipara Harbour (■); and (B) all *C. maximus* populations alone, based on 38 RAPD loci and 142 ISSR loci

Table 2.4 **Geographic distance (km) above diagonal and Nei's genetic distance *(D)* below diagonal for 12 wild populations of *Clianthus*, based on 142 ISSR and 38 RAPD loci. Population codes 1-11: *Clianthus maximus;* population code 12: *Clianthus puniceus* after Heenan (2000)**

Population	1	2	3	4	5	6	7	8	9	10	11	12	Mean (SD)
1	/	49.9	61.7	76.9	140.3	169.2	170.2	173.7	207.3	210.5	212.4	405.9	170.7 (98.1)
2	0.180	/	12.9	28.7	92.1	130.2	131.5	135.7	167.7	170.8	172.1	413.9	136.9 (108.1)
3	0.132	0.123	/	15.8	79.3	118	119.4	123.7	155.3	158.5	159.6	411.1	128.7 (107.5)
4	0.151	0.169	0.128	/	63.6	103.7	105.2	109.7	140.6	143.7	144.7	408.9	122.0 (104.8)
5	0.203	0.232	0.197	0.211	/	63.2	65.3	70.4	91.6	94.3	93.9	415.3	115.4 (101.9)
6	0.196	0.243	0.184	0.191	0.149	/	2.1	7.1	38.1	41.3	43.3	361.8	98.0 (102.3)
7	0.227	0.230	0.234	0.184	0.176	0.069	/	5.1	37.2	40.4	42.5	359.9	97.6 (102.7)
8	0.241	0.221	0.180	0.187	0.138	0.063	0.082	/	34.2	37.3	39.9	355.9	99.3 (101.6)
9	0.221	0.234	0.222	0.164	0.217	0.127	0.134	0.151	/	3.2	6.3	362	113.0 (108.6)
10	0.260	0.224	0.226	0.231	0.183	0.106	0.118	0.121	0.149	/	4.3	362.1	115.1 (108.7)
11	0.257	0.205	0.250	0.218	0.186	0.132	0.095	0.132	0.140	0.074	/	366.1	116.8 (109.2)
12	0.130	0.224	0.189	0.191	0.265	0.249	0.231	0.246	0.264	0.316	0.324	/	384.0 (26.3)
Mean D	0.200	0.208	0.187	0.184	0.196	0.155	0.162	0.160	0.184	0.182	0.183	0.239	
(SD)	0.045	0.035	0.042	0.029	0.034	0.060	0.062	0.058	0.046	0.072	0.073	0.054	

The UPGMA analysis, across all plants samples from the 12 wild populations, grouped all individuals of the same population very closely together to form a distinct cluster from other populations, indicating that the inter-population variation was far more important than intra-population variation, and that there was no or little interaction between populations. The strong effect of geographic location on genetic composition was also supported by this analysis, clustering the populations into two main groups (Fig. 2.5) and reflecting the more north-eastern and south-western distributions of *C. maximus* populations (Fig. 2.1, populations 1-4 and 6-11, respectively). The population of *C. maximus* that is geographically relatively isolated from each of these two groupings (population 5), associated with those of more inland distribution (Fig. 2.1). The population of *C. puniceus* from Kaipara Harbour (population 12) was grouped together with the northeastern populations (1-4) of *C. maximus*, and not with the more inland and southwestern populations of *C. maximus*.

The UPGMA analysis carried out at the population level resulted in a very similar dendrogram to that carried out at the individual level (Fig. 2.6). Again, the population of *C. puniceus* was grouped together with the northeastern populations of *C. maximus*, and most strongly with that from Te Araroa (population 1).

Both mean and overall values for polymorphism, gene diversity, Shannon Index and number of multilocus genotypes were consistently lower in the samples from cultivated sources than for wild populations, while the number of ramets per genet was higher. However, although the overall variation at the locus level in samples from cultivated plants represents a small subset of the variation in the wild, none of the cultivated genotypes was identical to any of the samples taken from wild populations. In the UPGMA analysis, samples from three cultivated accessions were closely grouped, and associated with the wild *C. maximus* population from Koutunui (population 2), while two other cultivated accessions were associated with the *C. puniceus* (population 12). The other two cultivated accessions formed a distinct sub-cluster between that of wild populations.

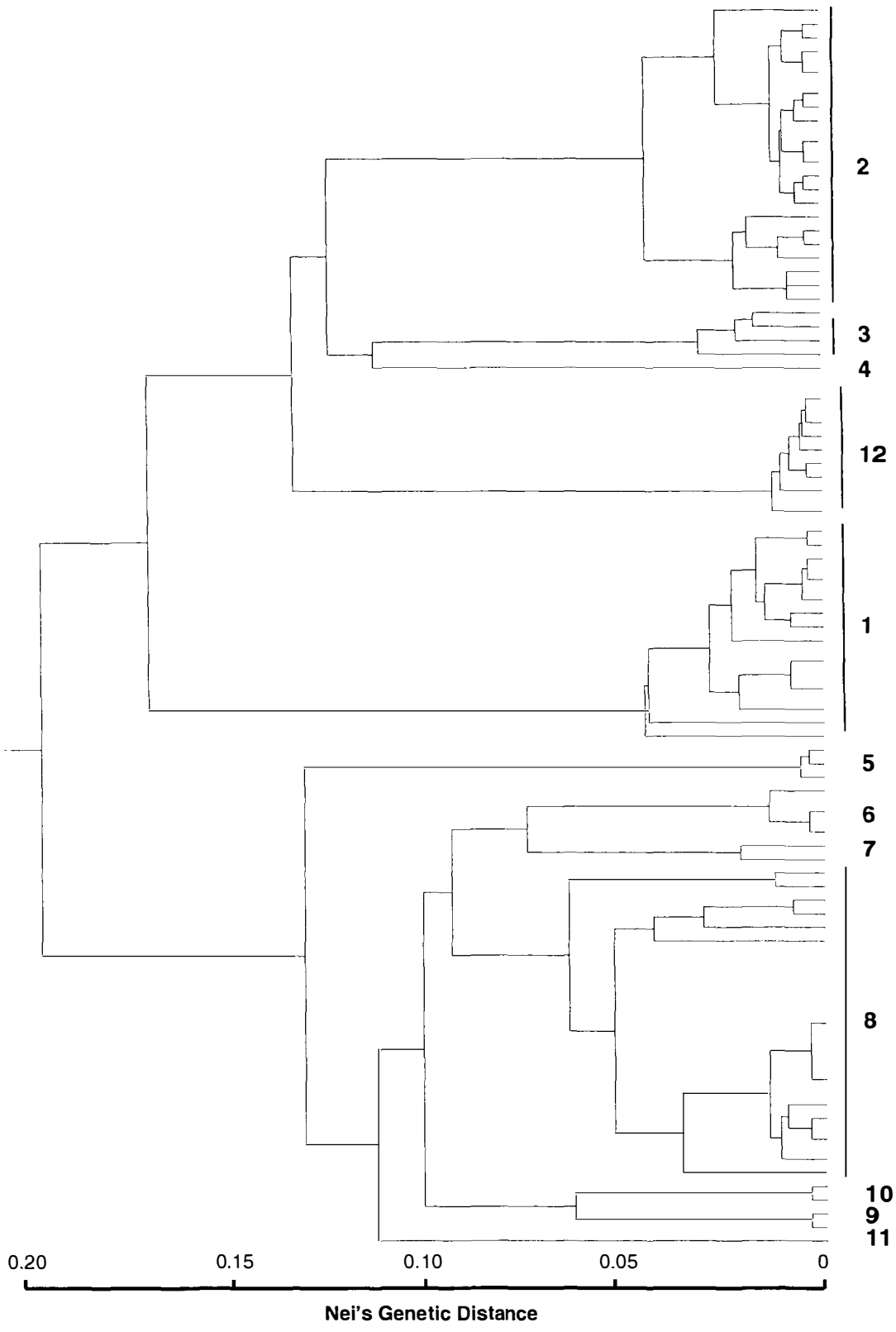

Figure 2.5 Genetic relatedness dendrogram for individual plants of 12 wild *Clianthus* populations generated by UPGMA analysis based on 142 ISSR and 38 RAPD loci

1: Waipuhatuhatu; 2: Koutunui; 3: Mangahauini; 4: Southern Woods; 5: Bartletts; 6: Ngamoko; 7: Waitio Bay; 8: Rosie Bay; 9: Heidi Track; 10: Shines Falls; 11: Te Heru o Tureia; 12: Moturemu Is.

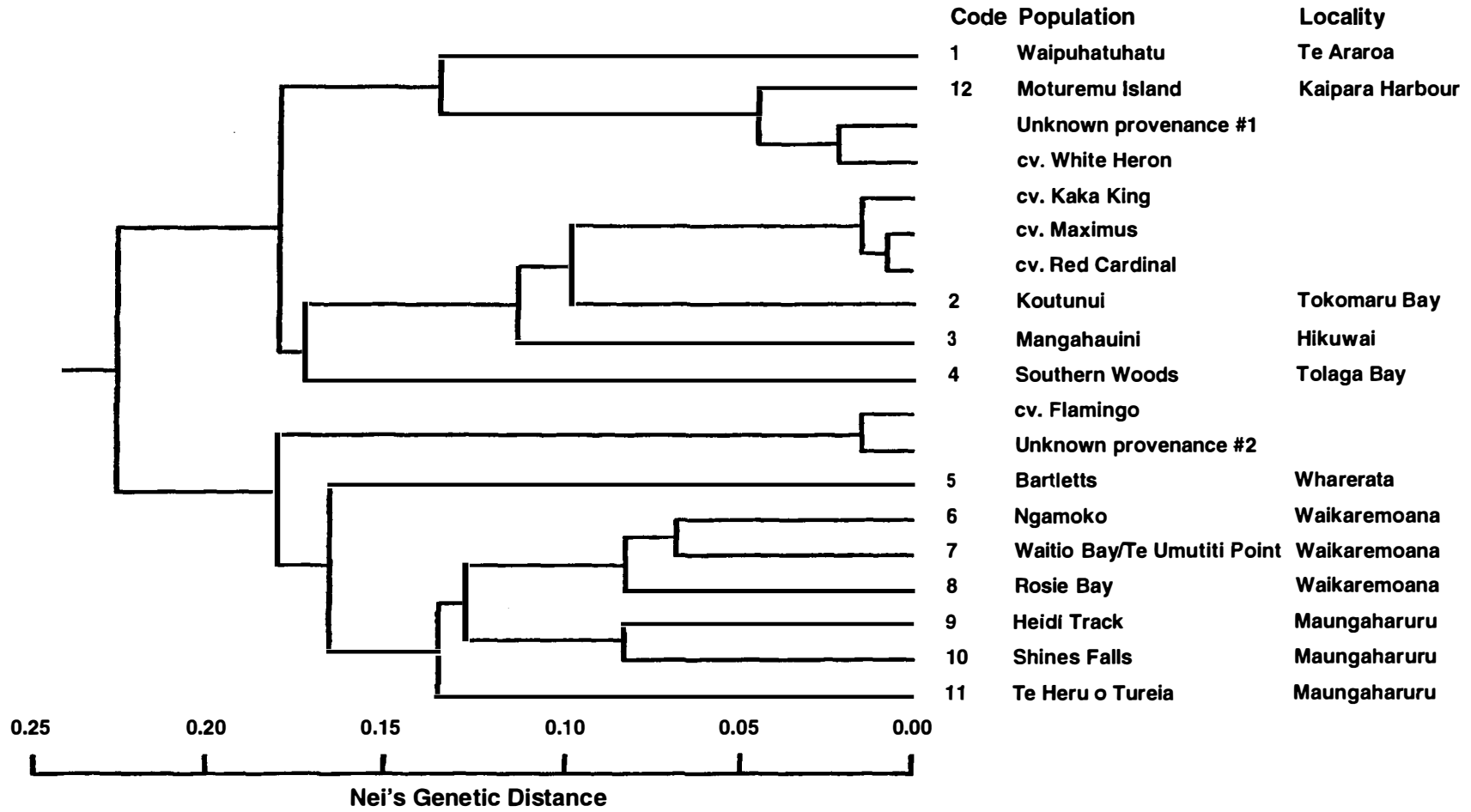

Figure 2.6 Genetic relatedness dendrogram of 12 wild *Clianthus* populations and seven commercial cultivars generated by UPGMA analysis based on 142 ISSR and 38 RAPD loci

2.3.2 Genetic diversity analysis of *Clianthus* populations using AFLP markers

Fifty-six combinations of selective amplification primers were screened using three pre-amplification products, from different population groups, as templates. Eight primer combinations (E-AAA/M-CAG, E-AAT/M-CTA, E-AGC/M-CAA, E-AGG/M-CAA, E-AGG/M-CAAG, E-ATA/M-CAT, E-ATT/M-CAA, E-ATT/M-CTA) were selected for further analysis of the full sample set of 46 individual plants. The choice of primer combinations was based on the visual clarity of banding patterns generated and the polymorphism presented among three bulked templates.

In general, 50-150 scorable bands were generated from each primer combination. However, a great number of bands were considered noisy for the current analysis since polymorphism was exhibited at both the population level and among individuals in the same population. As the main objective for this study was to obtain information about genetic diversity and relatedness at the population level, only major bands representing polymorphism in the population level were scored for the current study. In total, 125 bands generated from eight primer combinations were used for further analysis, of which 45 bands were polymorphic, with 36% polymorphism. The E-AAA/M-CAG and E-ATA/M-CTA primer combinations produced the greatest number (9) of valuable polymorphic fragments, while the E-ATT/M-CAT primer combination yielded only one valuable polymorphic band (Table 2.5). While PCR fragments were generated across the entire size range of 100 to 1500 bp, the sizes of the majority of scored fragments were in the 500-1000 bp range (Fig. 2.7).

The total gene diversity across all populations (H_T) was 0.106 and the within population diversity (H_S) was 0.014 (Table 2.5). Low H_S relative to H_T is consistent with the result from the UPGMA dendrogram generated for RAPD and ISSR markers, showing distinctive sub-clusters for individuals in each population. The genetic distances among populations varied from 0.010 to 0.194 (Table 2.6), with very similar overall distributions to that revealed by the combination RAPD and ISSR markers (Table 2.4).

The UPGMA dendrogram (Fig. 2.8) was also very similar to that generated for RAPD and ISSR data, grouping the 12 wild populations into two major clusters. Again, the population of *C. puniceus* from Kaipara Harbour (population 12) was grouped together

with the northeastern populations of *C. maximus*, and was most strongly associated with population 1 from Te Araroa. The only major difference between the two results was that the geographically relatively isolated population 5 was grouped together with the northeastern populations for AFLP data, instead of with the south-western populations grouped by RAPD and ISSR UPGMA. The AFLP UPGMA result could be clearly illustrated by the primer combination E-AAA/M-CAG, for which six of the nine major bands could clearly separate the north-eastern populations from south-western ones, with population 5 having the same band pattern as those of the north-eastern populations in most cases (Fig. 2.1; Fig. 2.7).

Table 2.5 Summary of AFLP amplification results for *Clianthus*

Primer combination	No. loci	Polymorphic loci	H	I	H_T	H_S	G_{ST}
E-AAA / M-CAG	14	9	0.3179	0.442	0.3153	0.0591	0.8135
E-AAT / M-CTA	11	2	0.0253	0.0467	0.0308	0.0068	0.7554
E-AGC / M-CAA	20	8	0.0786	0.1352	0.067	0.0042	0.911
E-AGG / M-CAA	17	5	0.1024	0.152	0.1103	0.0068	0.9434
E-AGG / M-CAAG	20	8	0.1171	0.18	0.1168	0.0104	0.8779
E-ATA / M- CTA	20	9	0.1177	0.1868	0.1134	0.0126	0.9155
E-ATT / M-CAA	13	3	0.0624	0.0993	0.0479	0.0096	0.7366
E-ATT / M-CAT	10	1	0.0499	0.0692	0.05	0	1
Mean	15.63	5.63	0.1089	0.1639	0.1064	0.0137	0.8692
Overall Mean		0.112	0.1705	0.1096	0.0135	0.8768	
SD			0.1794	0.2576	0.0294	0.0009	

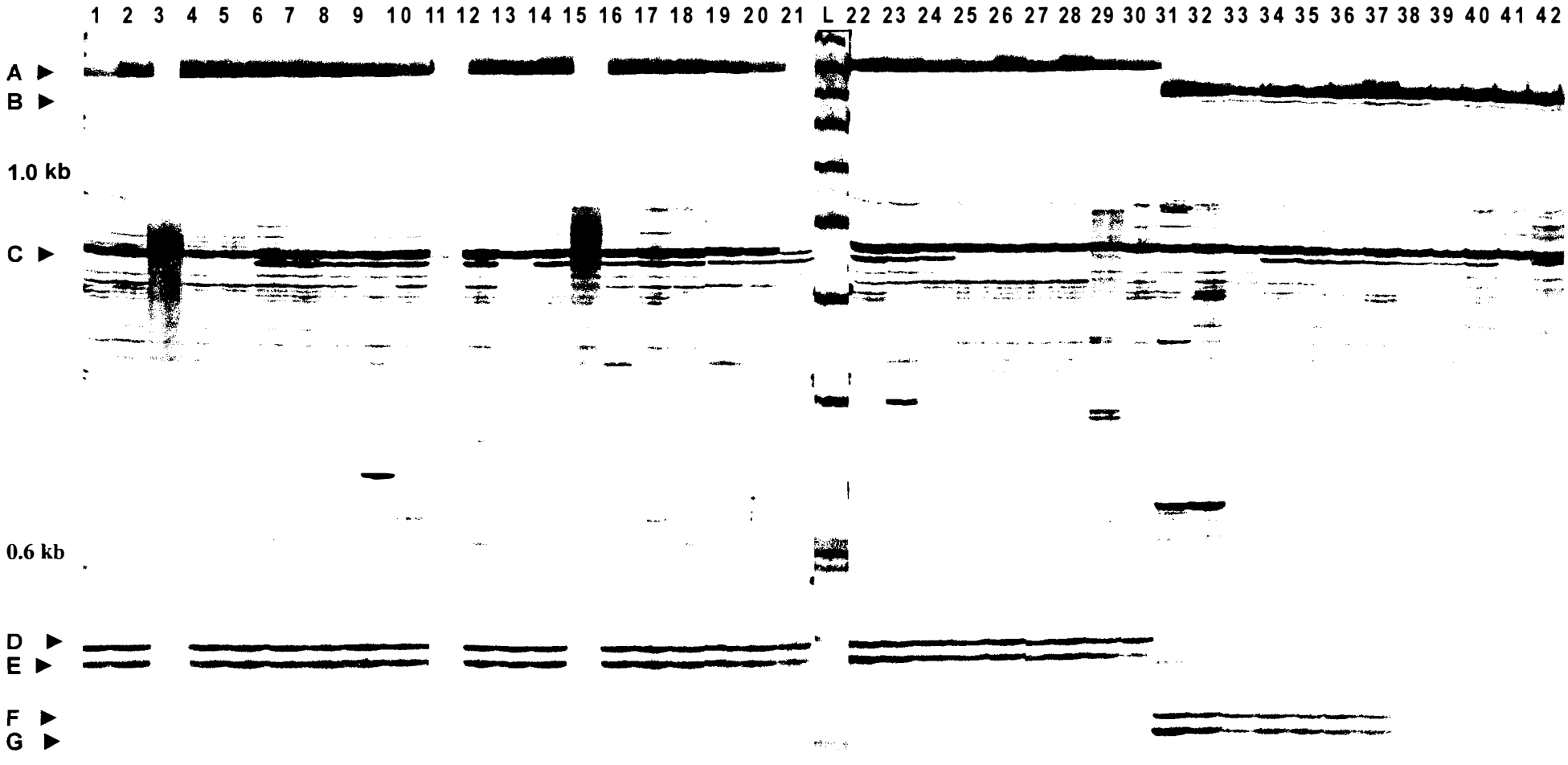

Figure 2.7 Representative AFLP profiles generated using the E-AAA/M-CAA primer combination

Lanes 1-42 contain DNA amplified from the representative single plants of the 12 wild populations. 1-5: Koutunui; 6-12: Mangahauini; 13: Southern Woods; 14-19: Moturemu Is; 20-25: Waipuhatuhatu; 26-28: Bartletts; 29-31: Ngamoko; 32-34: Waitio Bay; 35-37: Rosie Bay; 38-39: Shines Falls; 40-41: Heidi Track; 42: Te Heru o Tureia. L: 100-bp DNA ladder. Arrow-heads (A-G) on the left indicate the AFLP fragments that were scored along with fragments from other primer pairs to examine variation within and among populations of *Clianthus*.

Table 2.6 Nei's genetic distance for 12 wild populations of *Clianthus*, based on 125 AFLP loci. Population codes 1-11: *Clianthus maximus;* population code 12: *Clianthus puniceus* after Heenan (2000)

Population	1	2	3	4	5	6	7	8	9	10	11	12
1	****	0.8870	0.8870	0.9376	0.9157	0.8777	0.8748	0.8875	0.8311	0.8419	0.8656	0.9751
2	0.1199	****	0.9373	0.9144	0.8382	0.8740	0.8769	0.8724	0.8331	0.8260	0.8724	0.8900
3	0.1199	0.0647	****	0.9386	0.8717	0.8772	0.9018	0.8992	0.8452	0.8699	0.8983	0.8915
4	0.0644	0.0895	0.0634	****	0.9021	0.8584	0.8554	0.8679	0.8240	0.8267	0.8555	0.9378
5	0.0881	0.1765	0.1373	0.1031	****	0.9112	0.8939	0.9074	0.8540	0.8730	0.9017	0.9020
6	0.1305	0.1347	0.1311	0.1527	0.0930	****	0.9694	0.9753	0.9258	0.9214	0.9681	0.8706
7	0.1338	0.1314	0.1033	0.1562	0.1122	0.0310	****	0.9900	0.9488	0.9462	0.9896	0.8795
8	0.1194	0.1365	0.1063	0.1417	0.0972	0.0250	0.0101	****	0.9323	0.9270	0.9783	0.8906
9	0.1850	0.1826	0.1681	0.1936	0.1579	0.0771	0.0525	0.0701	****	0.8893	0.9518	0.8236
10	0.1721	0.1911	0.1393	0.1904	0.1358	0.0818	0.0553	0.0758	0.1174	****	0.9421	0.8424
11	0.1443	0.1365	0.1073	0.1560	0.1035	0.0324	0.0105	0.0220	0.0494	0.0596	****	0.8600
12	0.0252	0.1165	0.1149	0.0642	0.1032	0.1386	0.1284	0.1158	0.1941	0.1715	0.1508	****
Mean	0.1184	0.1347	0.1140	0.1250	0.1189	0.0936	0.0839	0.0837	0.1316	0.1263	0.0884	0.1108
SD	0.0457	0.0385	0.0310	0.0499	0.0290	0.0478	0.0534	0.0470	0.0594	0.0518	0.0552	0.0196

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

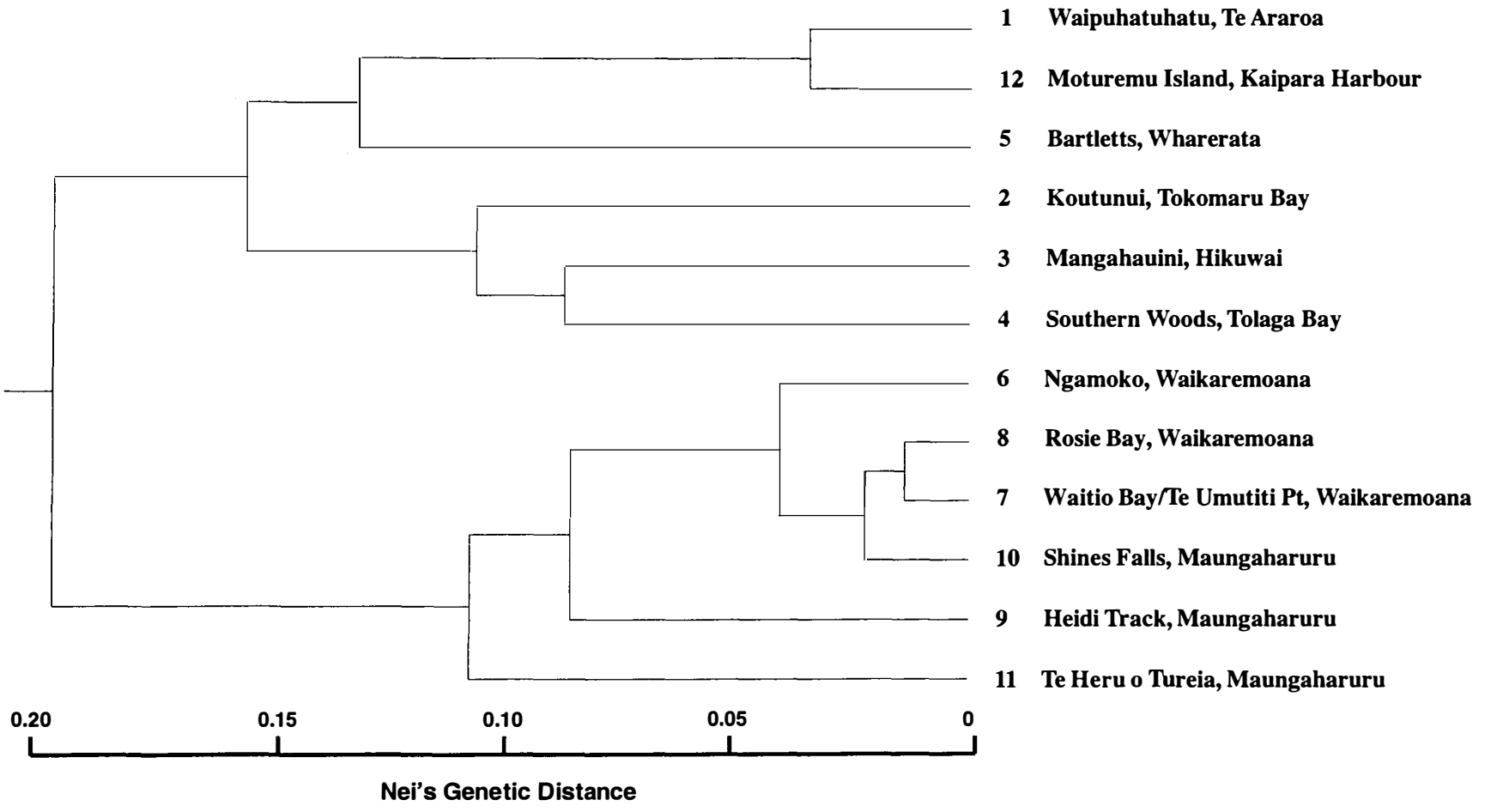

Figure 2.8 Genetic relatedness dendrogram of 12 wild *Clianthus* populations generated by UPGMA analysis based on 125 AFLP loci

2.3.3 Genetic diversity analysis of *Sophora* species using ISSR markers

Forty primers from the University of British Colombia ISSR primer set #9 were initially screened using genomic DNA from a small set of *Sophora* species. Six of these primers (811, 822, 828, 841, 844, and 868) resulted relatively more scorable and polymorphic bands on silver stained polyacrylamide gels, and were selected for further analysis of the 67 individual plants from eight New Zealand *Sophora* species and one closely related *Sophora* species from Chile. Between six and 11 scorable bands, ranging in size from 200 to 1200 bp, were generated from each of these primers, resulting in a total of 53 loci with 77.4% polymorphism. In general, a high proportion of polymorphism occurred not only among species, but also among different individuals within the same species. In many cases, the same band that varied within species could be shared by some samples from different species. This was especially the case for species previously classified under species *Sophora microphylla*, such as *S. chathamica*, *S. fulvida*, *S. godleyi*, and *S. longicarinata* (Fig. 2.9).

Genetic distances among genotypes varied from 0.051 to 0.489 (Table 2.7), suggesting considerable genetic differentiation across the nine tested species. Very low genetic distances (<0.01) between *S. microphylla* and species such as *S. chathamica*, *S. godleyi*, and *S. longicarinata* were found, while the highest genetic distances (>0.30) existed between *S. prostrata* and species such as *S. cassioides*, *S. fulvida* and *S. tetraptera*. *Sophora prostrata* and *S. cassioides* had remarkably high mean genetic distances, being 0.30 and 0.283, respectively.

The UPGMA analysis carried out based on the individual level across all plants from the nine species resulted in an ambiguous dendrogram, except in the case of *S. cassioides*, *S. tetraptera* and *S. prostrata*, for which all individuals were grouped together to form distinct clusters (Fig. 2.10). All individual samples from the other six species were dispersed across different species, despite some individuals in the same species grouping together to form small sub-clusters.

The UPGMA analysis at the species level (Fig. 2.11) did not yield any distinct group, with one species at the basal position of the others. *Sophora prostrata* was the species most distant from the others, followed by *S. cassioides*, and then *S. molloyi*. Unexpectedly, *S. tetraptera* was placed among species of the *Sophora microphylla* complex.

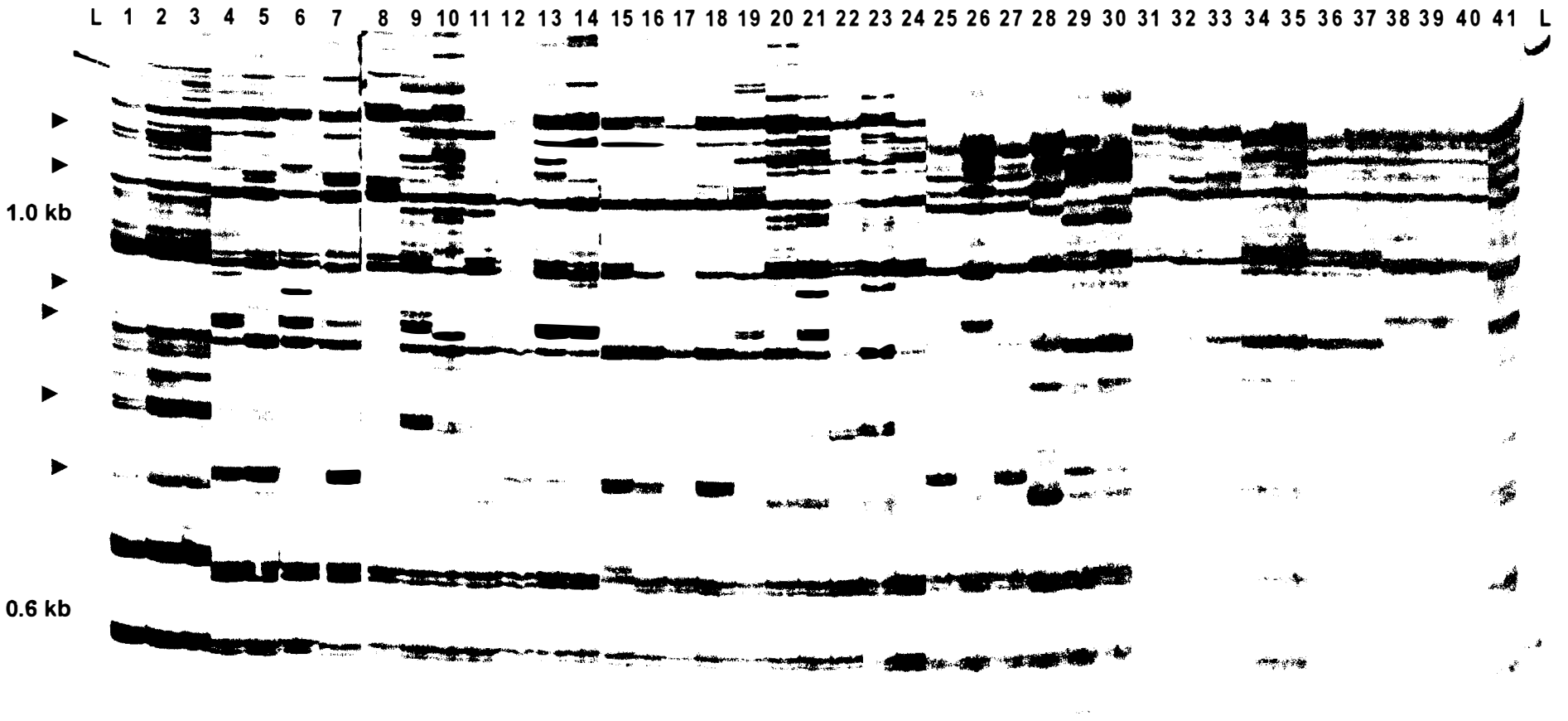

Figure 2.9 Representative ISSR profiles of nine *Sophora* species from section *Edwardsia* using UBC primer 866

Lanes 1-41 contain DNA amplified from the representative single plants of the nine *Sophora* species. Lanes: 1-3, *Sophora cassioides*; 4-6, *S. chathamica*; 7-11, *S. fulvida*; 12-14, *S. godleyi*; 15-18, *S. longicarinata*; 19-30, *S. microphylla*; 31-33, *S. molloyi*; 34-37, *S. prostrata*; 38-41, S. *tetraptera*. L: 100-bp DNA ladder. Arrow heads show bands that varied within species but were shared by individuals from different species.

Table 2.7 Genetic distance (below diagonal) and genetic identity (above diagonal) among nine species of *Sophora* Section *Edwardsia* based on Nei's (1978) unbiased measures

Population	1	2	3	4	5	6	7	8	9
1	/	0.7877	0.7238	0.7861	0.7227	0.8206	0.7626	0.6134	0.8352
2	0.2387	/	0.9310	0.9397	0.8831	0.9507	0.8467	0.7713	0.8797
3	0.3232	0.0715	/	0.9066	0.8703	0.8904	0.7968	0.7324	0.8383
4	0.2406	0.0622	0.0981	/	0.9030	0.9482	0.9204	0.7927	0.8738
5	0.3248	0.1244	0.1389	0.1021	/	0.9081	0.8534	0.7417	0.8297
6	0.1977	0.0505	0.1160	0.0532	0.0964	/	0.8793	0.8305	0.8945
7	0.2710	0.1664	0.2272	0.0829	0.1585	0.1286	/	0.7447	0.7943
8	0.4887	0.2597	0.3114	0.2323	0.2988	0.1857	0.2948	/	0.7179
9	0.1801	0.1282	0.1764	0.1349	0.1867	0.1114	0.2303	0.3314	/
Mean	0.2833	0.1377	0.1827	0.1258	0.1789	0.1175	0.1950	0.3004	0.1848
SD	0.0982	0.0793	0.0956	0.0728	0.0875	0.0540	0.0728	0.0895	0.0704

Population code: 1, *Sophora cassioides;* 2, *S. chathamica;* 3, *S. fulvida;* 4, *S. godleyi;* 5, *S. longicarinata;* 6, *S. microphylla;* 7, *S. molloyi;* 8, *S. prostrata;* 9, *S. tetraptera.*

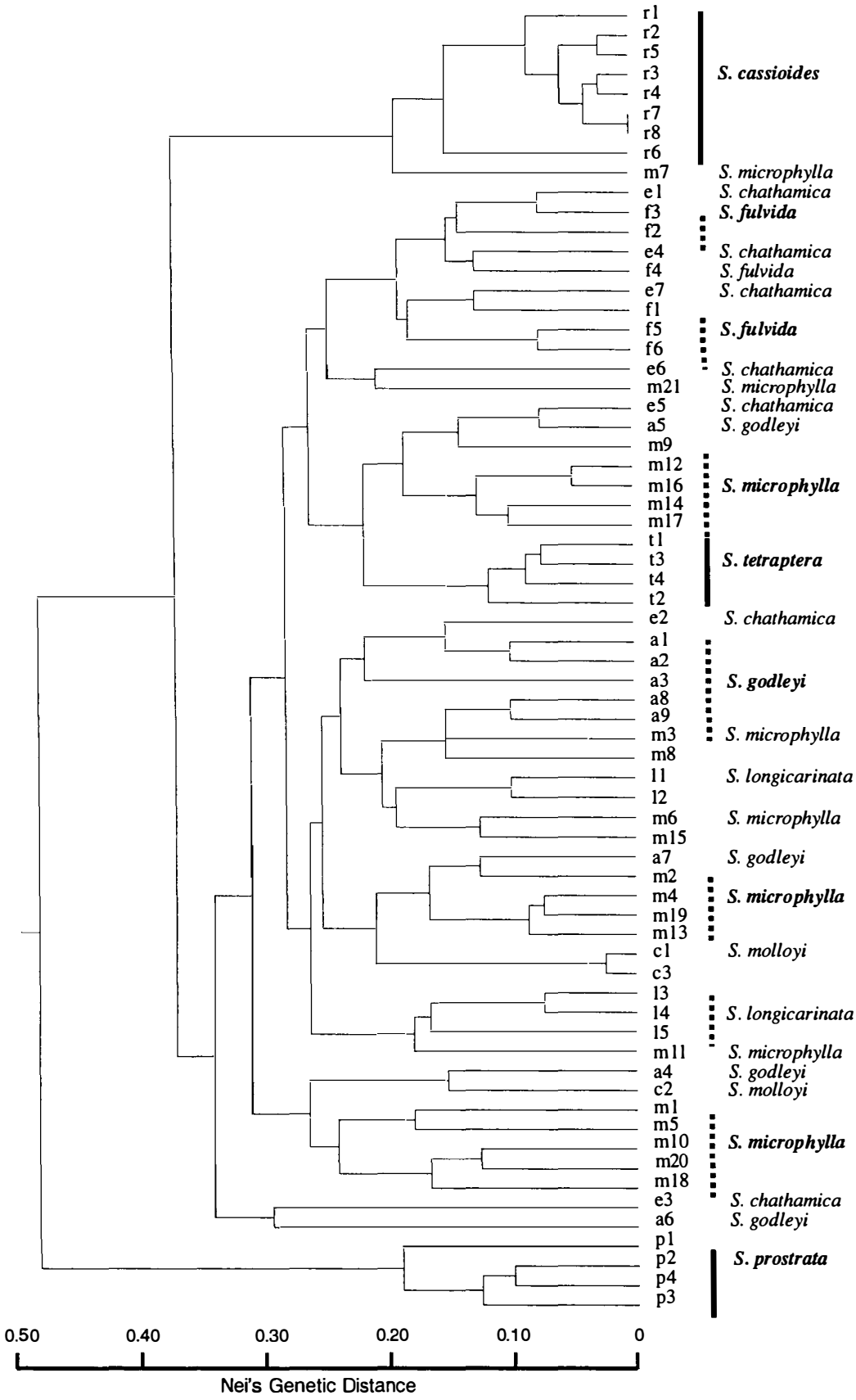

Figure 2.10 Genetic relatedness dendrogram of nine *Sophora* species using 67 individual samples from section *Edwardsia* generated by UPGMA analysis based on 53 ISSR loci

Vertical lines indicate species with all individuals grouped together. Broken lines indicate species with some of the individuals grouped together.

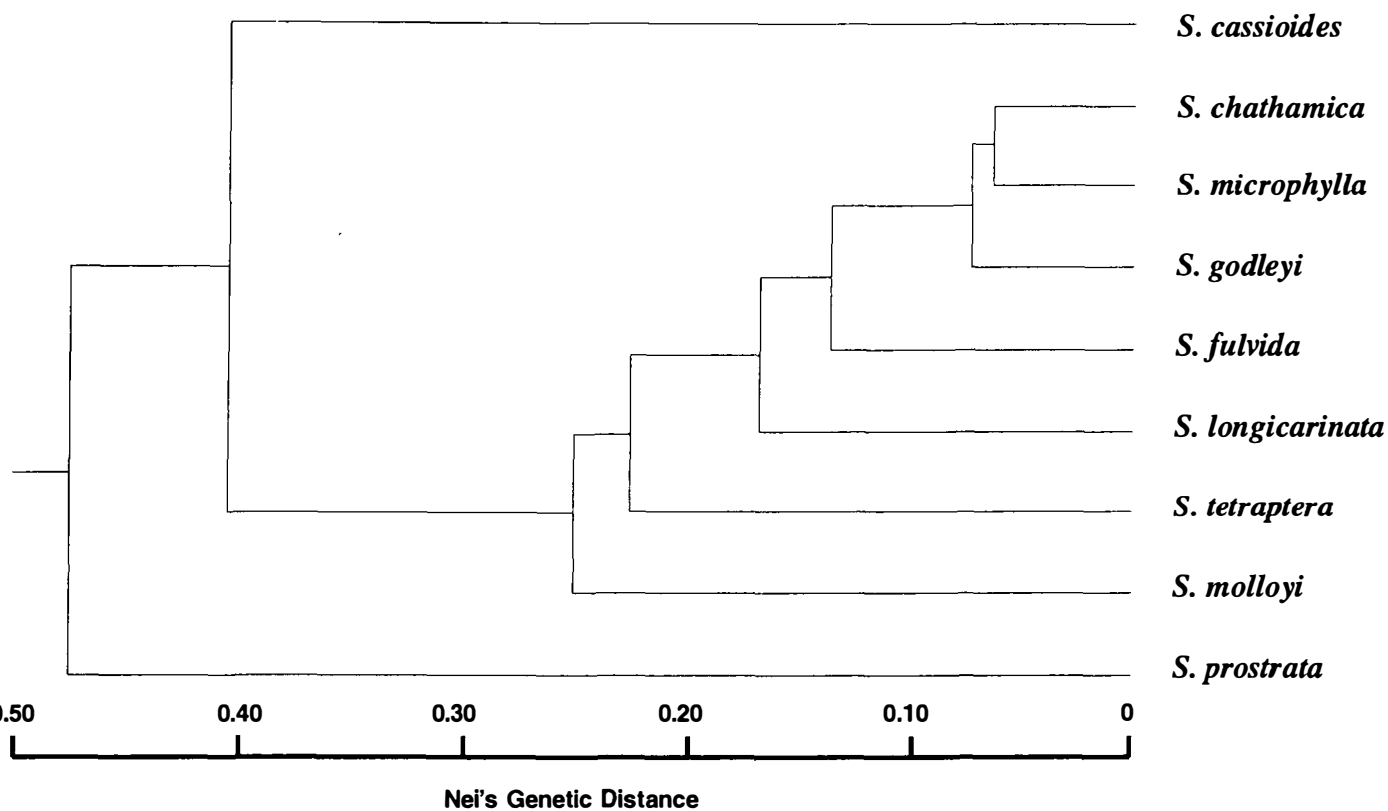

Figure 2.11 Genetic relatedness dendrogram of nine *Sophora* species from section *Edwardsia* generated by UPGMA analysis based on 53 ISSR loci

2.4 Discussion

2.4.1 Genetic diversity among *Clianthus* populations

The high genotype diversity and low ramet to genet ratios generally observed in the wild populations of *Clianthus* strongly suggests that natural populations reproduce sexually, with vegetative reproduction, though possible, being infrequent in large populations. On average, levels of polymorphism, gene diversity and Shannon Index in *Clianthus* are similar or lower than those found in other endangered or restricted plants, such as *Astragalus cremnophylax* (Travis et al., 1996), *Eryngium alpinum* (Gardeul et al., 2000) and *Prunus mahaleb* (Jordano and Godoy, 2000) that have been studied using dominant markers. While the low genetic variation observed in *Clianthus* may not be surprising given the low population sizes (<7 individuals in eight of the 12 populations), this was an accurate reflection of the situation in the wild because, in all but one case, all plants in a population were sampled.

The lower polymorphism and genotypic richness observed in small populations is probably due to the founder effect and random genetic drift, but might also be related to increased vegetative reproduction in mate limited situations (Bergelson et al., 1998; Lammi et al., 1999; Austerlitz et al., 2000). The linear relationship between population size and genetic diversity is unusual, with most other studies having identified logarithmic associations or no relationship between size and variation as is expected due to the loss of low frequency alleles in small populations (Ellstrand and Elam, 1993; Godt et al., 1996; Landergott et al., 2001). This may indicate that even the biggest of the *C. maximus* populations examined here was too small to carry equilibrium levels of genetic variation.

The association between genetic differentiation and geographical distance among wild *C. maximus* populations is indicative of isolation by distance. The continued presence of such structure might mean that patterns of gene flow by pollen and seed dispersal have been little affected by habitat loss and fragmentation. As a species that is thought to be generally adapted to disturbance (Shaw and Burns, 1997), such habitat loss might be less of a genetic threat than for other longer-lived New Zealand plants. For example, *Metrosideros excelsa* is a woody, perennial species that has also been subject to habitat loss, and for which little geographical genetic structure can currently be detected (Young et al., 2001).

The apparent exception to the pattern of isolation by distance is the population of *C. puniceus* (Heenan, 2000) originating from Kaipara Harbour, which shows strongest affinity with the Te Araroa population of *C. maximus*. One possible explanation for this is that the Kaipara Harbour population has been established at some unspecified time in the past using material from Te Araroa, or nearby. This would be consistent with the cultivation of *Clianthus* by Māori, which, although not documented in detail, appears to have been extensive and well established at the time of European settlement (Taylor, 1855; Colenso, 1885). A second possibility is that this magnitude of inter-population distance is simply beyond the range of gene flow most of the time. Differences in degree of isolation by distance over different spatial scales have been observed previously for shrub species, e.g. *Daviesia mimosoides* (Young and Brown, 1996). Isolation by distance breaks down at this scale, with gene flow moving from what is essentially a matrix model of gene diffusion to an island model.

The results in the present study are not consistent with the revision of *Clianthus* by Heenan (2000), who reported that the plants from Kaipara Harbour were of *C. puniceus*, and were morphologically distinct from those of *C. maximus*. It is unlikely that the techniques employed in the present study would not have been able to discriminate between the two closely related *Clianthus* species (indeed, two species that were long regarded as forms of a single species). This assertion was made because analyses in the present study were sufficiently discriminating to detect genetic structure within *C. maximus*. Moreover, the extent of variation across the range of *C. maximus* populations was greater than that between the plants of *C. puniceus* from Kaipara Harbour and those of *C. maximus* from Te Araroa.

The apparent discrepancy between the results in the present study and those of Heenan (2000) might be resolved by further analysis of the morphological variation that is anecdotally reported to exist within *C. maximus* (Shaw and Burns, 1997), when it might be found that there is not a clear distinction between north-eastern populations of *C. maximus* and the remaining individuals of *C. puniceus*. In support of this suggestion, Colenso (1885) did not note a difference between *C. puniceus* plants growing wild in northern New Zealand (corresponding to population 12 of *C. puniceus* from Kaipara Harbour in the present study) and *Clianthus* growing wild in the Hick's Bay-Poverty Bay coast (corresponding to populations 1-4 of *C. maximus* in the present study). However, Colenso did report a difference between northern *C. puniceus* and *Clianthus* from

Hawke's Bay (corresponding most closely with populations 6-11 in the present study) (Colenso, 1885). The explanation of common ancestry between the two *Clianthus* species with little gene flow between the separated populations seems unlikely. In addition to the effects of cultivation and seed exchange of *Clianthus*, which might have been practiced in North Island New Zealand for several centuries, there are reports of *Clianthus* (albeit not identifiable to the species level because of the absence of voucher specimens) occurring in the wild in the region that now separates the remaining *C. maximus* populations from the last known wild population of *C. puniceus* (Shaw and Burns, 1997). These intervening populations died out within the last 100 years, and *C. maximus* continues to suffer local extinctions (D. King pers. comm. to J. Clemens).

While the UPGMA analyses indicated that cultivated material has been sourced from different wild populations, the overall low level of genetic diversity in the cultivated samples relative to the wild populations suggests that cultivated stock does not broadly represent the genetic base of *Clianthus* in the wild. Indeed the generally high *R/G* ratios, especially for cv. Kaka King® and one population of unknown provenance, indicate that there has probably been repeated propagation of single clones, as one would expect for named cultivars.

Further sampling from the wild to introduce new breeding stock into cultivation should be structured so as to take account of the strong geographical trends in inter-population genetic differentiation.

Because *C. puniceus* possibly no longer exists in the wild, and its existence in cultivation is founded on such a limited collection from a rare distribution, no conclusion can be drawn about the effects of cultivation and seed exchange on genetic diversity of this species. However, it is concluded that human intervention has had little effect on the current distribution of genotypes of *C. maximus*.

Interestingly, early observers believed that *Clianthus* would have become extinct had it not been for its cultivation (e.g. Colenso, 1885). The species might well have occurred at a number of locations in the North Island as a direct result of its cultivation, and its distribution might have contracted as traditional Māori habitation and methods of cultivation have become less common. It is then suggested, based on the results of the present study, that the current distribution of *C. maximus* in the wild may correspond to local geological and climatic conditions that best suit the perpetuation of the species in

the absence of cultivation. Moreover, the fact that genetic structure is evident in *C. maximus* today might be a direct result of the contraction of the species to sites that are relatively unaffected by cultivation.

2.4.2 Genetic relatedness among New Zealand *Sophora* species

The relatively broad range and high value of inter-species genetic distance (0.05-0.49) detected in the present study using ISSR markers suggests that there exists considerable genetic differentiation across New Zealand *Sophora* species. However, the UPGMA dendrogram supported the monophyletic nature of the tested species as a whole, with *S. prostrata* at the most basal position, followed by *S. cassioides*. This result is consistent with other studies using morphological characters, but not with those using molecular tools, which detected very low inter-species genetic distances. Heenan et al. (2001) observed notable variation in leaf characters and growth habits among different species, using the same set of plants sampled in the present study. However, Mitchell and Heenan (2002) did not find any difference in nrDNA ITS sequences among 13 *Sophora* species of section *Edwardsia*, including the eight New Zealand endemic species, even at a genetic distance level of 10^{-8}. Similarly, Hurr et al. (1999) only detected very low genetic difference, using cpDNA sequence, among different *Sophora* species of section *Edwardsia* from New Zealand, South Pacific and South Atlantic regions, and were unable to resolve the relationships among these species. The result in the present study should be comparable to those of cpDNA sequence (Hurr et al., 1999) and nrDNA ITS sequences (Mitchell and Heenan, 2002) data, since many plants used in these three studies were identical.

Result in the present study also reflected, at least partially, the complexity and problematic situation of New Zealand *Sophora* taxonomy. The difficulties associated with establishing relationships among species from section *Edwardsia* has been well documented, and no phylogenetic or biogeographical hypothesis has been accepted unequivocally (Peña and Cassels, 1996; Ruiz et al., 1999; Mitchell and Heenan, 2002). The failure to construct well-supported phylogenetic or phenetic hypotheses for the species of section *Edwardsia*, several of which have distinct morphological attributes, highlights their recent origin (Hurr et al., 1999). This also provides evidence that morphological evolution is proceeding at a rate greater than the molecular (Mitchell and Heenan, 2002). This situation could even be extended beyond the section *Edwardsia* and

the tribe *Sophorea* when molecular evidence was used, illustrating the discrepancy between the traditional taxonomic treatment and the recent molecular evidence. In a study using chloroplast gene *rbc*L sequences, the closest relatives of the *Sophora* lineage that includes, for example, the New Zealand endemic *S. microphylla* are spread across the tribes Crotalariae, Podalyrieae, Liparieae, Thermopsideae, Eucresteae, and Genisteae (Kajita et al., 2001).

Despite the accepted high discriminating ability of ISSR markers, the present study was not able to establish the genetic relationship among some species such as *S. microphylla* and the newly recognized species *S. chathamica*, *S. godleyi*, and *S. longicarinata*, not only because of the low genetic distance among these species, but also because their individuals were spread across different groupings in the UPGMA dendrogram. This was clearly reflected by the ISSR profiles of these species, which had many bands that varied among individuals in the same species, but in which the same band was shared by individuals of other species. This may imply that these newly recognized species do not have sufficient genetic difference, at least of the molecular level, to be accepted at the species rank. On the other hand, this may also be a reflection of their heterozygous status resulting from natural hybridization and segregation. Given the bird pollinated nature of these species, interspecific gene exchange is probably a common phenomenon when two or more species occur in the area within the reach of pollinating birds. Interspecific hybridization has been indicated by the detection at many sites of F1 and F2 hybrids plants, and backcross plants having intermediate morphological characters on a continuum in variation between the putative parental species (Heenan et al., 2001).

The present study provided more molecular evidence to reveal the phylogenetic relationship, at least partially, of the New Zealand *Sophora* species. This is also sound evidence for the high discriminating ability and the usefulness of ISSR markers in establishing the genetic relationship of closely related taxa.

Chapter 3 Developmental and postharvest characteristics in *Clianthus maximus* and *Sophora tetraptera*

3.1 Introduction

The conservation status of the two *Clianthus* species were classified as critically endangered or vulnerable, with wild populations already dying out or being decreased both in number and in size of the populations (Shaw and Burns, 1997; Heenan, 2000). As for endangered species in general (Bernardello et al., 2004), a comprehensive understanding of the vegetative and reproductive developmental behaviours, especially the reproductive biology such as flower phenology and floral ontogeny, is critical to successful conservation efforts of the species in question. Although some general observations and descriptions concerning the vegetative and flowering characters have been made and documented for *Clianthus* (Smith-Dodsworth, 1991; Shaw and Burns, 1997; Heenan, 1998a, 2000), a systematic investigation of vegetative and reproductive growth characters has not yet been carried out for this genus. Furthermore, little is known about the details of its floral ontogeny.

The same is true for the eight New Zealand native species of *Sophora.* There is a lack of comprehensive information on the vegetative and reproductive growth characters, and floral ontogeny, although the general vegetative growth and flowering characters of these species has been well documented (Godley, 1975; Salmon, 1996; Godley and Smith, 1977; Heenan et al., 2001). Therefore, such investigations would be of great value.

In herbaceous model species, such as *Arabidopsis*, the order of floral organ initiation is: sepals, petals, stamens, and carpels. The four floral whorls do not overlap in time of initiation (Smyth et al., 1990). However, an unusual order of organ initiation is often observed in leguminous species, especially in the sub-family Papilionoideae, including the overlapping of carpels with stamens in tribe Sophoreae (Tucker, 2003a), common primordia in *Pisum* (Tucker, 1989; Ferrándiz et al., 1999), and ring meristems in tribe Swartzieae (Tucker, 2003b). This contrast in the pattern of organ initiation offers an opportunity to compare control of floral organ initiation (Tucker, 2003a).

Clianthus and *Sophora* species were cultivated long before the arrival of European

settlers, and have been considered valuable ornamental tree plants or pot plants in New Zealand in recent times (Godley, 1975; Salmon, 1996; Show and Burns, 1997; Heenan, 1998b, 2000; Heenan et al., 2001). However, the use of neither *Clianthus* nor *Sophora* species as cut flowers has been reported to date, possibly because the flowers of plants in these two genera abscise soon after being cut and put in a vase. The successful commercialization of a closely related plant, the Australian Sturt's Desert pea, *Swainsona formosa* (formerly *Clianthus formosus*), as a new cut flower (Williams and Taji, 1991; Zulkarnain, 2002) suggests that the vase life of *Clianthus* and/or *Sophora* cut flowers might be enhanced after appropriate postharvest treatment, and that these plants could eventually be commercialized as new cut flower species.

Postharvest treatments with silver thiosulfate (STS) and sucrose are commonly used to enhance the vase life of many cut flower species (Nowak and Mynett, 1985). Sugars such as sucrose play an important role as substrates for respiration, materials for cell wall synthesis, as well as for maintaining osmotic potential in cut flowers, and are often used to promote flower bud opening, and to prolong the vase life of cut flowers in a wide range of species (Halevy and Mayak, 1979; Borochov and Woodson, 1989; Ichimura and Hiraya, 1999; Eason et al., 2004).

One of the major causes for cut flower wilting and abscission is endogenous ethylene (Woltering and van Doorn, 1988). Since some legume cut flowers such as sweet pea and lupin are sensitive to ethylene (Mor et al., 1984; Ichimura and Hiraya, 1999; Mackay et al., 2001), it was then hypothesed that *Clianthus* and *Sophora* would be sensitive to ethylene too. To overcome the detrimental effect of ethylene, STS, an ethylene action inhibitor, is commonly used to extend the vase life of many cut flowers including legume cut flowers sweet pea and lupin (Veen, 1979; Nowak and Mynett, 1985; Reid and Wu, 1992; Ichimura and Hiraya, 1999; Mackay et al., 2001; Ichimura et al., 2002; Eason et al., 2004).

In view of the above, the objectives of this chapter were to test the possibility that vase life of *Clianthus* and *Sophora* flowering shoots could be enhanced using sucrose and STS treatment; and to conduct a systematic investigation of vegetative and reproductive growth characters, including the development of a calendar of floral ontogeny, through microscopic examination of bud samples.

3.2 Materials and methods

3.2.1 Monitoring of vegetative and reproductive development

Vegetatively propagated 2-3 year-old plants of *Clianthus maximus* Kaka King® and *Sophora tetraptera* were obtained from commercial nurseries in 2000 and were grown in the Palmerston North nursery of the New Zealand Institute for Crop and Food Research Limited.

Two complete growth cycles of vegetative and reproductive development were monitored between November 2000 and October 2002. Five shoots from each of the five randomly selected representative plants were labeled for shoot length, leaf number and node number monitoring. One of the five shoots from each plant was selected for leaf and inflorescence development monitoring. Monitoring data were recorded at two-week intervals. The vegetative and reproductive growth characters of adult *Sophora tetraptera* trees growing on Massey University Turitea campus were also observed for comparison.

3.2.2 Bud sampling and histological preparation

Inflorescences or individual flower buds of *Clianthus* and *Sophora* were collected, during the 2001-2002 growing cycle at 1- to 2-week intervals, from the same plot but different plants from the growth monitoring study. *Sophora* samples were taken in mid October as soon as the inflorescence primordia were visible in the axils of leaves on the new growth shoot. For the first 3-4 samplings, the whole shoot or the part of the shoot from first inflorescence bud to the shoot tip was collected until the inflorescence length reached 5 mm, then only inflorescences were collected. Individual flower buds were collected when their size reached 2-3 mm. A similar sampling procedure was conducted for *Clianthus*.

Samples were put directly into 15 ml plastic centrifuge tubes containing freshly made FAA and Camoy's fluid fixatives (Johansen, 1940; Table 3.1). The procedure of fixation and histological preparation was carried out based on the protocol described by Sreekantan (2002) with minor modifications.

Centrifuge tubes with collected bud samples were placed under partial vacuum for 2 h and then in held under normal atmospheric pressure for 20-24 h fixation. The bud samples

were washed with 70% (v/v) ethanol at least three times at 2-hourly intervals. Fixed samples were stored in 70% (v/v) ethanol at 4-7°C or were taken through serial dehydration solutions of tertiary butyl alcohol (Fig. 3.1). After dehydration, the samples were embedded in paraplast using an embedding machine (Leica EG1160). The embedded blocks were hardened on the chill tray of the embedder, and then kept at 4-7°C till sectioning.

Blocks of embedded tissue bud samples were microtomed to serial sections of 10 μm thickness on a Leica RM2145 microtome. Ribbon pieces were floated on water at 42°C in a Leica HI 1210 water bath for 1 min to take the compression out of the tissue. Superfrost plus treated microscope slides (Biolab Scientific) were positioned in the water under the ribbon pieces and the ribbons were scooped on to them. Water was drained off the slides and they were incubated on a slide warmer set to 45°C for 24-48 h. Slides were then taken through a staining process (Fig. 3.2, Table 3.1) that was a modification of the schedule of Johansen (1940). Sections were then mounted in DPX mountant (BDH). After drying in the fume hood for at least 2-3 h, sections were viewed under the light microscope.

Table 3.1 Composition of fixatives and stains

Fixative / Stain	Composition	Quantity (ml)
FAA	70% (v/v) ethyl alcohol	90
	glacial acetic acid	5
	37% (v/v) formalin	5
Carnoy's Fluid	100% ethyl alcohol	60
	glacial acetic acid	10
	chloroform	30
Safranin	1% (w/v) safranin in 70% (v/v) ethyl alcohol (w/v)	
Picro- alcohol	0.5% (x/v) picric acid in 95% (v/v) ethyl alcohol (w/v)	
Fast green	clove oil	15
	methyl cellosolve	15
	95% (v/v) ethyl alcohol	90
	glacial acetic acid	30
	fast green	0.3 g
Clove oil/Absolute alcohol/Histoclear		50:25 :25
Histoclear/Absolute alcohol		50: 50
Methylene blue	1.5% (w/v) in 96% (v/v) alcohol	

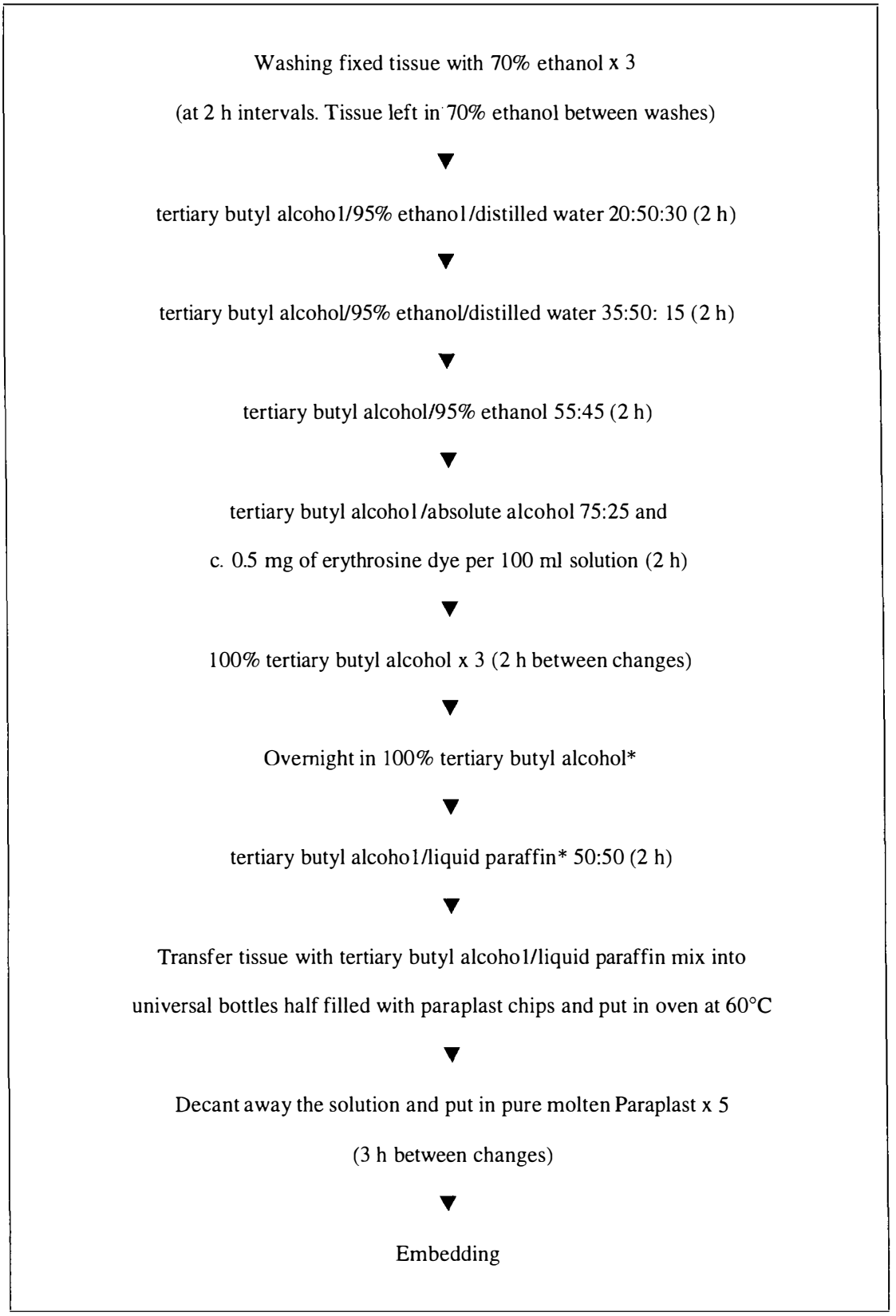

Figure 3.1 Flow chart of tissue dehydration and wax infiltration for paraffin embedding of *Clianthus* and *Sophora* samples

Adapted from Sreekantan (2002). *Temperature to be kept c. 30-35°C using a water bath or using a fan heater to prevent solutions from solidifying at cooler temperatures.

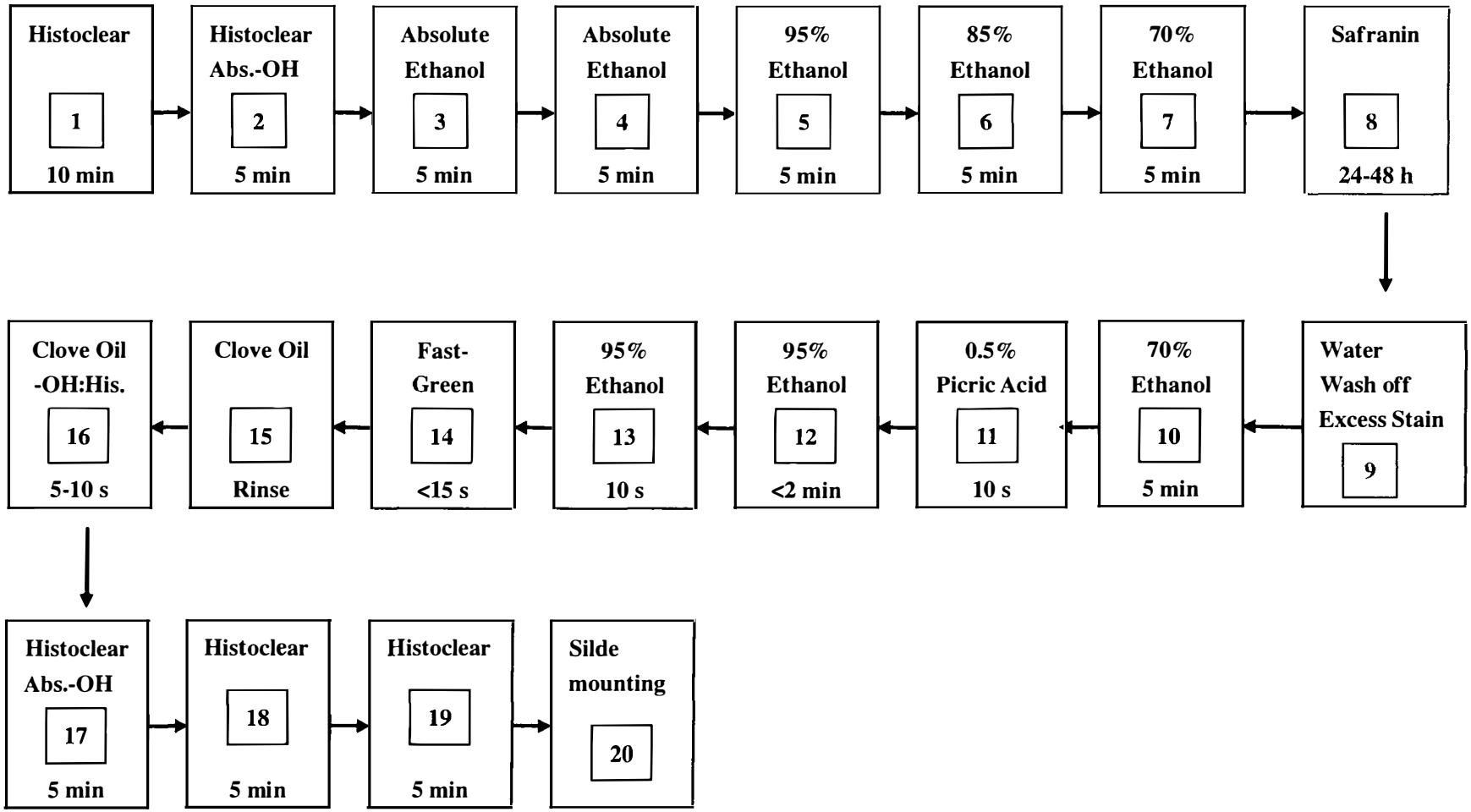

Figure 3.2 Staining schedule with safranin and fast green

Adapted from Sreekantan (2002). Histoclear Abs.-OH: Histoclear/Absolute ethanol (50/50). Clove oil–OH:His.: Clove oil/Absolute ethanol/Histoclear (50/25/25).

3.2.3 Postharvest treatment of cut shoots

Harvest and experimental preparations

The postharvest experiments were conducted during the 2001 and 2002 flowering seasons, in September for *Clianthus* and end of September to early October for *Sophora*, using the same plants of *Clianthus maximus* Kaka King® and *Sophora tetraptera* used for developmental mornitoring as sources of flowering shoots for cutting.

For *Clianthus*, flowering shoots 30-40 cm in length bearing similar numbers and developmental stages of flower buds (equivalent to stages 3-5 in Fig. 3.3) were selected for the experiments. Shoots were harvested in the early morning (8:00-9:00) from the experimental plants and the cut ends were immediately put in containers filled with distilled water. After being transported to the experimental lab, all shoots used for the same replicate in the same experiment bearing the similar number and developmental status of flower buds were trimmed to approximately the same length, and were randomly assigned to each treatment. In order to avoid the trapped air bubbles around the cut stem, the selected shoots were re-cut under water immediately before being placed in a 50 ml centrifuge tube or a 125 ml flask filled with each experimental solution.

Similar preparation was conducted for *Sophora* flowering shoots c. 20 cm in length bearing flower bud at stages 3-5 (Fig. 3.4).

Throughout the experiments, all cut flower shoots were maintained under controlled conditions in a vase life evaluation room at Crop and Food Research Ltd, Palmerston North. The room was maintained at a constant temperature of 20°C with a relative humidity (RH) at approximately 70%. Overhead lighting was provided by four cool white fluorescent tubes at c.20 μmol m^{-2} s^{-1} at the bench top level, on a 12 h photoperiod. Distilled water served as the control for all experiments unless otherwise stated. In general there were 4 or more replicates of all treatments.

Figure 3.3 Mid- and late developmental stages of *Clianthus* flower buds

1:End August. 2-7: September.

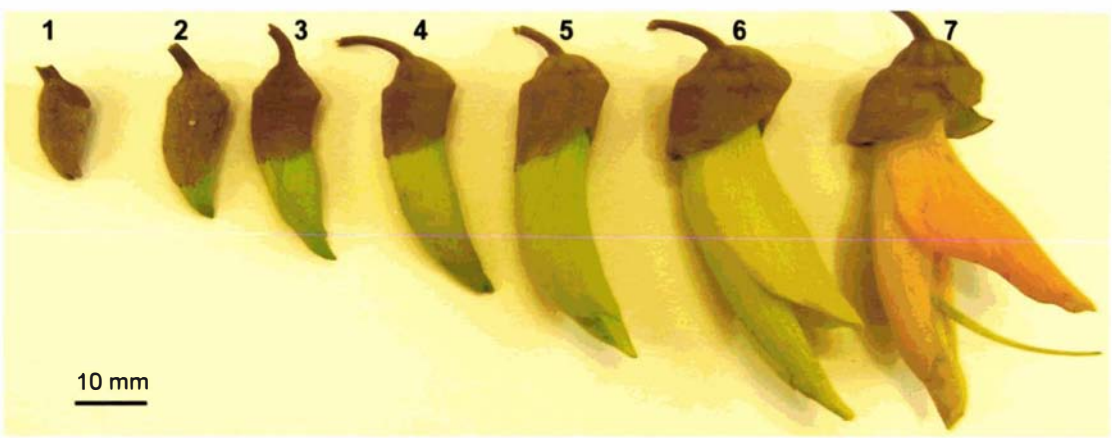

Figure 3.4 Mid- and late developmental stages of *Sophora* flower buds

1: End August. 2-6: September. 7: Early October.

3.2.3.1 Effects of sucrose holding solutions

For a sucrose holding solution experiment, shoots were placed in 1%, 2%, 4%, or 8% sucrose solutions throughout the experimental period. In addition, 200 mg l^{-1} 8-hydroxyquinoline sulfate (HQS) and 300 mg l^{-1} citric acid in distilled water were also included in this experiment to test their effects.

3.2.3.2 Effects of sucrose pulse solutions

For a sucrose pulse experiment, shoots tips were firstly immersed in 4%, 8%, or 16% sucrose solutions for 2, 4, or 24 h, in factorial combination. After the sucrose pulse, shoots were transferred to and held in distilled water throughout the rest of the testing period. Separate sets of shoots were held continuously in 2% sucrose or distilled water as controls.

3.2.3.3 Effects of STS treatments

In a preliminary experiment to test the effect of an STS treatment, 0.5 mM, 1.0 mM, or 2.0 mM STS in distilled water were used as holding solutions. In a separate experiment, 2.0 mM STS was used as a pulse treatment for 24 h, with or without the combination with 2% sucrose in the holding solution. The third experiment was conducted using 2.0 mM STS as a pulse treatment for 30 min, 60 min or 120 min, followed by distilled water or 2% sucrose in the holding solution for the rest of the experimental period. Separate sets of shoots were held continuously in distilled water as control.

3.2.3.4 Effect of repeated stem cutting and solution change

As a pretreatment for all treatments, shoots were pulsed by 2.0 mM STS for 60 min, and then placed in 2% sucrose holding solution. The experiment was conducted by changing the holding solution every 3 days, or by cutting the stem ends every 3 days, or by combining the solution change and stem cutting treatment.

3.2.3.5 Vase performance evaluation

Water uptake, shoot weight, and vase performance characters such as petal length, number of open flowers, and vase life were recorded daily.

For daily water (solution) uptake, containers were weighed before and after adding new solution. For petal length measurement, five flower buds 25-30 mm in length (c. stage 3 of Fig. 3.3 and Fig. 3.4 for *Clianthus* and *Sophora*, respectively) were labeled for each treatment and the length from the end of calyx to the tip of the flower bud was measured. A single flower was considered as open when the standard petal was separated by >5mm from other petals (c. stage 6 in Fig. 3.3 and Fig. 3.4 for *Clianthus* and *Sophora*, respectively). The normally senescent flowers after opening were also counted and added to the total numbers of open flowers. The vase life of the cut flowering shoot was regarded as the period from harvest to the time when 50% of the initial flowers had senesced, wilted or faded petals, or had abscised.

3.2.3.6 Statistical methods

Each replicate, with shoots harvested on different days, served as a block in randomized block design. Experimental data were analysed by analysis of variance, using SPSS version 12.0 software.

3.3 Results

3.3.1 Developmental characteristics in *Clianthus*

3.3.1.1 Vegetative development characteristics

In *Clianthus*, shoot growth (elongation and node number increase) and leaf emergence occurred continually throughout the year. The shoot length of the plants pruned back in October extended by nearly 600 mm with more than 40 nodes from November to May (Fig. 3.5). During the wintertime (from May), both shoot growth and leaf emergence rate were greatly slowed down. Vegetative growth almost ceased during the flowering season in September, and resumed in October after flowering.

Leaves expanded throughout year and reached their full size in one to two months (Fig. 3.6), and began to abscise four to five months after their emergence. Fully expanded leaf size varied from 60-70 mm to 120-130 mm in length depending on their time of emergence, the longest emerging between late summer and early autumn (Fig. 3.6). During wintertime and flowering season, old leaves abscised more rapidly than new leaves emerged, causing the number of leaves per shoot to decrease rapidly (Fig. 3.5).

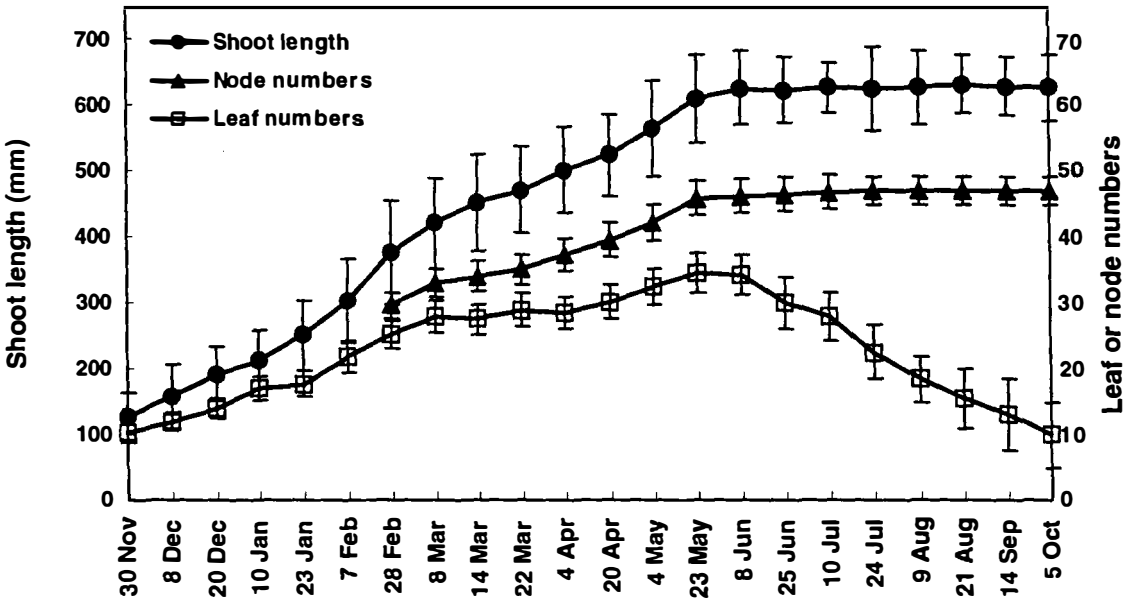

Figure 3.5 Annual mean shoot growth and leaf number per shoot in *Clianthus maximus*

Values are means (± SE) of five plants.

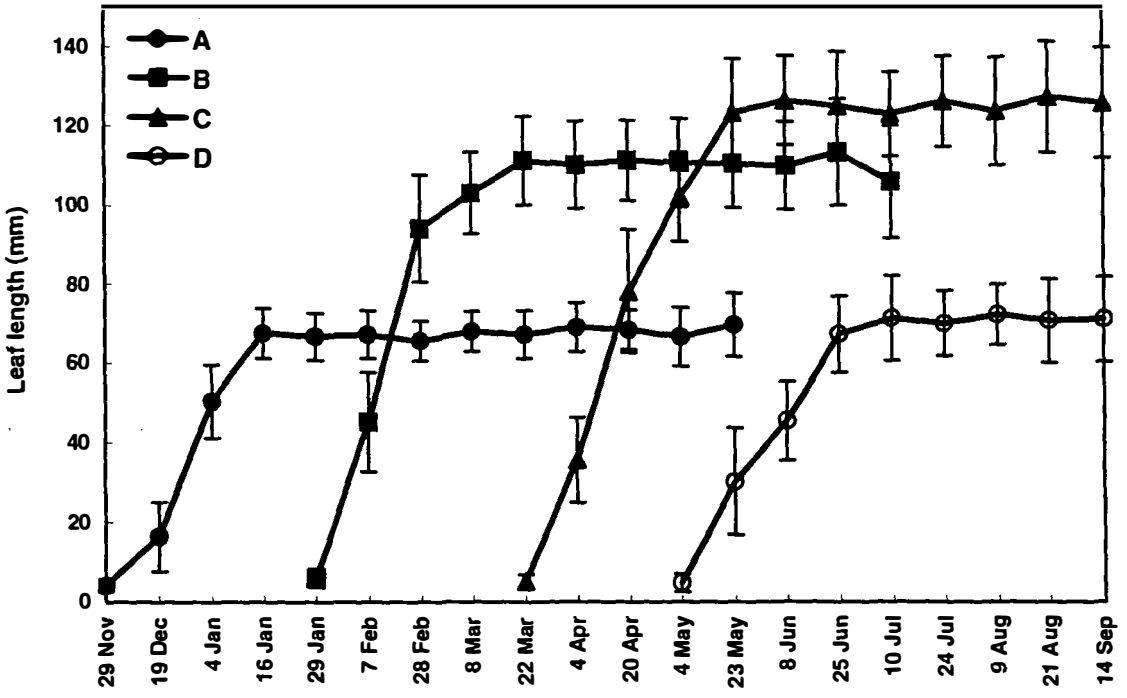

Figure 3.6 Leaf emergence and development in *Clianthus*

Values are means (± SE) of five plants. A: leaves emerged before 29 November; B: leaves emerged between 16 and 29 January; C: leaves emerged between 8 and 22 March; D: leaves emerged between 20 April and 4 May.

3.3.1.2 Reproductive development characteristics

Inflorescences emerged continually from axils of undeveloped leaves within the shoot tip almost throughout year, from shortly after the previous flowering season in October until a short time before the next flower season in August. The early-emerged inflorescences normally abscised during the development period. Inflorescences that emerged after May rarely reached the flowering stage, and abscised before or during the flowering season. Only those that emerged from April to early May (mid-autumn) continued to develop fully, and individual flowers developed to maturity (Fig. 3.7). Generally, these inflorescences were situated from nodes 40 upward on the main shoots of pruned plants, and from nodes 5-10 upward on the secondary branches. Inflorescences reached the flowering stage about four months after their emergence, with individual flower buds undergoing a series of developmental stages before opening in mid-September.

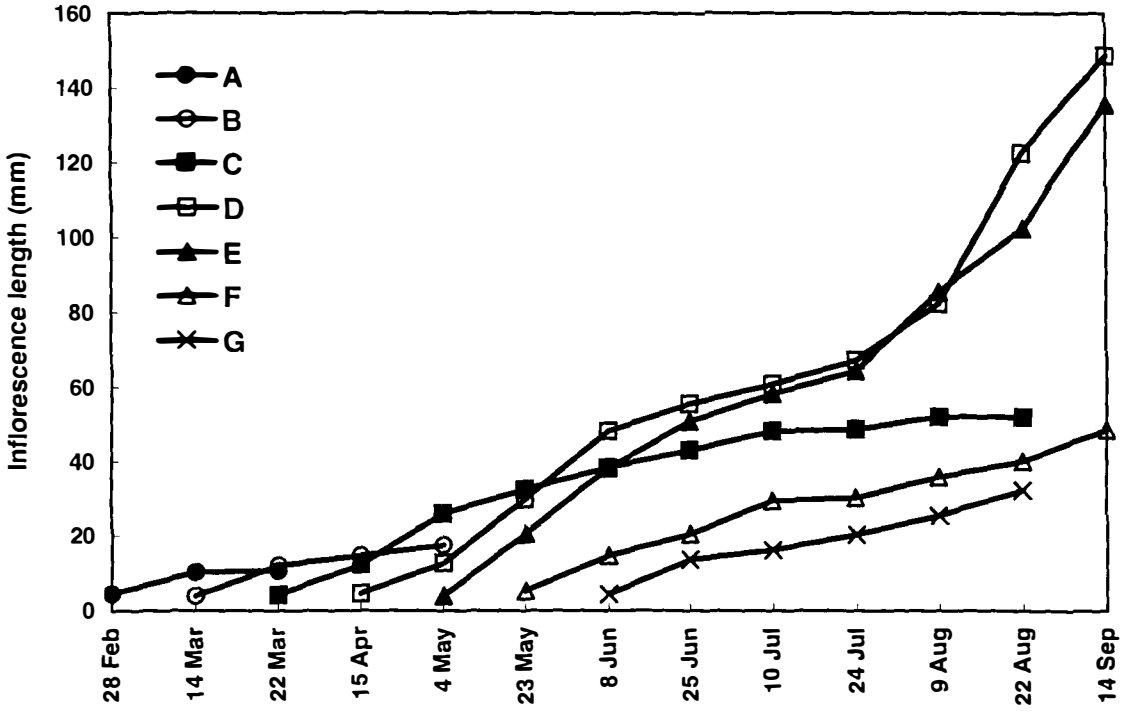

Figure 3.7 Inflorescence emergence and elongation in *Clianthus*

Values are means of five plants. A-G: Groups of inflorescences emerged 3-5 days before the date shown, and were >4 mm in length.

Seven developmental stages of flower buds were distinguished and were used as a reference for further studies. Stage 1: Flower buds with the sepals of the calyx completely enclosing the corolla petals. Stage 2: Sepals have begun to separate and petal tips are visible. Stage 3: Petal extending beyond the calyx to the same length as that of the calyx teeth. Stage 4: Length of petals 2-3x that of the calyx. Stage 5: Flower reaches its full size while all petals are still closely bunched. Stage 6: The standard petal separates from the other petals. Stage 7: Petals start to lose colour and to wilt (Fig.3.3)

There were 10-20 flowers in an inflorescence with the developing order from base (proximal) toward the tip (distal). The length of a mature flower bud before opening was 60-70 mm. Petals began to show typical red colour and to have ornamental value when 25-30 mm in length, 5-8 days before flower opening. Wilting occurred 5-7 days after flower opening, which marked the end of ornamental value (Fig. 3.3).

3.3.1.3 Microscopic observation of reproductive development in Clianthus

Results of microscopy studies confirmed observations made in the field that inflorescences initiated continually from leaf axils a couple of nodes back from the shoot apex from November to August (Fig. 3.8 A). Flower primordia were initiated within the inflorescence shortly after the initiation of the latter (Fig. 3.8 B). Although sepals could occasionally be seen during November- March, no further organogenesis was observed in flower primordia from inflorescences harvested before the end of April. At this time organogenesis of the floral organs began, with the carpel initiated at the same time as, if not before, stamens (Fig. 3.8 C). Organogenesis progressed very slowly from April to June, with carpel development much more advanced than that of stamens. In early June, the length of the carpel was 1.5- to 2-times greater than that of the outer stamens, and 3- to 4-times greater than that of the inner stamens (Fig. 3.8 D). By July, carpel and stamens began to differentiate, and extended quickly, with obvious formation of stigma, ovary and anthers, with the height of gynoecium still much higher than that of androecium. Even at this stage no petals were observed (Fig. 3.8 E). In early August, the volume of floral organs further expanded quickly, petals were first observed and began to elongate rapidly, and pollen grains and ovules could be distinguished (Fig. 3.8 F). It was only from this time that the androecium became higher than the gynoecium. At this time, the length of the flower bud was c. 5-7 mm, equivalent to Stage 1 of flower development (Fig. 3.3).

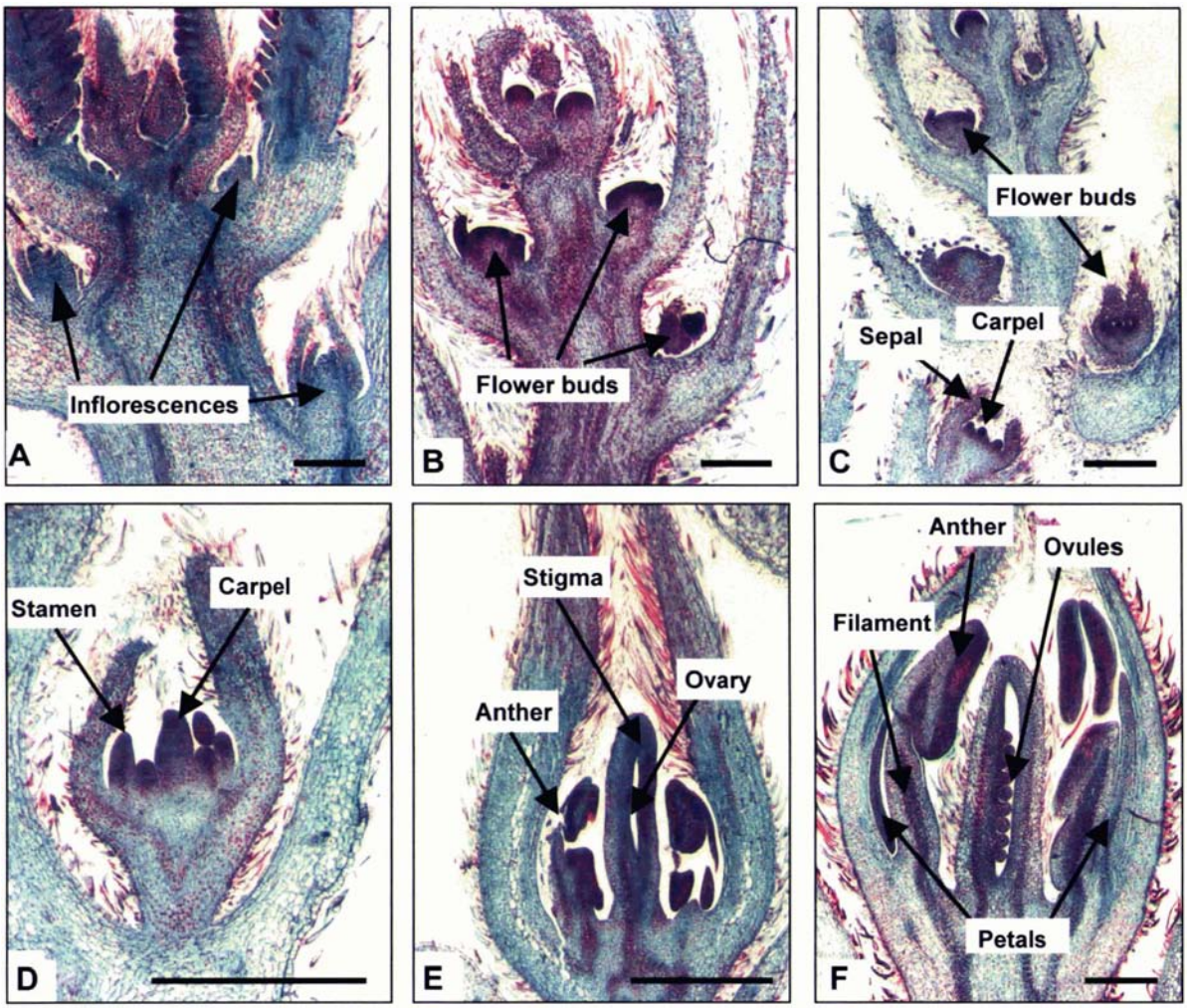

Figure 3.8 Inflorescence and flower bud initiation and development in *Clianthus*

A: Inflorescence initiation, January to August. B: Flower bud initiation, February to August. C: Floral organ initiation, April to June. D: Floral organ development, June. E: Floral organ differentiation, July. F: Floral organ elongation and gametocyte development, August. Bar=1mm.

3.3.2 Vegetative and reproductive development cycles in *Sophora*

3.3.2.1 Vegetative development characteristics

For adult *Sophora* trees, shoots began to grow from the previous season's apex or axillary buds shortly before the flowering season in early October, and ceased growth in December with 8-15 new nodes and subtending leaves, leaving the axillary buds and the apical bud dormant for most of the year until the following September.

For the vegetatively propagated two- to three-year old *Sophora* plants, two different types of shoots were recognised according to their growth characteristics. Some of them grew vigorously for a longer period than those described above, from September to June, and kept growing slowly during the winter time. These shoots could reach a length of over 900 mm with more than 40 nodes. These types of shoots were not included for further monitoring because they were present only in young trees and were much less frequently observed during the second growth cycle. In more typical shoots, growth started in late September and stopped in early December, reaching the new growth length of 200-300 mm with around 20 nodes (Fig. 3.9). These shoots remained inactive until the next growing season in the following September.

Leaves emerged from late September before the flower season to November, and elongated rapidly to reach their full size in 5-6 weeks. Leaf length varied from 60-70 mm to 90-100 mm according to their initiation order, with later initiated leaves having larger size (Fig. 3.10). In the shoot tip, 2-4 leaf primordia formed in November but ceased to develop beyond <2-3 mm in length and remained undeveloped for the next 9-10 months. Occasionally, some leaves could reach >5 mm in length before they ceased to develop further, resuming their development in the next growing season (Fig. 3.10). The majority of adult leaves remained attached to the shoot for 8-9 months and abscised during a period of mass leaf fall which occurred from July to September (Fig. 3.9).

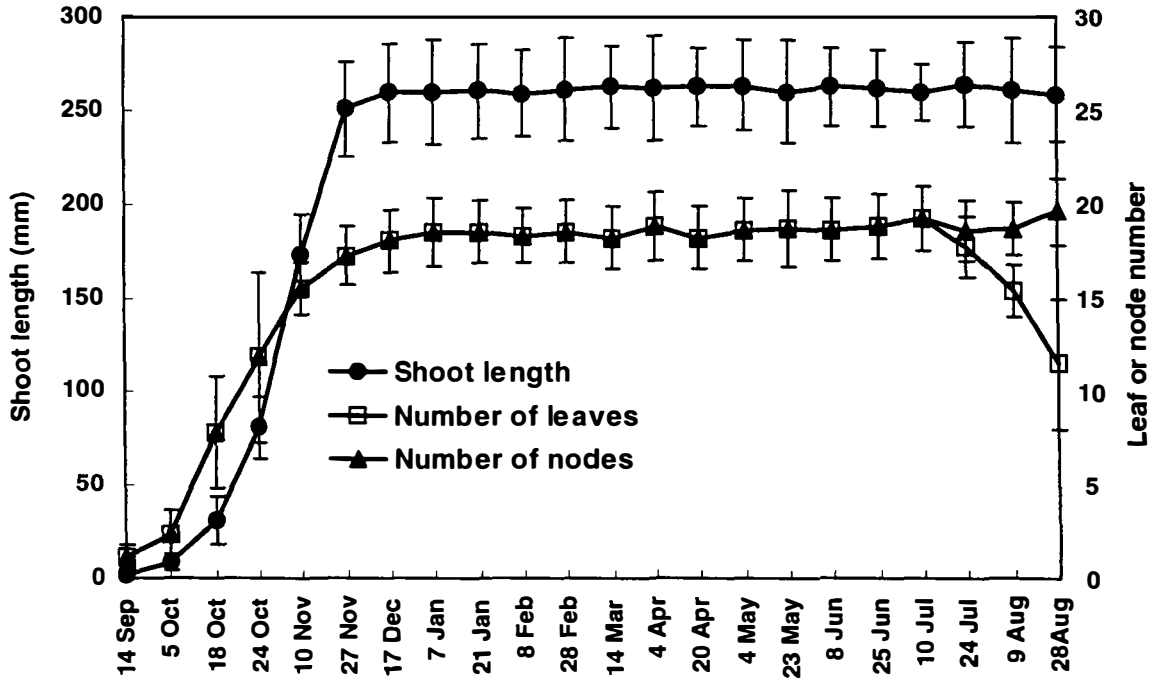

Figure 3.9 Annual mean shoot growth and leaf number per shoot in *Sophora teptratera*

Values are means (± SE) of five plants.

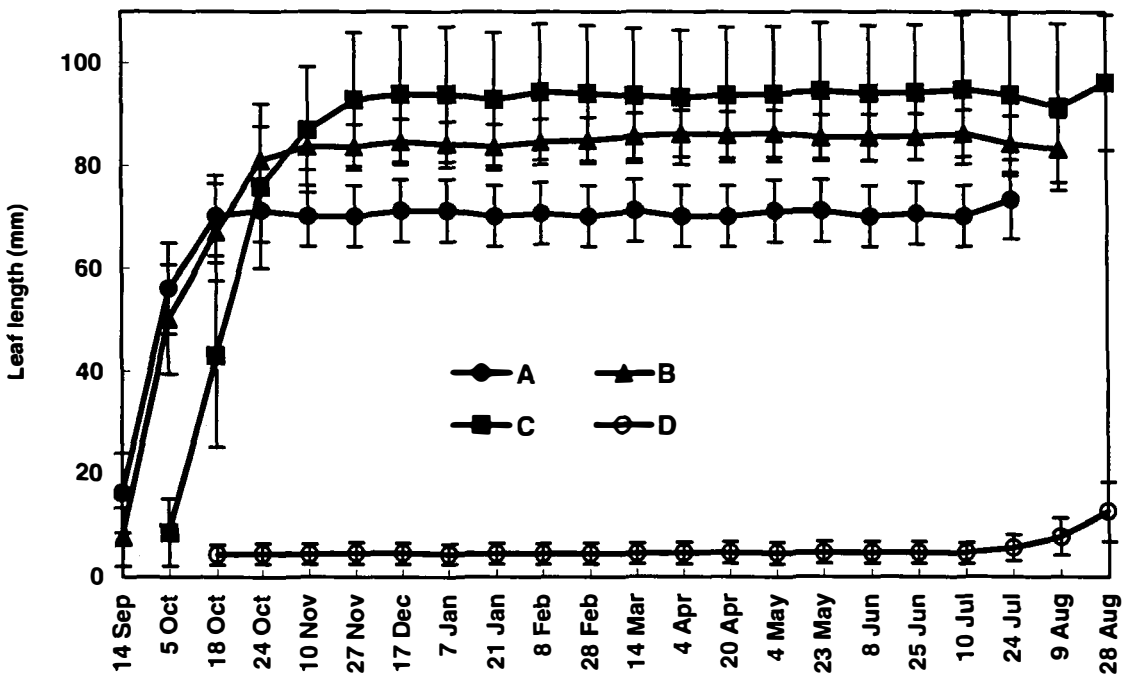

Figure 3.10 Leaf emergence and development in *Sophora*

Values are means (± SE) of five plants. A-B: leaves were 4-6 mm and 10-15 mm in length, respectively, on 14 September; C: leaves emerged between 14 September and 5 January; D: leaves 4-6 mm in length on 18 October.

3.3.2.2 Reproductive development characteristics

Inflorescences initiated from the leaf axils of the growing shoots during a very short time, from mid-October to November. These inflorescences developed quickly, and reached a size of 30-40 mm in length with individual flower buds c.1 mm in length in the axils by the end of December. These inflorescences and flower buds then ceased to further develop and remained dormant for 6-7 months, from late summer through autumn to early winter, until July when individual flower buds resumed development. These flower buds then increased their volume progressively through a series of stages toward their full size at flowering time in early October. An inflorescence consisted of 5-10 individual flowers 40-50 mm in length when fully developed. An open individual flower normally lasted for 4-6 days on the parent plant, and the flowering period for a tree lasted 2-3 weeks.

Seven developmental stages of flower buds were distinguished. Stage 1: Flower buds with the sepals of the calyx completely enclosing the corolla petals. Stage 2: Sepals have begun to separate and petal tips are visible. Stage 3: Petal parts outside the calyx are about the same visible length as the calyx. Stage 4: The length of petals are 2- to 3-times that of the calyx. Stage 5: Flower reaches its full size while all petals are not parted. Stage 6: The standard petal separates from the other petals by > c. 4 mm. Anthers and pistil are visible. Stage 7: Petals start to lose colour and to wilt (Fig. 3.4).

3.3.2.3 Microscopic observation of reproductive development in Sophora

Microscopic observation revealed that soon after the initiation of inflorescences in October, individual floral primordia initiated from the axils of the inflorescences (Fig. 3.11 A). Organogenesis of floral buds occurred from November to December, with sepals initiated firstly (Fig. 3.11 B). Stamens and carpels initiated at approximately the same time and started to develop soon after their initiation, without any apparent structure of petals being observed at this stage (Fig. 3.11 C). Floral organs ceased their development at this stage and remained in similar developmental status for the next 5-6 months, until June. At that time, stamens and carpels resumed their development and started to differentiate. Petals were also distinguishable (Fig. 3.11 D) at that time. In July, stamens and carpels well differentiated, with distinct anthers, filaments, stigma and ovaries. Petals also started to elongate (Fig. 3.11 E). By August, ovules and pollen grains were well formed and petals elongated quickly (Fig. 3.11 F). At this time, the length of the flower bud was c. 10-12 mm, equivalent to Stage 1 of flower development (Fig. 3.4).

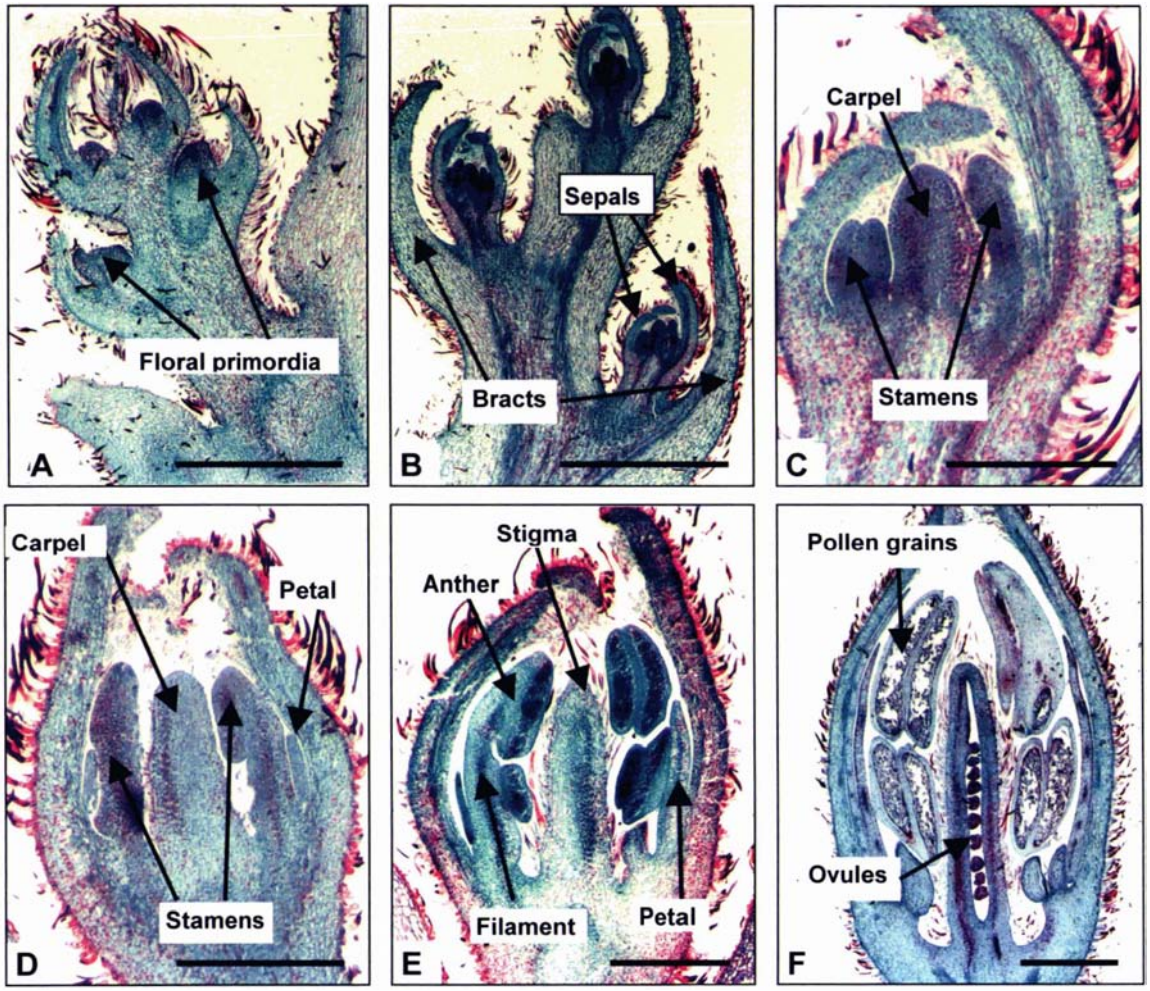

Figure 3.11 Inflorescence and flower bud initiation and development in *Sophora*

A: Inflorescence with initiated flower buds, October to November. B, C: Floral organ initiation, November to December. D: Floral organ differentiation, June. E: Floral organ elongation, July. F: Gametocyte development, August. Bars in A, B, F: 2 mm; C, D, E, 0.5 mm.

3.3.3 Postharvest treatment of cut *Clianthus* flowering shoots

3.3.3.1 Effect of sucrose holding solution on vase performance

Flower buds on the cut flowering shoots of *Clianthus* began to lose their bright colour and/or to wilt after 2-3 days in the control vase containing distilled water. At day 4-5, most buds had abscised and the occasional open flower began to wilt and lose its ornamental value. Vase performance (opening of flower buds and vase life) was notably improved by adding 1-8% sucrose to the holding solution. Among the different concentrations, 2% sucrose extended the vase life from 4.3 days to 8.8 days, and increased the percentage of open flowers to 80.4% from 14.7% in the distilled water control. Significantly better results were observed in 4% and 8% sucrose holding solutions than that of 1% sucrose, although the latter enhanced the vase performance significantly compared with the control (Fig. 3.12).

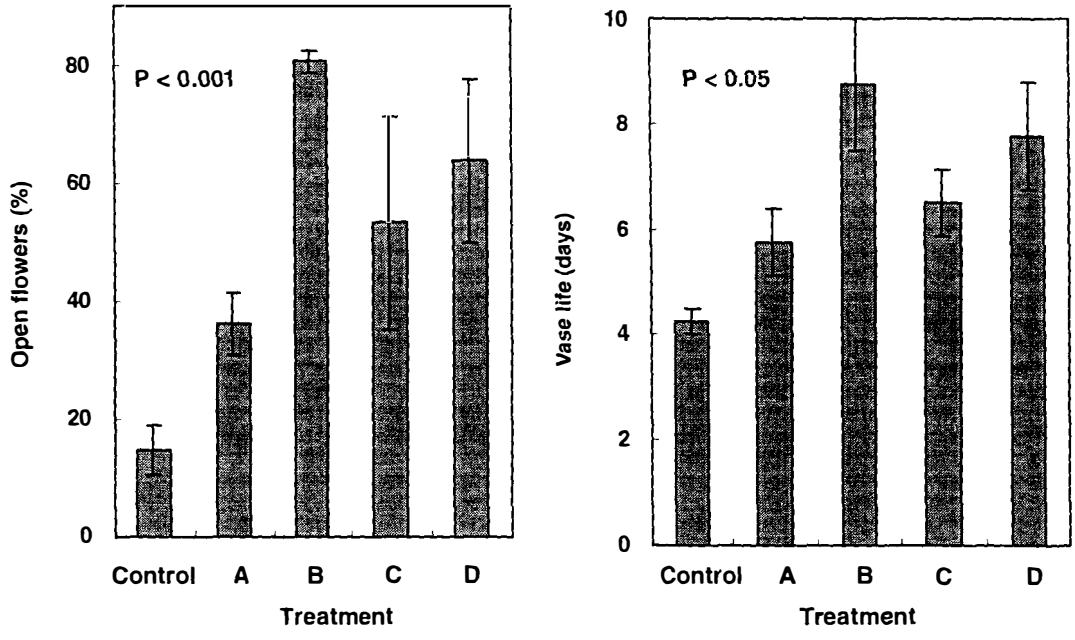

Figure 3.12 Effect of sucrose holding solution treatment on the percentage of open flower and vase life in *Clianthus*

Control: distilled water. A: 1% sucrose; B: 2% sucrose; C: 4% sucrose; D: 8% sucrose. Values were the mean of four replicates (± SE).

Marked effects on water uptake, shoot weight and petal length were observed (Fig. 3.13). During the first days in the vase, the water uptake rate declined sharply as the sucrose concentration increased, with 40% decrease in the 8% sucrose solution compared with that in distilled water from day 1 to day 3. However, 2% sucrose had a water uptake rate similar to that of the distilled water control and maintained a high rate later in the experiment. Shoot fresh weight and petal length was greatly promoted by sucrose holding solutions, with 2% yielding the highest values, followed by 4% sucrose solution (Fig. 3.13).

Application of 300 mg l^{-1} citric acid and 200 mg l^{-1} 8-hydroxyquinoline sulfate (HQS) did not yield significant results in improving vase performance (data not shown).

3.3.3.2 Effect of sucrose pulse on vase performance

Similar to the sucrose holding solution experiment, all concentrations and pulsing times of the sucrose pulse treatments extended the vase life, and increased the percentage of open flowers. Treatment also decreased the percentage of bud abscission. These beneficial effects became greater as sucrose concentration increased and pulsing time was more prolonged. The percentage of open flowers, percentage of bud abscission, and the vase life were 73%, 7.8%, and 7.3 days, respectively, after pulsing with 16% sucrose for 24 h, representing 6.6- 8.7- and 1.7-fold increases or decreases compared to those of the control. Compared to the 2% sucrose holding solution treatment, this pulse treatment also gave much better results in increasing the percentage of open flowers and decreasing the percentage of bud abscission, although the vase life remained the same (Fig. 3.14).

While the daily water uptake rate was lower in all other treatment than that of the 2% sucrose holding solution, the 8% and 16% pulse treatments applied for 24 h greatly increased the weight of the cut flowering shoots, especially during the later days of the experiment. For instance, the shoot weight for these two treatments (27.2 g and 26.8 g) were almost double that of the water control on day five (Fig. 3.15).

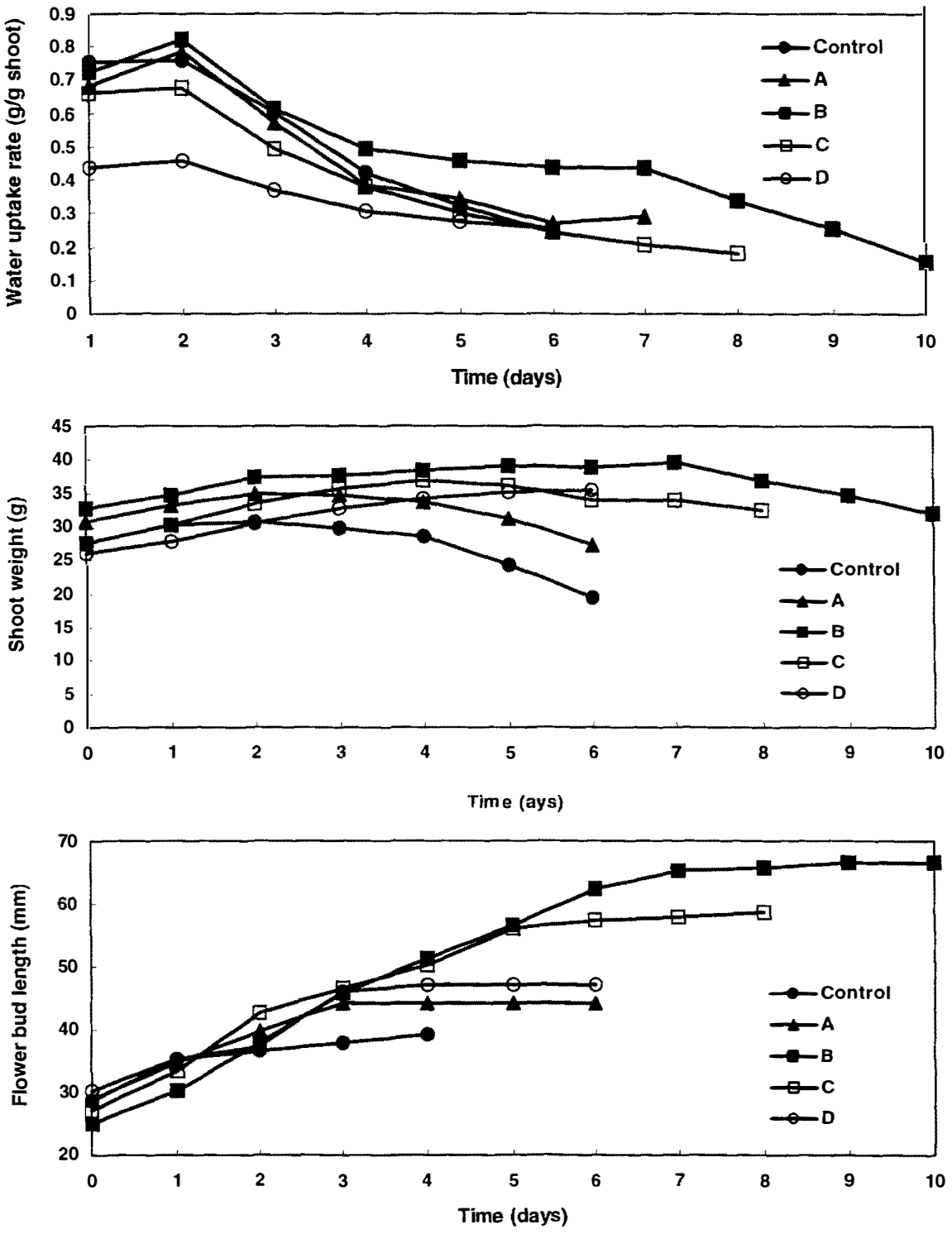

Figure 3.13 Effect of sucrose holding solution treatment on water uptake, shoot weight and petal growth rate in *Clianthus*

Control: distilled water. A: 1% sucrose in holding solution. B: 2% sucrose in holding solution. C: 4% sucrose in holding solution. D: 8% sucrose in holding solution. Values were the mean of four replicates.

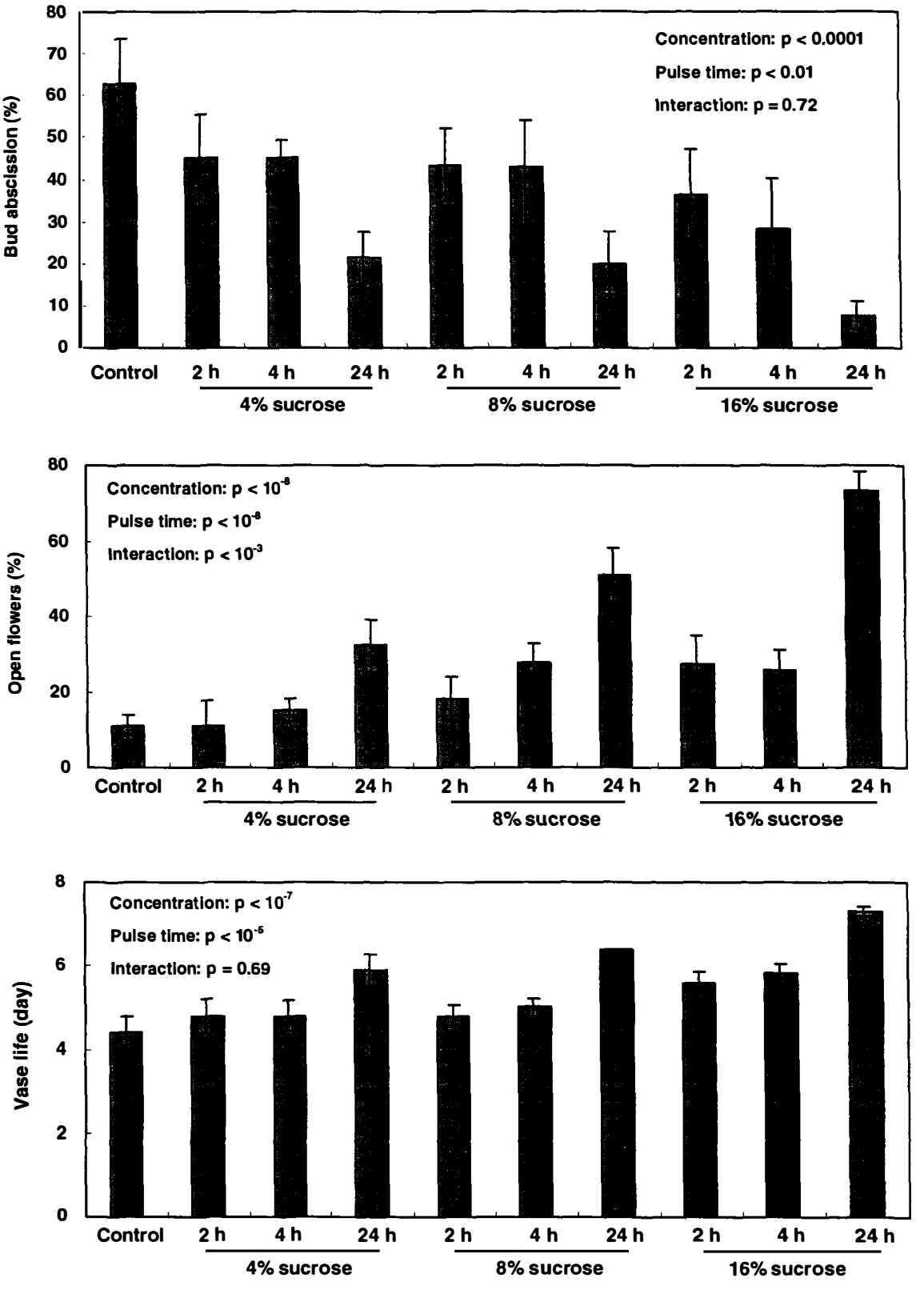

Figure 3.14 Effect of sucrose pulse treatment on flower bud abscission, the percentage of open flowers, and vase life in *Clianthus*

Control: distilled water. Values are the mean (± SE) of four replicates.

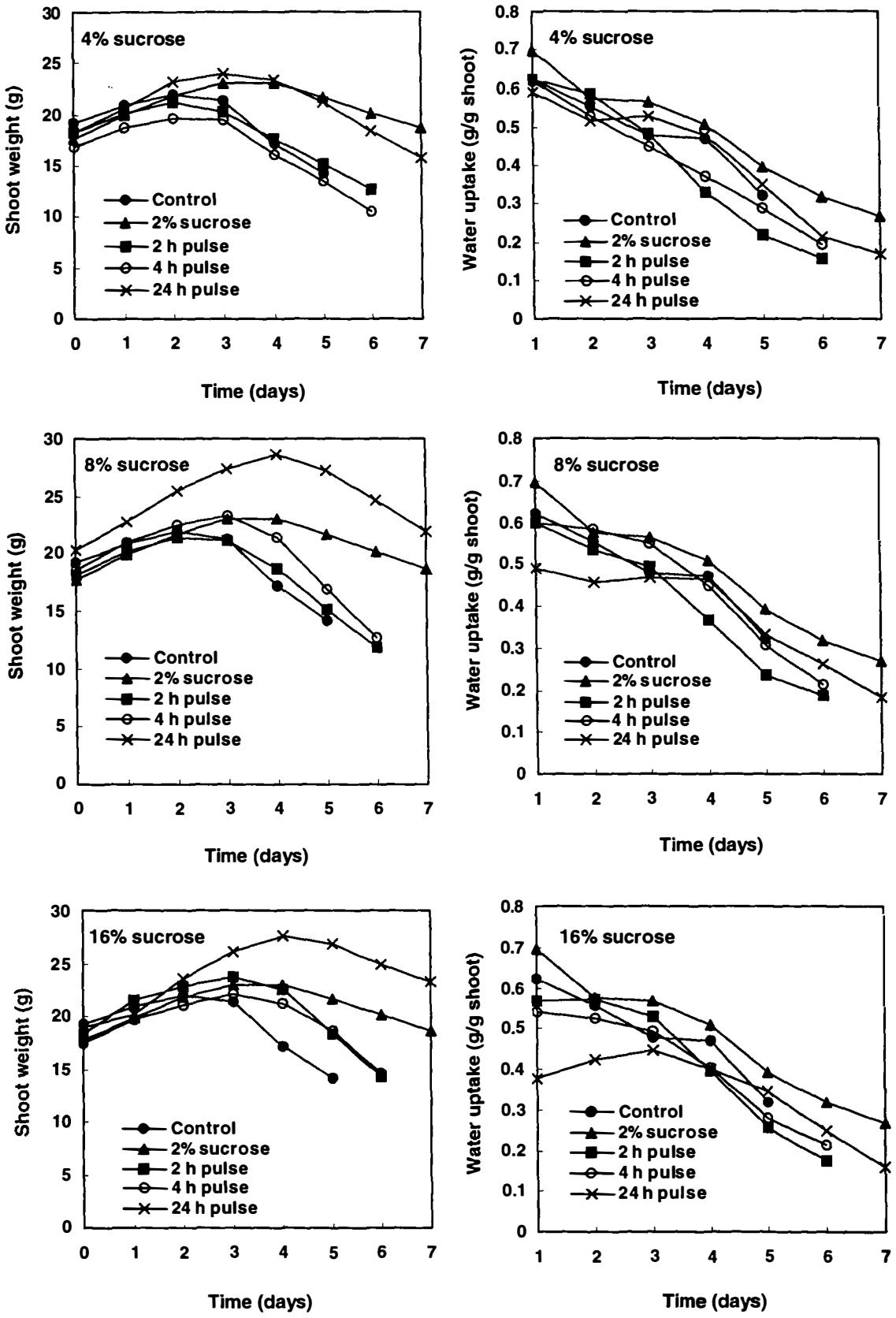

Figure 3.15 Effect of sucrose pulse treatment on shoot weight (left) and water uptake (right) in *Clianthus*

Values were the mean of four replicates.

3.3.3.3 Effect of STS treatments on vase performance

Addition of 0.5-2.0 mM STS to the holding solution was harmful to vase performance by decreasing the vase life and the number of open flowers. Flower buds were deformed and petal elongation was significantly slowed down with STS in the holding solution. No flowers opened when the STS concentrations were higher than 0.5 mM. Pulse treatment with 2 mM STS for 24 h was also harmful both in a water holding solution and in a 2% sucrose holding solution (data not shown).

However, 2mM STS pulse for a shorter time (30-120 min) had a beneficial effect and significantly improved the vase performance in most cases. The combination of STS pulse with 2% sucrose extended vase life and increased flower opening more than in either STS or sucrose treatments alone. STS pulse for 60 min followed by a 2% sucrose holding solution gave the highest values of these variables (11 days vase life and 85.4% open flowers, being 2.1- and 4.2-times higher than those of the water control) (Fig. 3.16).

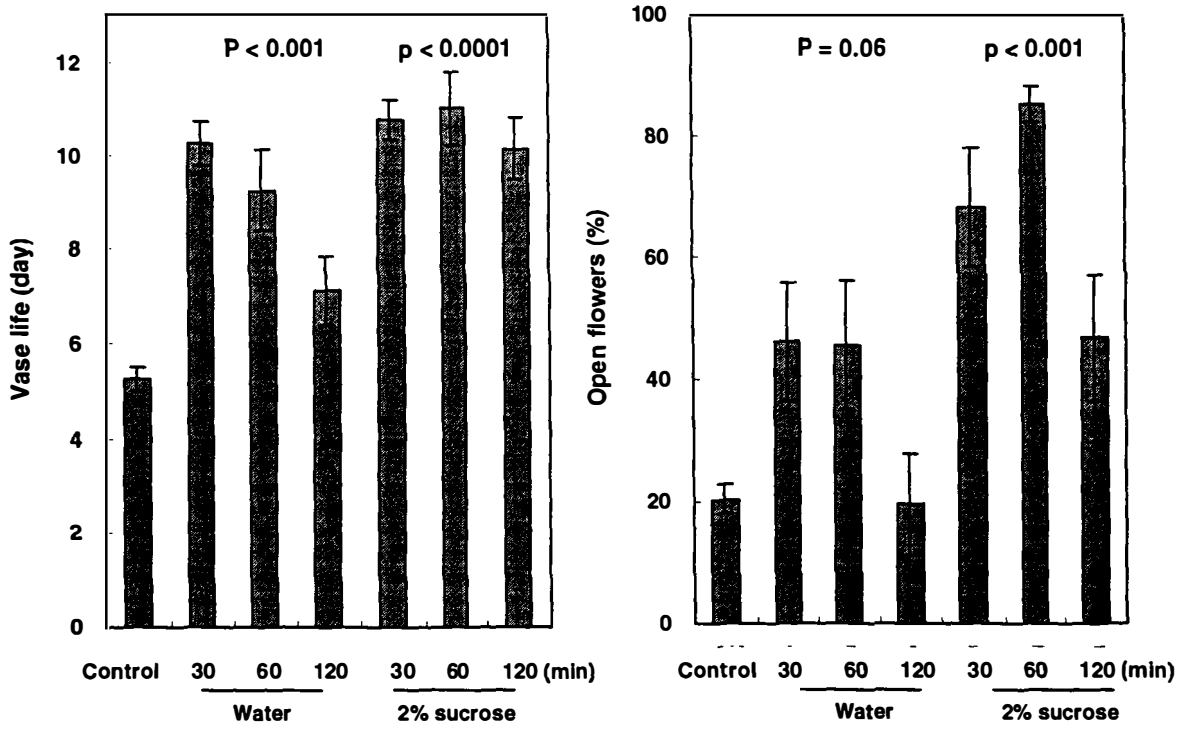

Figure 3.16 Effect of 2 mM STS pulse treatment on vase life (left) and percentage of open flowers (right) in *Clianthus*

After STS pulse, shoots were placed in distilled water or 2% sucrose. Values are the mean (± SE) of four replicates.

All STS pulse treatments decreased water uptake, at least during the first two days after treatment. The water uptake rate greatly varied for different pulsing times if the cut flowers were placed in distilled water after STS pulse, with the longest time having the lowest water uptake ability. However, this variation was almost eliminated if the cut flowers were placed in 2% sucrose after STS pulse (Fig. 3.16). The combined STS and sucrose treatments also showed the highest weight of cut flowering shoots during the whole vase period (Fig. 3.17).

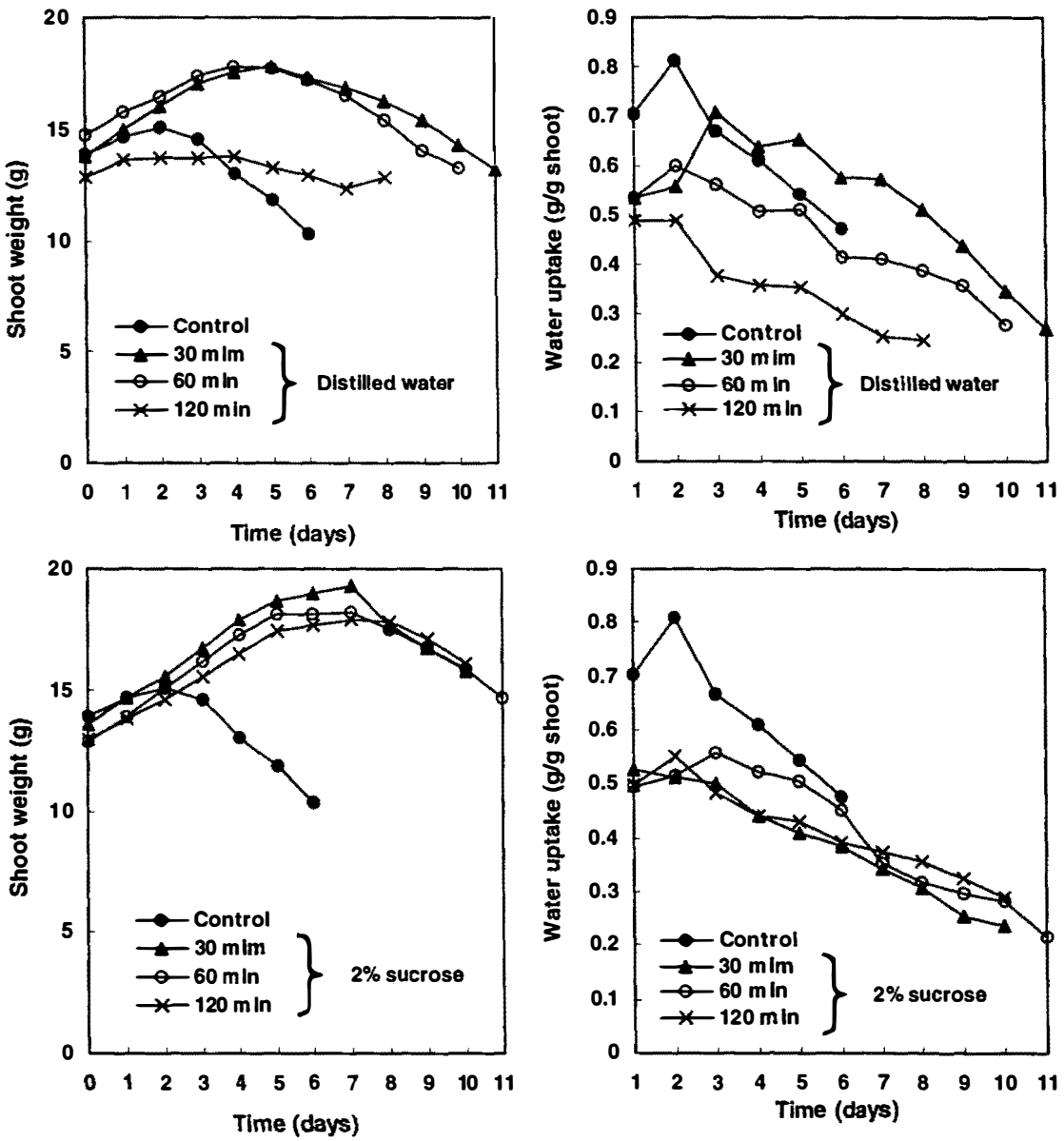

Figure 3.17 Effect of 2 mM STS pulse treatment on shoot weight and water uptake in *Clianthus*
After STS pulse, shoots were placed in distilled water or 2% sucrose. Values were the mean of four replicates.

3.3.3.4 Effect of repeated stem cutting and solution change

In the previous experiments, it was observed that water uptake ability decreased rapidly during the vase period. This might have been due to the blockage of water uptake by the accumulation of microorganisms or other substances around the cut surface. In order to test if this was the case, repeated changing of the 2% sucrose holding solution and/or stem end re-cutting were conducted every 3 days during the vase period.

While repeated solution changing or stem cutting alone did slow down the decline of the water uptake when applied during the vase period, their combination greatly increased the water uptake ability and shoot weight during the whole vase period. This treatment extended the vase life to 12 days compared with the water control (5.4 days) and the normal 2% sucrose holding control (8.8 days). A remarkably increased water uptake was observed immediately after every stem cut (Fig. 3.18).

In summary, 2% sucrose in the holding solution, 16% sucrose pulse for 24 h, and 2mM STS pulse for 30-60 min significantly enhanced the vase performance of cut flowering shoots of *Clianthus*. The combination of these treatments further increased their beneficial effects. Cut flowering shoots pulsed with 2 mM STS for 60 min and placed in 2% sucrose holding solution lasted as long as 10-12 days, with high ornamental value (Fig. 3.19).

3.3.4 Postharvest treatments of cut *Sophora* flowering shoots

Similar postharvest treatments were carried out for cut flowering shoots of *Sophora tetraptera*. No change of vase performance was observed for any treatment, all having wilted and dried out petals within five days of cutting. The best treatment combination for *Clianthus* (2 mM STS pulse for 1 h then 2% sucrose holding solution) did not extend the vase life of *Sophora* for more than one day (Appendix 1).

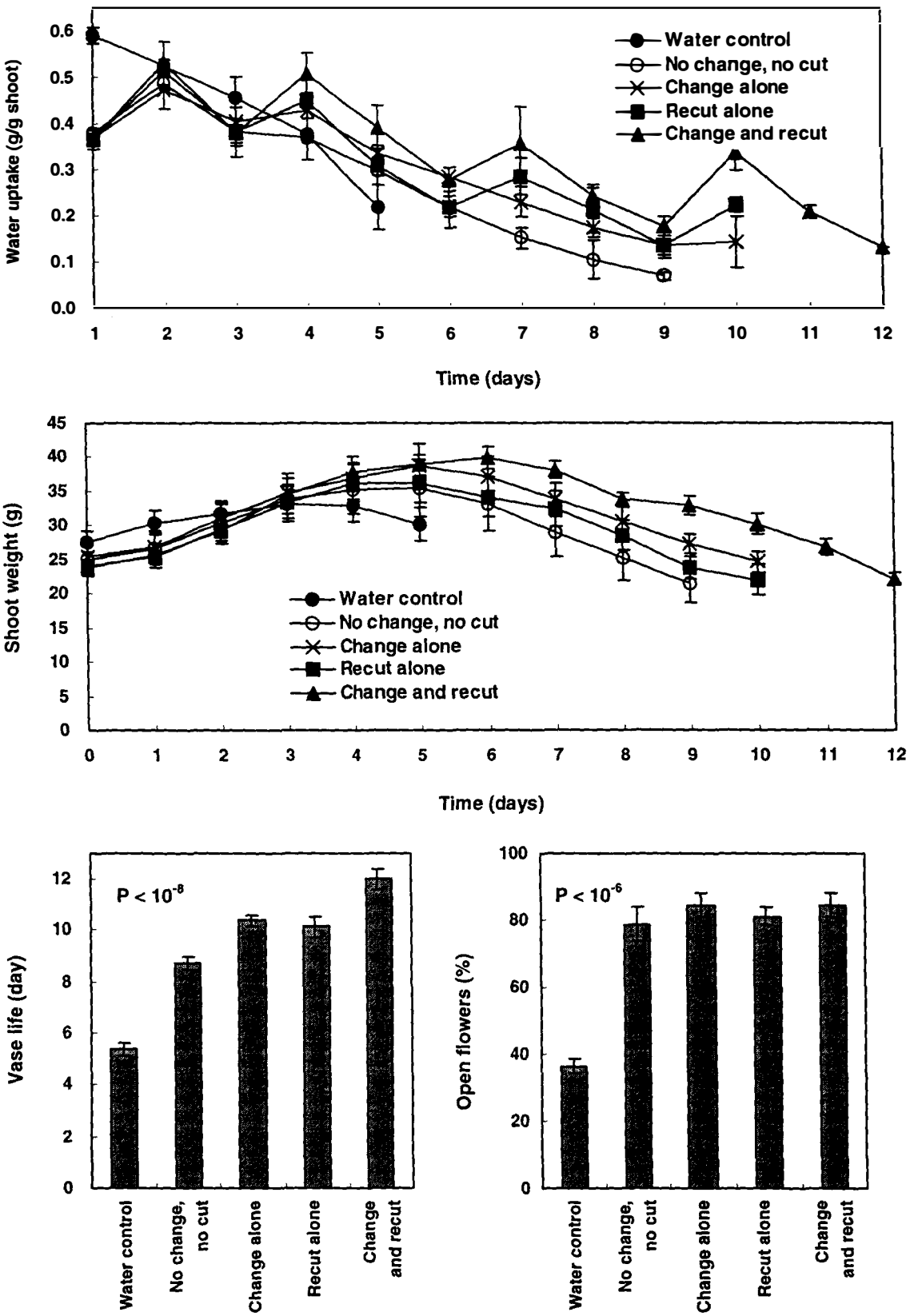

Figure 3.18 Effect of solution change and stem cut on water uptake, shoot weight, vase life and percentage of open flowers in *Clianthus*

2 mM STS pulse for 60 min then in 2% sucrose holding solution. Solution change, and stem recut were conducted every 3 days. Values are the mean (± SE) of four replicates.

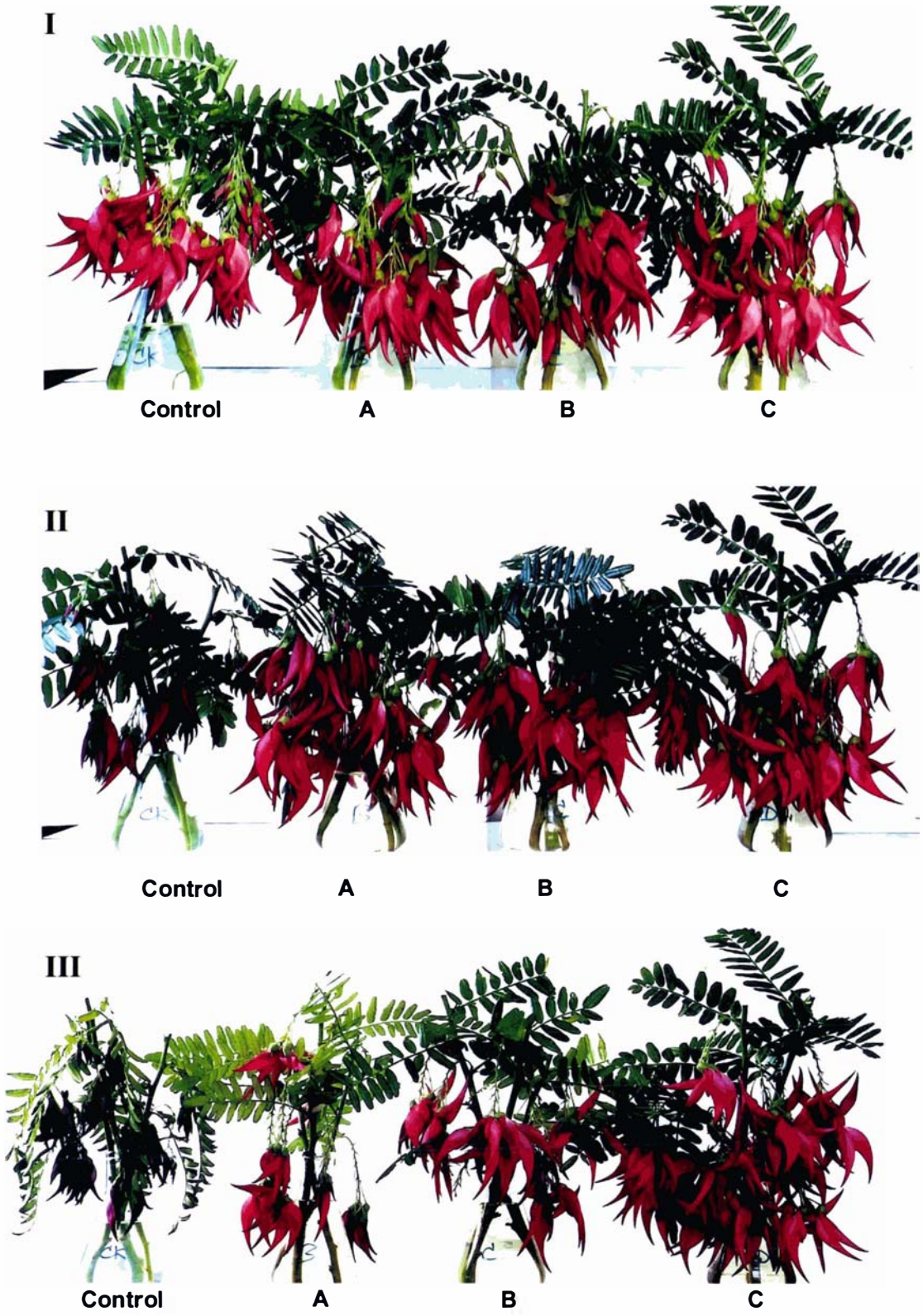

Figure 3.19 Postharvest treatments for *Clianthus* cut flowers

I: before treatment. II: 5 days after treatment. III: 10 days after treatment. Control: distilled water. A: 2% sucrose holding solution. B: 2 mM STS pulse for 1 hour then in distill water. C: 2 mM STS pulse for 1 hour then in 2% sucrose holding solution.

3.4 Discussion

3.4.1 Vegetative and reproductive development

Results in the present study, based on the systematic investigation during two complete growing cycles, provided comprehensive information and detailed developmental calendars of vegetative and reproductive growth behaviours, and microscopy characteristics of floral ontogeny for both *Clianthus maximus* and *Sophora tetraptera.* To my knowledge, this is the most comprehensive study of its type to date for all *Clianthus* and New Zealand *Sophora* species.

The vegetative and reproductive growth characters of *Clianthus* revealed by the present study supported the general finding made by Smith-Dodsworth (1991), Shaw and Burns (1997) and Heenan (1998a, 2000). However, no detailed developmental calendar, either vegetative or reproductive, has yet been established to date for this species. The year-round emergence and the mass abortion of inflorescences have not been described in other studies. Furthermore, no microscopic observation of floral ontogeny has been reported so far in the genus *Clianthus* and other closely related genera in the tribe *Galegeae*, including *Swainsona* and *Carmichaelia.*

In the present study, a very unusual floral organogeny and ontogeny process was observed in *Clianthus maximus,* with the following aspects. Firstly, the initiation of the carpel was before, or at least simultaneous with, the initiation of the outer stamens. Secondly, the development of the carpel was much more advanced than that of the other floral organs in whorl two and whorl three during most developmental stages, and the height of the former remained superior until the formation of gametocytes (Fig. 3.8F). Furthermore, petal development was markedly delayed until a late developmental stage, when other floral organs were well differentiated (Fig. 3.8F).

This process of floral ontogeny is in contrast to that most commonly reported in other species, including those from the family Fabaceae and the sub-family Papilionoideae, in which carpels are initiated after all other three whorls, or occasionally overlapped with the inner stamens (Tucker, 1994, 2003a; Prenner, 2004). The only similar situation was described by Tucker (2002) in *Cadia purpurea* in the sub-family Papilionoideae. In this species, the carpel initiated directly after petal initiation and its development was more

advanced than other floral organs till the differentiation of anthers and ovary. The petal primordia were the same length as the stamen primordia (c.75 μm) at the time when all petal and stamen primordia had been initiated, and remained the same size until their enlargement in the late stage of floral ontogeny, when all other floral organs had enlarged, and differentiated.

It did not appear that the same situation occurred for the petals of *Clianthus* because no floral structure of the same size was observed during organogenesis and in the early stages of organ enlargement. Given the fact that the inner stamens were also of the length of 80-100 μm and were clearly distinguishable after organogenesis was completed, and that the outer stamens were c. 200 μm in length at this stage (Fig. 3.8 D), it was very unlikely that petal primordia were overlooked or confused with stamens in the current study. It is then suggested that either petal initiation occurred in the normal sequence and these petals remained small, much smaller than in the case of *Cadia purpurea*, or petal initiation occurred at a later stage. Since there is no information of floral ontogeny available in other species within the tribe *Galegeae*, no conclusion could be made at this stage as to whether this unusual process of floral ontogeny is specific to the genus *Clianthus* or is shared by other closely related species, such as in the genera *Swainsona* and *Carmichaelia*.

In *Sophora tetraptera*, the vegetative and reproductive growth characters revealed by the present study were consistent with some more general observations made by Salmon (1996) and Heenan et al. (2001). However, little information about the detailed flower developmental process and floral ontogeny for this species is available to date. No detailed vegetative and reproductive developmental calendar has yet been established. The phenomenon of inflorescence and flower bud initiation and development during a short time in spring, and a long period of summer-autumn dormancy revealed in the present study has also not been described. The same is true for all New Zealand *Sophora* species and even for the whole section *Edwardsia* that comprises at least 19 species occurring across the Pacific, the South Atlantic, Indian Ocean and in South America (Polhill, 1981, Maunder et al., 1999; Heenan et al., 2001; Bernardello et al., 2004). Since the genus *Sophora* is considered one of the most specialized elements of the Sophoreae (Polhill, 1981; Tucker, 1994), the results revealed in the present study may provide some useful indication of behaviour in other species, especially in the section *Edwardsia*.

In fact, the section *Edwardsia* is distinct from the other 10 sections in the genus *Sophora,* because its members have adaptations for bird pollination. The general vegetative growth and flowering characters of the species in this section and their distinctness from other members of the genus *Sophora*, including larger flower size, yellow petals, nearly equal petal size and shape, long exserted stamens, and lack of typical papilionoid corolla structure, has been well documented (Godley, 1975; Godley and Smith, 1977; Polhill, 1981; Heenan, 1998a; Peña et al., 2000; Heenan et al., 2001; Bernardello et al., 2004).

Microscopic study in *Sophora tetraptera* revealed a similar but varied process of floral ontogeny to that of *Clianthus maximus*. Delayed petal development was observed as for *Clianthus*. However, the development of outer stamens remained more advanced than that of the carpel at all stages, in contrast to the situation in *Clianthus*. Furthermore, a long developmentally inactive period (from January to June) of initiated floral organs was observed, which was coincident with the summer-autumn dormancy observed at the whole plant level. During this rest or dormant period, carpel and stamens remained un-elongated and un-differentiated, although their size increased a little but very slowly.

This process of floral ontogeny varied from that of many related species in the sub-family Papilionoideae, such as *Pisum sativum* (Tucker, 1989), *Trifolium repens* (Retallack et al., 1990) and *Daviesia cordata* (Prenner, 2004). No obvious precocious initiation of carpels and delay of petal development were observed in these studies. It even contrasted with that of many other species in the tribe Sophoreae and genus *Sophora,* such as *Myroxylon balsamum*, *Castanospermum australe* and *Sophora japonica* (Tucker, 1993, 1994). In a study including eight species from six of the eleven sections in the genus *Sophora*, Tucker (1994) concluded that the majority of investigated *Sophora* species had essentially uniform organogeny, with predominantly acropetal order of floral organs initiation. The sepals initiated in the first place, followed by petals. Carpels initiated precociously at the same time as the outer stamens, and the inner stamens initiated at the end of organogenesis. In this generalised process of organogeny represented by *S. japonica*, the petals remained longer and more superior than stamens during all stages of organogenesis and floral organ development.

One exception observed in Tucker's study (1994) was *Sophora chrysophylla,* a species from section *Edwardsia,* in which initiation overlap occurred in three inner whorls. Three outer stamens and two inner stamens initiated before the last petal initiated. Carpels

initiated before inner stamens. At the time all organs were initiated, the length of the largest stamen was similar to that of the carpel, while the length of the largest petal was 2/3 that of the carpel. The smaller petals and smaller stamens were similar in length, which was half of that of the carpel. A slightly delayed petal development was also observed. Although this process of organogeny is similar to that of *S. tetraptera*, the petal initiation and development of *S. chrysophylla* was much in keeping with the generalized process for the genus *Sophora* (Tucker, 1994). Interestingly, the organogeny of another species from the section *Edwardsia* included in the study by Tucker (1994), the New Zealand *S. microphylla*, was considered as normal by the author because no special note of an unusual order of organogeny was made for this species, and no comparison of this species with *S. chrysophylla* was made.

Furthermore, none of these studies described the interruption of floral ontogeny by a prolonged dormant period as revealed in the present study, which should have been applicable at least to *S. microphylla.*

In summary, unusual floral organ ontogeny, with precocious carpel initiation and delayed petal development, were observed in both *Clianthus maximus* and *Sophora tetraptera*. More advanced carpel development than all other floral organs was also observed in *C. maximus*. An interruption of floral organ ontogeny occurred for a prolonged time (January to June) in *S. tetraptera.*

The unusual floral organogeny and ontogeny revealed in the present study, and the successive and overlapping order of organ initiation or other anomalies occurring in many other leguminous species (Tucker, 1989; Ferrándiz et al., 1999; Tucker, 2003a) conflict with the prevailing interpretation of the ABC model hypothesis of floral organ identity applied to herbaceous model species such as *Arabidopsis*, in which all organs of a whorl initiate simultaneously (Meyerowitz et al., 1991; Irish, 1999; Jack, 2001). This strongly suggest that the genetic control of the floral process in these leguminous species, especially woody legumes such as *Clianthus* and *Sophora,* varies from that in the herbaceous model species. Therefore, it is of great significance to investigate whether the key regulatory genes involved in flowering function the same way in these species as in the model species, *Arabidopsis* and *Antirrhinum.*

The validity of applying the herbaceous model of flowering to woody leguminous species *Clianthus* and *Sophora* was assessed by isolating the floral identity genes including *LFY*

and *ABC* class genes such as *AP1, PI* and *AG* homologues in these two species (Chapter 4), and then by investigating the temporal and developmental expression patterns of these genes (Chapter 5).

3.4.2 Postharvest treatment

Postharvest experiments indicated that *Clianthus* has several features suggesting commercial cut flower potential if appropriate postharvest treatment could be applied. Cut *Clianthus* flowering shoots lasted as long as 10-12 days, which is comparable to many other popular cut flower species (Halevy and Mayak 1979; Nowak and Mynett, 1985).

Since this is the first reported study of the vase life characteristics of cut *Clianthus* flowering shoots, direct comparison with other studies of these species was not possible. In postharvest studies of other species, the end point of vase life has been variously regarded as occurring at the start of wilting or fading, through all the intermediate stages, to the senescence of all flowers (Halevy and Mayak 1979). The time until 50% of flowers had senesced was found to be the preferred measure for vase life of cut flowers, and was therefore used in the present study.

The effect of a sucrose pulse or its continuous presence in the holding solution by prolonging vase performance revealed in the present study has been documented for a great number of species, including legume cut flower species such as sweet pea (Halevy and Mayak, 1979; Nowak and Mynett, 1985; Sexton et al., 1995). Ichimura and Hiraya (1999) reported that treatment with sucrose doubled the vase life, and promoted pigmentation of petals in sweet pea cut flowers. In non-leguminous species, while best vase performance of cut *Eustoma* flowers was obtained by continuous treatment with vase solutions containing 3% or 6% sucrose (Cho et al., 2001), Eason et al. (2004) was able to double the vase life of the *Gentiana* cultivar 'Late Blue' using pulsing solutions that contained 5% sucrose.

For the sucrose pulse treatment, most reports used a sucrose concentration of less than 10% (Sexton et al., 1995; Ichimura and Hiraya, 1999). Eason et al. (2004) found that pulsing solutions that contained sucrose higher than 10% caused reddening of the leaves and therefore did not increase the vase life of the whole stem. Zhang (1996) also noted that 10% sucrose pulse for 24 h enhanced the quality of cut stems, whereas 15% sucrose

pulse for 24 h caused leaf burn. Results from these reports were not consistent with those of the present study, for which the 16% sucrose pulse for 24 h resulted in the enhancement of vase performance compared to that of 8% and 4% sucrose. This suggests that the sensitivity and requirement of sugar may vary notably among different species.

The role of sucrose in cut *Clianthus* flowers might be as osmolyte and substrate for respiration, as for the majority of cut flower species (Halevy and Mayak, 1979; Ichimura and Hiraya, 1999). An alternative role of sucrose may be to reduce the ethylene sensitivity and/or to inhibit ethylene production in cut flowers. Sucrose has been found to reduce the sensitivity to ethylene in cut carnation flowers (Mayak and Dilley, 1976). Ichimura and Hiraya (1999) reported that treatment with sucrose alone extended the vase life of florets of cut sweet peas, accompanied by the inhibition of ethylene production. They assumed that treatment with sucrose could increase the sugar concentration in the flowers, which inhibited ethylene production, leading to the extension of the vase life of flower.

Ethylene has long been associated with abortion, abscission, and premature death of buds and flowers of many plant species. For sensitive species, ethylene induced senescence and abscission of flowers may result from endogenous production of ethylene or from exposure to exogenous ethylene (Halevy and Mayak, 1979). Thus, in cut flower species showing ethylene-sensitivity, vase life could be improved by treatment with ethylene inhibitors such as silver thiosulfate (STS), which prevents ethylene action (Beyer, 1976; Veen and van de Geijn, 1978; Reid and Wu, 1992). Given the fact that untreated cut flowers of *Clianthus* abscised soon after being put in a vase containing water, and that STS treatment largely enhanced the vase life of *Clianthus* flowers in the present study, it is strongly suggested that this species is ethylene sensitive, as are its closely related cut flower species sweet pea and lupin. Treatment of the latter species with STS also markedly extended the vase life of cut flowers (Mor et al., 1984; Mackay et al., 2001; Ichimura et al., 2002). In addition, STS is effective in extending the vase life of many other cut flowers (Veen, 1979; Nowak and Mynett, 1985).

The beneficial effect of STS treatment on postharvest quality of cut flowers is mainly due to its binding to ethylene receptors (Sisler et al., 1986), increasing the opening of immature buds that would otherwise abort due to carbohydrate depletion. The effectiveness of STS compared with other ethylene inhibitors appears to be due to its high

mobility within the plant over a relatively long period (Veen and van de Geijn, 1978; Ichimura and Hiraya, 1999). This effectiveness of STS is also applicable in woody species. For example, ethylene effects were effectively negated in cut *Metrosideros collina* flowers by 2.0 mM STS treatment (Sun et al., 2000).

The present study also showed that high concentrations of ,or prolonged pretreatment with, STS could be detrimental, causing blackening of flower buds and yellowing of leaves, presumably due to phytotoxic effects of too much STS being absorbed. A similar situation was observed by Han and Miller (2003) in *Lilium* cut flowers pretreated using 1 mM STS for longer than 1 h, in which early leaf yellowing developed in treated stems. Interestingly, these phytotoxic effects were reduced, in the present study, by combining the STS pulse pretreatment with sucrose in the holding solution, which resulted in the highest number of open flowers and the longest vase life. This synergistic effect has been found in a number of other studies. In cut sweet pea flowers, vase life was also further extended when sucrose was added to vase water together with STS (Mor et al., 1984). Similarly, Ichimura (1998) observed that the addition of STS followed by sucrose was more effective than that of STS alone in improving the vase life of cut sweet pea flowers. Pretreatment with 0.2 mM STS for 2 h followed by 10% sucrose pulse for 16 h was the most effective in promoting flower opening as well as extending the vase life. Meir et al. (1995) reported that vase life could be extended in mini-gladiolus cut spikes treated with combined sucrose and STS pulsing.

In contrast to *Clianthus* and the above mentioned species, no beneficial effect was observed in the present study by either sucrose or STS treatment for *Sophora* cut flowers using similar treatment to those for *Clianthus*. This suggests that this species might not be sensitive to ethylene, and that *Sophora* cut flowers might belong to the type II flower group described by Woltering and van Doorn (1988), in which wilting is apparently not mediated by ethylene. According to these authors, senescence of this type of flower is not affected by treatment with exogenous ethylene or ethylene inhibitors. Similar situations were observed by Evans et al. (2002) for *Ranunculus lyallii* for which sucrose treatments did not enhance bud opening.

Chapter 4 Isolation and characterization of floral identity genes in *Clianthus* and *Sophora*

4.1 Introduction

Knowledge of molecular mechanisms involved in the flowering process could greatly enhance our understanding of the reproductive behaviour of plants, which has particular significance for the conservation of endangered or potentially endangered species, such as *Clianthus* and New Zealand *Sophora.*

The flowering process consists of several steps including the formation of the floral meristem, the establishment of unique organ identities, and the differentiation of floral structures. The meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) in *Arabidopsis*, and *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*) in *Antirrhinum* are necessary for the determination of floral meristem identity and the formation of floral primordia after their floral induction (Coen et al., 1990; Weigel et al., 1992; Weigel and Nilsson, 1995; Mandel and Yanofsky, 1995). After individual flowers have been initiated, floral organs arise in four concentric rings or whorls. The fate of these organs is dependent on floral organ identity genes, which fall into three classes and fit the ABC model of floral organ development. These genes act in a combinatorial manner to specify each organ type (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994).

In *Arabidopsis*, *LFY* protein is necessary and sufficient for the vital switch from vegetative to reproductive development (Weigel et al., 1992; Weigel and Nilsson, 1995). After initiating the meristem identity switch, *LFY* has a second role in the activation of the floral homeotic genes that specify the identity of organs in the flower (Weigel and Meyerowitz, 1994). *LFY* regulates the transition to flower development by inducing *AP1* expression in regions of the shoot apical meristem that give rise to flower primordia (Parcy et al., 1998).

Apart from its role in meristem identity, *AP1* also act as an A class gene that specifies sepal and petal identity during flower development (Bowman et al., 1993; Schultz and Haughn, 1993). Two B class genes, *PISTILLATA* (*PI*) and *APETALA3* (*AP3*) in *Arabidopsis* and *GLOBOSA* (*GLO*) and *DEFICIENS* (*DEF*) in *Antirrhinum*, are required for specifying petal and stamen identity (Trobner et al., 1992; Jack et al., 1992; Goto and

Meyerowitz, 1994; Riechmann et al., 1996a, b). C class genes *AGAMOUS* (*AG*) in *Arabidopsis* and *PLENA* (*PLE*) in *Antirrhinum* are necessary for the identity of petals and carpels (Drews et al., 1991; Ma, 1994).

Although these floral identity genes have been isolated and characterized in a variety of plant species, including some woody species such as the New Zealand *Metrosideros* (Sreekantan et al., 2004), little is known about these genes in leguminous species, especially woody legumes. Only a few *LFY* and *AP1* homologues, such as *UNIFOLIATA* (Hofer and Ellis, 1998) and *PEAM4* from *Pisum sativum* (Taylor et al., 2002), have been isolated and studied for their role in floral identity, although *LFY* homologues were recently isolated from *Brownea* and other leguminous species for objectives other than the study of floral development (Archambault and Bruneau, 2004). Homologues of B and C class genes in legumes have not yet been reported, although the first *PI* homologue sequence of *PEAM1* from *Pisum sativum* has recently been submitted to GenBank, and the data still remain unpublished (Ferrándiz and Beltran, 2004). These studies showed that floral identity genes in *Pisum sativum* have the same basic roles as those for herbaceous model species, with some variation. The applicability of the ABC model still has to be tested for other leguminous species, especially perennial woody legumes.

As described in Chapter 3, *Sophora* and *Clianthus* flowering processes are distinct from those of annual species and other woody perennial species. Contrasting characters between these two genera were also observed. Some unusual sequences of events in organogenesis and floral organ development, such as the precocious initiation of the carpel at the delayed development of petals were also observed, conflicting with the hypotheses of the ABC model in the timing of determination of organ identity.

Given the fact that floral identity genes, especially those of the ABC class genes, are highly conserved across angiosperm species, it was hypothesised that there would be homologues of all herbaceous floral identity genes expressed in the reproductive organs of both *Sophora* and *Clianthus*. In the current chapter, a description of how partial homologues of *LFY/FLO*, *AP1/SQUA*, *PI/GLO* and *AG/PLE* were isolated from *Sophora* and *Clianthus* is provided. cDNA and deduced protein sequences were analysed and compared to their homologues from a broad taxonomic range of species.

4.2 Materials and methods

4.2.1 Extraction of RNA

For RNA extraction, inflorescences and flower buds were sampled from three year-old *Clianthus maximus* Kaka King® and *Sophora tetraptera* plants grown in the Palmerston North site nursery of the New Zealand Institute for Crop and Food Research Limited. At the time of harvest, tissue samples were immersed in liquid nitrogen on site and stored at -80°C after being transferred to the laboratory.

4.2.1.1 Hot-borate extraction method

Initially, total RNA was isolated using the hot-borate extraction protocol for total RNA isolation based on the method described by Wilkins and Smart (1996) and adapted by Sreekantan (2002) with minor modification. The hot-borate extraction (XT) buffer contained:

0.2 M Na borate decahydrate (borax)
30 mM EDTA
2% (w/v) SDS
10 mM dithiotrietol (DTT)
1% Nonidet P-40 (NP-40)
2% (w/v) polyvinylpyrrolidone (PVP)

To dissolve, components were added to pre-warmed RNase-free milliQ water, pH adjusted to 9 with NaOH, and the stock solution was autoclaved. DTT, PVP and Nonidet P-40 were added only immediately before the extraction.

Approximately 1 g of frozen *Sophora* or *Clianthus* tissue was ground to a fine powder in a pre-chilled mortar and pestle using liquid nitrogen as needed. Pre-warmed (80°C) hot-borate extraction (XT) buffer was dispensed into a mortar and pestle at room temperature and the ground tissue was transferred into it (2.5 ml g^{-1}). After blending well for 1 min the homogenate was quickly decanted into a 50 ml centrifuge tube with 105 µl of the proteinase K (20 mg ml^{-1} in water) solution. The mortar was rinsed with 1 ml of XT buffer, and this was also transferred to the proteinase K homogenate in the centrifuge tube.

The tube was then incubated at 42°C for 1.5 h with gentle swirling and mixing every 10 min. After that, 280 µl of 2 M KCl was added to a final concentration of 160 mM. The mix was gently swirled and then incubated on ice for 1 h. It was then centrifuged at 12000g for 20 min at 4°C to remove the debris. The supernatant was transferred carefully to another clean centrifuge tube, 1/3 volume of 8 M LiCl was added (to a final concentration of 2 M LiCl) and the mixture incubated overnight at 4°C.

Precipitated RNA was then pelleted by centrifugation at 12000g for 20 min at 4°C. The supernatant was decanted and discarded. The pellet was washed in 5 ml of ice-cold 2 M LiCl four times, each time centrifuging at 12000g for 10 min at 4°C and discarding the supernatant. After the fourth wash, the pellet was suspended in 2 ml of 10 mM Tris-HCl (pH 7.5) by gentle vortexing. The sample was warmed to room temperature to dissolve. The insoluble material was pelleted by centrifugation at 12000g for 10 min at 4°C. The supernatant was transferred to another centrifuge tube, 1/10 volume of 2 M potassium acetate (pH 5.5) was added and the mix incubated on ice for 15 min. This was followed by centrifugation at 12000g at 4°C for 10 min to remove the polysaccharides and insoluble material. The supernatant was transferred to a further clean centrifuge tube and RNA was precipitated with the addition of 2.5 x volume of 100% ethanol and left overnight at -20°C.

The next morning, the RNA was pelleted by centrifugation at 9800g for 30 min at 4°C. The ethanol was decanted and discarded. The RNA pellet was washed gently with 2 ml of ice-cold 70% ethanol and centrifuged at 9800g for 5 min at 4°C. The ethanol was decanted and discarded and the residual ethanol was removed under vacuum. The pellet was re-suspended in 200 µl RNase-free autoclaved milliQ water and transferred to a 1.5 ml centrifuge tube. The 50 ml centrifuge tube was rinsed with another 1 ml of water and that too was transferred to the 1.5 ml centrifuge tube. For further purification, the RNA was precipitated with 1/10 volume of 3 M sodium acetate (pH 6.0) and 2.5 x volume of ice-cold 100% ethanol at 20°C for 2 h. RNA was pelleted and collected by centrifugation (16000g) at 4°C for 20 min in a bench top centrifuge. The pellet washed with cold 70% ethanol and centrifuged for another 5 min. Residual ethanol was removed and the pellet dried under vacuum. The RNA was re-suspended in sterile RNase-free water.

The quantity and the quality of RNA were estimated based on the A_{230} nm, A_{260} nm, A_{280} nm, and A_{320} nm values in a spectrophotometer (Hitachi V-1100). Subtracting the A_{320}

nm values from the A_{260} nm readings gave the RNA concentrations corrected for polysaccharides and other contaminants. RNA quantification was carried out using the formula 1 A_{260} nm reading = 40 μg RNA. An A_{230} nm: A_{260} nm: A_{280} nm ratio of 1:2:1 indicated a preparation of high quality.

4.2.1.2 Mini-preparation method

To minimise the tissue sample needed and to simplify the RNA extraction procedure, a mini-preparation protocol was developed based on the standard protocol of the TRI Reagent method. 50-100 mg of frozen tissue sample was ground in a 1.5 ml centrifuge tube with a plastic mini pestle in the presence of liquid N_2. TRI reagent (800 μl) was added and vortexed for 20-30 s. The mixed sample was transferred to a new centrifuge tube and centrifuged for 5 min at 10000g at 4°C in a benchtop centrifuge. The supernatant was transferred to a new centrifuge tube and stored at room temperature for 5 min. Chloroform (500 μl, isoamylalcohol-free) was added, shaken vigorously for 15 s and stored for 10 min before being centrifuged for 15 min at 10000g at 4°C. The aqueous phase was transferred to a new centrifuge tube. Isopropanol (100 μl) was added, well mixed, stored for 10 min, and then centrifuged at 10000g for 8 min at 4°C. The RNA pellet was washed with 1 ml 75% ethanol and centrifuged at 4000g for 5 min at 4°C. Ethanol was removed and the pellet air-dried for 3-5 min. RNA was dissolved in 30 μl TE buffer or RNAse-free water with 1x RNAsecure™ (Ambion Inc) and heated to 65°C for 15 min to activate its RNA protection ability.

The quantity and quality of total RNA were measured using a NanoDrop spectrophotometer (Nyxor, ND-100). Its integrity was checked by visualization of ethidium bromide-stained RNA separated on 2% (w/v) agarose gel containing 2% (v/v) formaldehyde. Only RNA without detectable degradation of 18S and 28S rRNA was used for subsequent cDNA synthesis either immediately or following storage at –20°C.

4.2.2 Reverse transcription-polymerase chain reaction (RT-PCR)

One μg RNA and 0.2-0.3 μg (40-60 pmoles) oligo $(dT)_{15}$ primer was added to RNase-free sterile water in a centrifuge tube to make a final volume of 10 μl and was warmed at 65°C for 10 min in a water bath. The reaction mix was put on ice to cool and then 4 μl of 5 x buffer, 2 μl of 100 mM DTT, 1 μl of 20 mM dNTPs mix, 1 μlof RNase inhibitor (50 U/μl)

(Roche) and 1 μl of Expand Reverse Transcriptase (Roche) were added to make a final volume of 20 μl. The reaction mix was incubated at 48°C for 30 min. It was then cooled on ice and the synthesized cDNA stored at -20°C until the PCR reaction. The composition of the PCR mix is given below for a 20 μl PCR reaction:

10X Taq buffer	2 μl
dNTPs (2 mM)	2.5 μl
25 mM $MgCl_2$	1 μl
Primers (10 pmol/μl)	1 μl each
cDNA (5-fold diluted)	2 μl
Taq DNA polymerase (5 U/μl)	0.2μl
Water	10.3 μl

First run PCR was carried out to synthesize the second cDNA strand using 2 μl 5-fold diluted RT reaction. The PCR programme was: 94°C, 5 min; 40°C, 5 min; 72°C, 5 min (1 cycle). 94°C, 45 s; 48°C, 45 s; 72°C, 1 min (30 cycles). 72°C, 5 min. 4 °C hold. For PCR amplification, the second run PCR was conducted using 2 μl first run PCR product as template. The PCR program was: 94°C, 5 min. 94°C, 45 s; 56°C, 45 s; 72°C, 1 min (30 cycles); 72°C, 5 min. 4 °C hold.

4.2.3 Isolation of the partial homologues of floral identity genes and housekeeping genes from *Sophora* and *Clianthus*

All target genes in this study were isolated through direct sequencing of RT-PCR product using tissue specific cDNA as PCR template. Degenerate primers were used for isolation because there was no previous molecular information of floral genes and housekeeping genes available for these two species, and very little such information was available for leguminous woody species or even for the whole leguminous family in general.

4.2.3.1 Degenerate primer design

All the target genes in this study, except for the *LFY* homologue, belong to the sequence conserved MADS box gene family containing many subfamilies, and there normally exist several paralogues in the same organism. In order to isolate the coding sequences of each specific target gene whilst avoiding the false isolation of any of co-existent paralogues or genes from other subfamilies, a series of orthologue amino acid sequences for each gene

of interest obtained from GenBank was aligned using the Clustal X program (Version 1.83) (Thompson et al., 1997). Amino acid sequence segments conserved within the specific subfamily of the target gene, but not across the subfamilies in the MADS-box gene family, were identified. DNA sequences of these segments were then aligned again and the degenerate primers of 20-24 nucleotides were carefully designed. The suitability of these short sequences as PCR primers was checked using the Primer Premier program (Version 5.0). In general, two forward primers (F) and two reverse primers (R) were selected for each target gene. Sequences of these primers were as follows (N = ATGC, M = AC, R = AG, Y = CT, W=AT, K=GT, S=GC, H=ACT, B=CGT, V=CG, D=AGT):

LFY	F1: 5'	CAAGGCTGCHRTHMGRGC 3'
	F2: 5'	CAGAGCCWGGDGARSYKGC 3'
	R1: 5'	GCTTKGTDGGNACRTACC 3'
	R2: 5'	CATAGCARTGHACRTAGTSYCKC 3'
API	F1: 5'	GGTAGRGTNCARYTGAAGMG 3'
	F2: 5'	CTTCAGARTYTKGARCASC 3'
	R1: 5'	GAGTCAGDTCVAGMTCRTTCC 3'
	R2: 5'	CWYGRTAMWKMCCACCC 3'
PI	F1: 5'	GGMAAGATHGAGATMAAGMRG 3'
	F2: 5'	GARCTHAGGCAYYTKAARGG 3'
	R1: 5'	GCAGATTKGGCTCVAWNGG 3'
	R2: 5'	CCTCHCCYTTMARRTGCC 3'
AG	F1: 5'	GAGATHAAGMGVATHGARAAYAC 3'
	F2: 5'	GARGTBGCYCTYATYGTCTTCTC 3'
	R: 5'	CARMTCMAYYTCCCTYTTYTGC 3'
18S	F1: 5'	TACCGTCCTAGTCTCAACCATAA 3'
	F2: 5'	TATGGTCGCAAGGCTGAA 3'
	R1: 5'	AGAACATCTAAGGGCATCACA 3'
	R2: 5'	GCTGAGGTCTCGTTCGTTA 3'
β-actin	F1: 5'	GTGAAGGAAAAACATGCSTAYAT 3'
	F2: 5'	TATGCYAGTGGCCGTACA 3'

	R1: 5'	KGAACCACCACTCAAMACAATG 3'
	R2: 5'	CTTCACGTCTCTCACAATTTCT 3'
GAPGH	F1: 5'	GACARTGGAARMACSAYGA 3'
	F2: 5'	GGACTGGAGAGGTGGAAGA 3'
	R1: 5'	TTCCACCTCTCCAGTCCTT 3'
	R2: 5'	ACATCRTCTTCAGTGTAWCCRA 3'

4.2.3.2 Sequencing and sequence verification

After amplification, RT-PCR product was run on a 1.5% agarose gel and visualized after ethidium bromide staining. All bands of approximately the expected size were excised from the agarose gel, purified using the Concert™ Rapid gel extraction system (Life Technologies) and quantified using a mass ladder (High DNA Mass TM Ladder, Life Technologies).

The purified fragments were sequenced using the Big Dye Terminator 3.1 reaction in an ABS 3730 sequencer using the same degenerated primers as those used for PCR amplification. Both forward and reverse primer was used in separate sequencing reactions in order to obtain sequence information from both DNA strands. At least two independent sequences for each strand were obtained for every putative gene. Raw DNA sequence data were checked manually, compared with electropherograms, and further edited if necessary to improve the quality and reliability of the data, using the BioEdit program.

It was highly possible that sequences from the same sample could have been slightly different, representing alleles of a single gene (but not of different genes). Therefore, sequences for each target gene were aligned using Clustal X (Thompson et al., 1997) to determine how many different sequences were present. If the sequences showed slight nucleotide differences in a variable pattern across the whole sequence, those sequences were considered to represent alleles of the same gene, and a consensus sequence was used for further analysis.

To verify if the right gene homologues were obtained from each specific target gene, each edited sequence was translated in all three reading frames using the Primer Premier program (Version 5.0). Both cDNA sequences and amino acid sequences were searched against the GenBank database at the National Center for Biotechnology Information

(NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) using the BLAST search program (Altschul et al., 1990).

For each confirmed target gene, the structure of the gene fragment (exon/intron splice sites) and its relative position in the gene was deduced by comparing its sequence with the published cDNA sequence and the genomic sequence of its orthologues from the most related species.

4.2.3.3 Sequence comparison and phylogenic analysis

For each target gene, cDNA sequences of the two homologues for *Sophora* and *Clianthus* were first compared to test their similarity. The deduced amino acid sequences of the putative genes were then aligned with their homologues from *Arabidopsis*, *Antirrhinum* and other species of interest in the current study, using the Vector NTI (Version 9) program to generate the multi-sequence alignment data.

For phylogenetic analysis, around 15-20 homologous sequences in data banks gathered from phylogenically diverse species and some interesting species were used in alignments, together with the sequence of the putative target genes for both *Sophora* and *Clianthus*. Amino acid multiple alignments and phylogenetic analyses were constructed using the Clustal x program (Thompson et al., 1997). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei, 1987) using the same program. The graphic tree representation was given by TreeView software version 1.6.1 (Page, 1996). All analyses were performed with 1000 bootstrap replicates. Separate amino acid alignments were conducted for each target gene. In most cases, one or several paralogues of the target gene were included as out-group entries in the phylogenetic analyses in order to root the phylogenetic tree.

The sequences used for multiple alignment and /or phylogenetic analysis for putative *LFY* homologues from *Sophora* and *Clianthus* included *LFY/FLO* homologues across eudicot, monocot angiosperm species and gymnosperm species. They were *LFY* from *Arabidopsis thaliana*, *FLO* from *Antirrhinum majus*, *BLFL* from *Brownea leucantha*, *UNIFOLIATA* (*UNI*) from *Pisum sativum*, *AcLFY* from *Acacia caven*, *MEL* from *Metrosideros excelsa*, *ELF1* from *Eucalyptus globulus*, *PTLF* from *Populus trichocarpa*, *ALF* from *Actinidia deliciosa*, *AFL1* and *AFL2* from *Malus domestica*, *CFL* from *Chrysanthemum lavandulifolium*, *ZFL1* from *Zea mays*, *LFL* from *Lolium temulentum*, *NEEDLY* from

Pinus radiata, and *GinLFY* from *Ginkgo biloba* (Appendix 2).

AP1/SQUA homologues were used for multiple alignment and phylogenetic analysis of putative *AP1* homologues from *Sophora* and *Clianthus*. They were *AP1* from *Arabidopsis thaliana*, *SQUA* from *Antirrhinum majus*, *PEAM4* from *Pisum sativum*, *DcMADS1* from *Daucus carota*, *BOAP1* from *Brassica oleracea*, *NAP1* from *Nicotiana tabacum*, *MESAP1* from *Metrosideros excelsa*, *EAP1* from *Eucalyptus globulus*, *PTAP1-1* and *PTAP1-2* from *Populus trichocarpa*, *AAP1* from *Actinidia deliciosa*, *MdMADS2* from *Malus domestica*, *CsAP1* from *Citrus sinensis*, *VvMADS6* from *Vitis vinifera*, *LpMADS1* from *Lolium perenne*, and *RAP1B* from *Oryza sativa. PI* and *AG* from *Arabidopsis thaliana* were also used to indicate the inter-subfamily relationships and to serve as outgroup genes to root the phylogenetic tree (Appendix 2).

The following *PI* homologues and paralogues were included in multiple alignment and phylogenetic analysis of putative *PI* homologues from *Sophora* and *Clianthus*: *PI* from *Arabidopsis thaliana*, *GLO* from *Antirrhinum majus*, *PEAM1* from *Pisum sativum*, *NGL9* from *Medicago sativa*, *HaPI* from *Helianthus annuus*, *FBPI* from *Petunia hybrida*, *MdPI* from *Malus domestica*, *RbPI-1* from *Ranunculus bulbosus*, *DaPI* from *Delphinium ajacis*, *SvPI* from *Syringa vulgaris*, *PhPI* from *Peperomia hirta*, *OsMADS4* from *Oryza sativa*, *OrcPI* from *Orchis italica*, and *LrGLO* from *Lilium regale*. *PI* paralogues *AP3* from *Arabidopsis thaliana* and *DEF* from *Antirrhium majus* were also used to elucidate their relationship to putative genes and to serve as outgroup genes to root the phylogenetic tree (Appendix 2).

The sequences used for multiple alignment and phylogenetic analysis for putative *AG* homologues from *Sophora* and *Clianthus* were: *AG* from *Arabidopsis thaliana*, *PLE* from *Antirrhinum majus*, *BAG1* from *Brassica napus*, *HaAG* from *Helianthus annuus*, *NAG1* from *Nicotiana tabacum*, *PTAG1* and *PTAG2* from *Populus trichocarpa*, *MADS15* from *Malus domestica*, *TAG1* from *Lycopersicon esculentum*, *LAG* from *Liquidambar styraciflua*, *RAG1* from a *Rosa hybrida* cultivar, *MADS13* from *Oryza sativa*, *HvAG1* from *Hordeum vulgare*, *WAG* from *Triticum aestivum*, and *ZAG1* from *Zea mays*. *PI* and *AG* from *Arabidopsis thaliana* were also used as outgroup genes to root the phylogenetic tree, and to indicate the inter-subfamily relationship (Appendix 2).

4.3 Results

4.3.1 Analysis of *LFY* homologues *STLFY* and *CMLFY*

A single PCR product was obtained using *Sophora tetraptera* cDNA as PCR template (Appendix 3). When the purified PCR product was directly sequenced and the automatically generated sequences hand corrected, the phenomenon of two different nucleotide signals presenting at the same nucleotide site was observed in several sites along the obtained sequence. This problem was solved by sequencing several PCR products using cDNAs originating from different individual plants, and by using the consensus sequence resulted from multiple sequence alignment. A verified 403 bp sequence was finally obtained. This was considered to be a putative *LFY* homologue and was named *STLFY*. The confirmed cDNA sequence of this fragment was as follows:

CCTGGGGAGGTTGCACGTGGCAAGAAGAACGGCCTTGATTATCTCTTTCATCTCTACGAGC
AATGCCGTGAGTTCTTGATTCAGGTTCAGGCCATTGCCAAGGACCGAGGTGAAAAATGCCC
AACTAAGGTGACAAACCAAGTGTTTAGGTACGCCAAGAAAGCAGGAGCAAGCTACATCAA
CAAGCCCAAAATGCGACACTACGTGCACTGCTATGCGTTGCACTGTCTTGACGAGGAAGT
GTCGAATGAGCTGAGGAGAGCTTTCAAGGAGAGAGGAGAGAACGTTGGAGCGTGGAGAC
AAGCATGTTACAAGCCACTTGTGGTCATCGCAGCACGTCAAGGTTGGGACATTGATGCCAT
ATTCAATGCGCATCCTCGTCTTTCCATATGGTATGTCCCCA

Conceptual translation of the ORFs of this cDNA sequence yielded an amino acid sequence of 133 aa. The amino acid sequence was:

PGEVARGKKNGLDYLFHLYEQCREFLIQVQAIAKDRGEKCPTKVTNQVFRYAKKAGASYINK
PKMRHYVHCYALHCLDEEVSNELRRAFKERGENVGAWRQACYKPLVVIAARQGWDIDAIFN
AHPRLSIWYVP

Similarly, a 401 bp putative *LFY* homologue for *Clianthus* was obtained and named *CMLFY* (Appendix 3). The confirmed cDNA sequence of this fragment was as follows:

CCTGGGGAGGTTGCACGTGGAAAGAAAAACGGTCTCGACTATCTGTTTCACTTATACGATC
AATGCCGTGAGTTCTTGATTCAAGTTCAGACGATCGCTAAGGACCGCGGTGAAAAATGCCC
CACCAAGGTAACAAATCAGGTATTTAGGTATGCGAAGAAAGCTGGAGCTAGCTACATTAAC
AAGCCAAAAATGCGACACTACGTGCACTGCTATGCATTGCATTGTTTGGACGAAGAGGCGT
CGAATGAGCTGAGAAGGGCATTCAAGGAGAGAGGGGAGAATGTTGGGGCGTGGAGGCAA
GCATGTTACAAGCCACTTGTGGCAATTGCTGCACGTCAAGGTTGGGATATTGATGCCATATT

CAATGCGCATCCTCGTCTTTCTATATGGTATGTCCC

The deduced amino acid sequence of this cDNA fragment was as follows:
PGEVARGKKNGLDYLFHLYDQCREFLIQVQTIAKDRGEKCPTKVTNQVFRYAKKAGASYINK
PKMRHYVHCYALHCLDEEASNELRRAFKERGENVGAWRQACYKPLVAIAARQGWDIDAIFN
AHPRLSIWYV

The result of sequence alignment showed that *STLFY* and *CMLFY* shared 88.03% identity at the nucleotide level and 97.74% identity at the amino acid level. The BLAST searching result (Appendix 4) using *CMLFY* amino acid sequence against the GenBank amino acid database showed that the fragment had sequence characteristics typical of a *LFY* and *FLO* homologue. All of the first 360 sequences observed were LFY and FLO homologue proteins. The two most similar protein sequences to CMLFY were FLO/LFY-like protein (BLFL) for *Brownea leucantha* and UNIFOLIATA (UNI) protein for *Pisum sativa*, with 97.0% (129/133) and 96.24% similarity (128/133), respectively. The similarity between *CMLFY* and both LFY and FLO proteins were 94.7%. Blast search with STLFY yielded a very similar result with slightly different similarities between each gene.

Multiple alignment of STLFY and CMLFY with these homologue proteins (Fig. 4.1) showed that all of the six amino acid sequences were highly conserved. Both putative sequences were slightly more similar to those of the two leguminous species BLFL and UNI than to LFY and FLO. There were only two residue differences between STLFY and *Brownea* BLFL, and three residue differences between STLFY and CMLFY. Unexpectedly, the residue on position 20 of CMLFY differed from that of all other sequences, the glutamic acid changed to aspartic acid. STLFY and CMLFY were slightly more similar to FLO than to LFY, having six and four uniquely different residues, respectively. The similarity between LFY and FLO was also as high as 91%. In most cases, the differences of STLFY and CMLFY to FLO and LFY were shared by BLFL and UNI.

In order to determine the sequence similarity of *STLFY* and *CMLFY* to other reported homologues in a broader taxonomic range, 15 *LFY/FLO* homologues from eudicot, lower dicot, monocot and gymnosperm species were compared (Table 4.1). In general, the amino acid sequence region covered by that of *STLFY* and *CMLFY* was highly conserved across all the tested species, except for *ALF* (*Actinidia deliciosa*) and *MEL* (*Metrosideros excelsa*), which had shorter sequences than other entries and were excluded for direct

comparison. The similarities of *STLFY* and *CMLFY* to other *LFY/FLO* homologues were in the range 77–96%, with over 90% similarity found in a broad range of species, including herbaceous and woody species. The *UNI* protein (*UNIFOLIATA* from *Pisum sativum*) had the highest similarity to *STLFY* and *CMLFY* (95-96%). This was followed by *FLO*, *AFL* (*Malus domestica*), *LFY* and *PTLF* (*Populus trichocarpa*).

The topology of the neighbour-joining phylogenetic tree constructed using the above sequence data (Fig. 4.2) confirmed that both *STLFY* and *CMLFY* fell well into the *LFY/FLO* gene class, being closely grouped with homologues from eudicot species, especially taxonomically related species. *LFY/FLO* from monocots and gymnosperms formed distinctly isolated clusters far away from that of eudicots.

The possible structure of the *STLFY* and *CMLFY* gene fragments were deduced by comparing their cDNA and amino acid sequences with those of the *Arabidopsis LFY* gene (GenBank accession number: AF466791) (Fig. 4.3). Both *STLFY* and *CMLFY* span the second intron, with 129 bp at the end of the exon 1 and the rest of the sequence at the starting part of the exon 3.

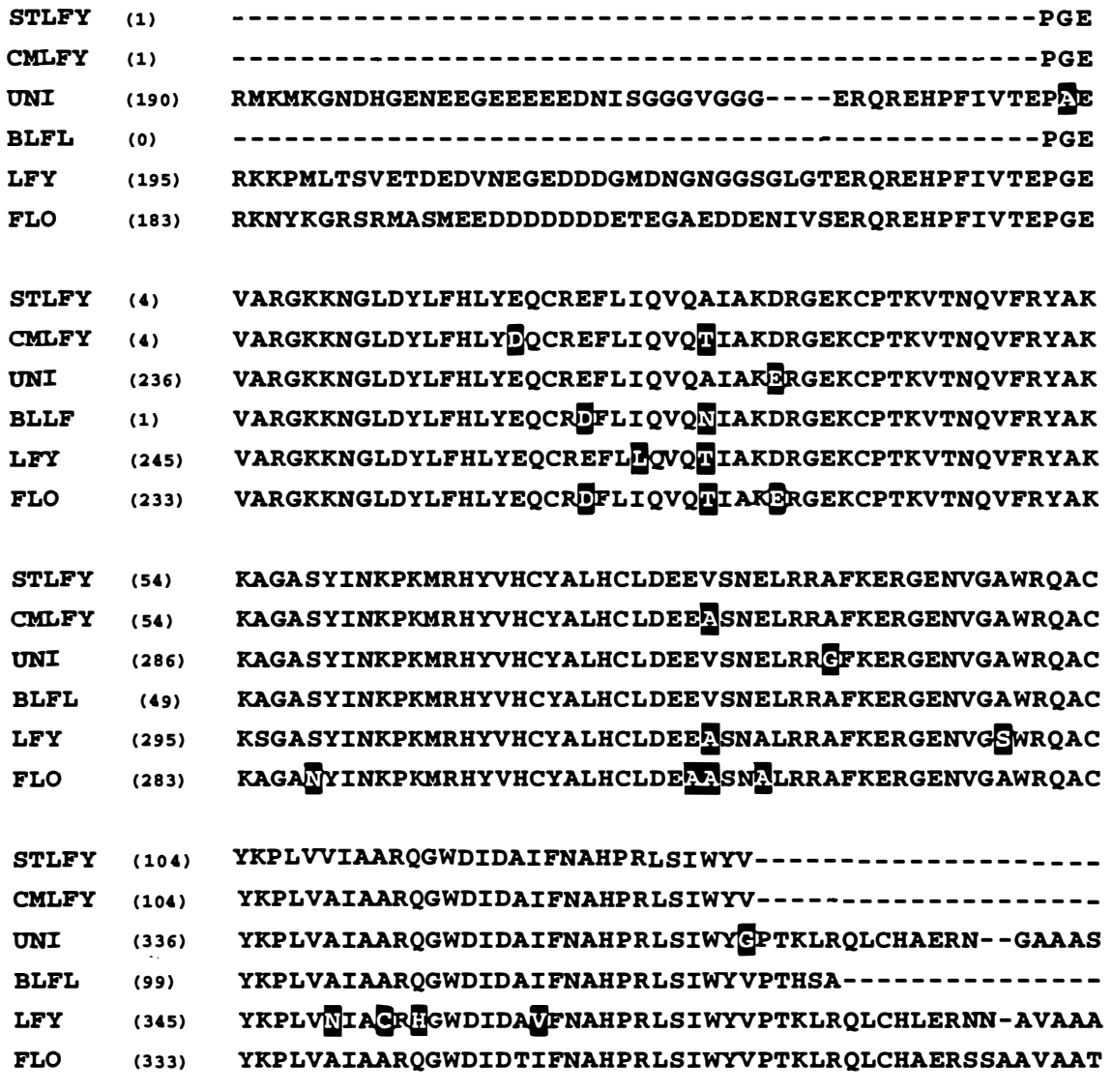

Figure 4.1 Comparison of amino acid sequences of STLFY and CMLFY with some FLO/LFY-like proteins

Amino acids that differed from the STLFY sequence are shaded. Sequence segments not covered by the STLFY and CMLFY fragments were not included for comparison.

Table 4.1 Sequence similarities (%) between *CMLFY*, *STLFY* and *LFY/FLO* homologue genes

	STLFY	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2 *CMLFY*	97															
3 *UNI*	96	95														
4 *FLO*	94	95	93													
5 *AFL2*	94	95	94	96												
6 *LFY*	93	94	90	91	91											
7 *PTLF*	93	94	92	96	95	91										
8 *AFL1*	92	91	90	91	95	89	91									
9 *CFL*	92	91	91	91	94	91	90	91								
10 *ELF1*	88	89	87	89	89	88	91	86	86							
11 *AcLFY*	87	86	88	86	86	83	84	84	86	81						
12 *ZFL1*	84	84	82	84	84	86	85	81	84	82	76					
13 *LFL*	81	81	79	81	83	84	82	80	81	80	75	91				
14 *GinLFY*	78	77	77	78	78	79	79	80	79	76	74	74	72			
15 *NEEDLY*	78	78	78	78	79	77	78	78	80	76	74	73	71	88		
16 *ALF*	60	61	59	61	61	58	62	58	58	56	61	56	53	47	48	
17 *MEL*	53	54	53	53	53	52	54	51	52	61	54	48	46	44	44	80

Similarities were calculated based on the sequence fragments of 133 amino acids that covered *STLFY* and *CMLFY* sequences for all genes other than *MEL* and *ALF* that had shorter sequences available in the GenBank database.

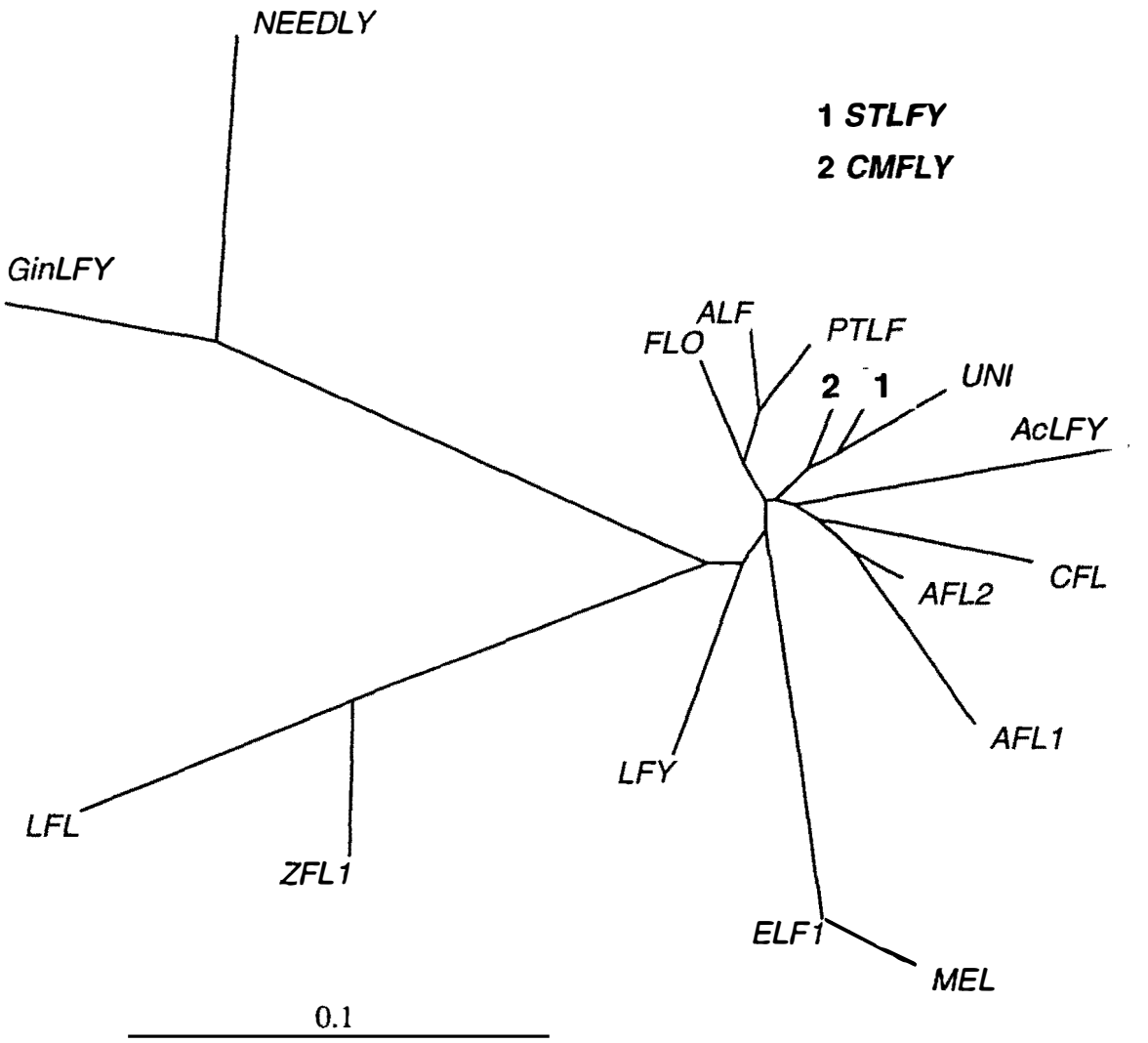

Figure 4.2 Phylogenetic relationship of some *LFY* homologues from a wide range of angiosperm and gymnosperm species

Bar represents 10% amino acid substitution along protein fragments of around 130 amino acid residues. Gene codes and species names are described in Section 4.2.3.3.

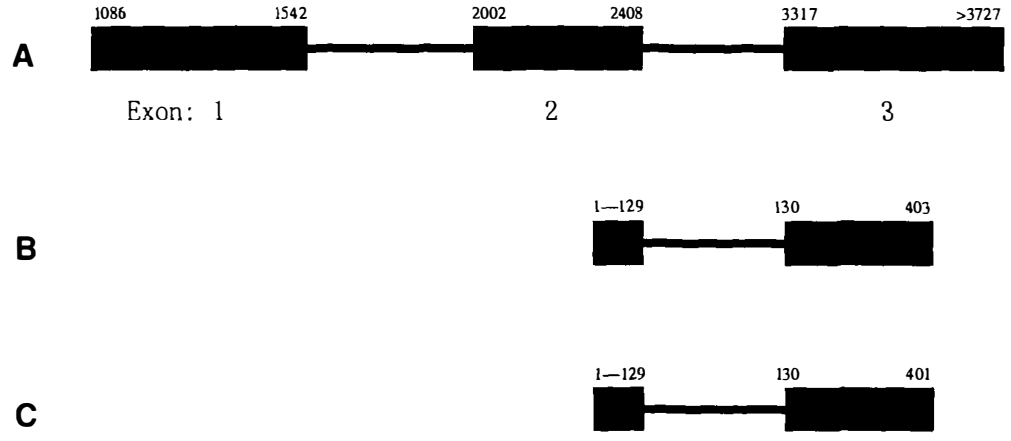

Figure 4.3 Deduced gene structure of *STLFY* and *CMLFY*

A: *Arabidopsis thaliana LFY* gene (GenBank access No: AF466791); B: Fragment of *Sophora* putative *LFY* homologue *STLFY*; C: Fragment of *Clianthus* putative *LFY* homologue *CMLFY*.

4.3.2 Analysis of *APETALA1* sequences, *CMAP1* and *STAP1*

The RT-PCR using degenerate *AP1* primers yielded a cDNA fragment of 441 bp for *Sophora* (Appendix 3). After sequencing and sequence confirmation, this putative *AP1* homologue was named *STAP1*. The cDNA sequence of this fragment was as follows:

AGGTTACTTTCTCCAAAAGGAGAGCTGGGTTACTCAAGAAAGCACATGAGATCTCTGTGCT
CTGCGATGCTGAGGTCGCTTTGATTGTCTTCTCCAACAAAGGAAAGCTCTTTGAATATGCC
ACTGATTCATGCATGGAGAAGATACTGGAACGCCATGAAAGGTATGCCTATGCAGAGAGAC
AGCTGGAGGCAAATAATTCTGAGACACAGGGAAACTGGACCATTGAATATACTAGACTCAA
GGCAAGGATTGACCTTTTGGAGAGAAACCACAGGCACTATATGGGAGAAGATTTGGGTTC
AATGAGCCTCAAAGAGCTTCAAAGTCTGGAGCAGCAGTTAGATACTGCTCTCAAAACCATT
CGTACACGCAGAAACCAACTCATGTACGAGTCCATATCTGAGCTTCATAAAGAGGAGAAAG
TGATACAAGAGCAGA

The deduced amino acid sequence from this cDNA fragment was:

VTFSKRRAGLLKKAHEISVLCDAEVALIVFSNKGKLFEYATDSCMEKILERHERYAYAERQLEA
NNSETQGNWTIEYTRLKARIDLLERNHRHYMGEDLGSMSLKELQSLEQQLDTALKTIRTRRN
QLMYESISELHKEEKVIQEQ

A 629 bp putative *AP1* homologue for *Clianthus* was obtained by a different primer-pair from that for *Sophora* (Appendix 3) and named *CMAP1*. The confirmed cDNA sequence

of this fragment was as follows:

AACAAGATCAATCGCCAGGTTACTTTCTCTAAAAGGAGAGCTGGACTTCTTAAGAAAGCTC
ATGAGATCTCTGTCCTCTGTGATGCTGAGGTTGCTTTGATTGTCTTCTCCAACAAAGGAAA
GCTCTTTGAATATGCCACTGATTCTTGTATGGAGAAGATACTGGAACGCTATGAAAGGTATT
CCTATGCGGAGAGACAGTTAGTTGCAAATGATTCTGAGTCACAGGGAAATTGGACCATTGA
GTATACTAGGCTCAAGGCAAAGATTGATCTTCTGCAGAGAAACTATAGGCACTACATGGGA
GAAGATTTGGGTTCAATGAGTCTGAAAGAGCTTCAAAGTCTGGAACAGCAGTTAGATACT
GCTCTGAAACAAATTCGTACCCGCAGAAACCAACTCATGTACGAGTCCATTTCTGAGCTTC
AGAAAAAGGAGAAAGTGATTCAGGAACAGAATAACATGCTTGCAAAGAAGATCAAGGAG
AAAGAAAAGATTGCAGCACAGCAGACACAGTGGGAGCACCACCAAAACCATGGAGTTAA
TCCATCTTTCTTGCTCCAGCAGCCACTTCCAAGTTTGAACATGGGTGGCAATTACCGTGAG
GAAGCACCAGAAATGGGGAGGAA

The deduced amino acid sequence was:

NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALIVFSNKGKLFEYATDSCMEKILERYERYSYA
ERQLVANDSESQGNWTIEYTRLKAKIDLLQRNYRHYMGEDLGSMSLKELQSLEQQLDTALKQ
IRTRRNQLMYESISELQKKEKVIQEQNNMLAKKIKEKEKIAAQQTQWEHHQNHGVNPSFLLQ
QPLPSLNMGGNYREEAPEMGR

The sequences of *STAP1* and *CMAP1* shared 90.03% identity at the nucleotide level and 92.5% identity at the amino acid level, along the overlapping sequence region. All of the sequences yielded by BLAST searching of *STAP1* aa sequence against the GenBank amino acid database were AP1 and SQUA homologue proteins. The protein most similar to STAP1 was PEAM4 for *Pisum sativum*, followed by AP1, DcMADS1 for *Daucus carota*, and SQUA, with similarities of 91.0%, 80.14%, 78.77% and 78.08%, respectively. BLAST search with CMAP1 revealed a result similar to that of STAP1 (Appendix 5).

Multiple alignment of STAP1 and CMAP1 with some representative AP1/SQUA-like proteins (Fig. 4.4) showed that sequence variation occurred randomly along the 146 overlapping sequence region, although the sequence at the 1-60 positions of STAP1 were more conserved than the rest of the region. CMAP1 and PEAM4 shared the highest sequence similarity, with only three residues differing. However, differences between STAP1 and CMAP1 were not evenly dispersed, with 70% (8/11) of the residue differences occurring at positions 52 to 91 of the STAP1 sequence, whereas no difference were detected along the first 51 amino acid positions.

To elucidate the relationship of *STAP1* and *CMAP1* with other homologues in a broad taxonomic range, 16 *AP1/SQUA* homologues from eudicot and monocot species were compared (Table 4.2), together with *AG* and *PI* serving as the inter-subfamily references. Similarities related to *MESAP1* (*Metrosideros excelsa*) and *AAP1* (*Actinidia deliciosa*) were excluded for direct comparison since they were under-estimated because of their shorter sequences in relation to other entries. At the amino acid level, the similarities of *STAP1* and *CMAP1* to *AG* and *PI* (35-42%) were remarkably lower than those to *AP1/SQUA* homologues, ranging from around 70% to over 90%. Generally, the similarities of the two putative genes to dicot homologues were much higher than to monocot homologues. Consistent with the results of direct sequence comparison, the highest sequence similarity of 98% was found between *CMAP1* and *PEAM4*, which was higher than the similarity (93%) between *STAP1* and *CMAP1*. The similarity between *AP1* and *SQUA* was 77%. The two putative sequences had slightly higher sequence similarity to *AP1* than to *SQUA*. In all cases, *CMAP1* had higher similarity to other *AP1/SQUA* homologues than to that of *STAP1*, being 37-98% and 35-91%, respectively.

The phylogenetic tree (Fig. 4.5) constructed using the above sequence information clearly indicated the relationship of these genes. While *AG* and *PI* played their role of tree rooting very well, all *AP1/SQUA* homologues of dicot species were grouped together and well separated from those of monocot species. *STAP1* and *CMAP1* were closely related to the eudicot *AP1* homologues, especially *PEAM4*, which was the only member available from the leguminous family.

In comparing the cDNA and amino acid sequences of several *AP1* homologues from taxonomically related species available from the GenBank database, the intron/exon excide sites were deduced and the possible gene structures were reconstructed for *STAP1* and *CMAP1* (Fig. 4.6). *PEAM4* (GenBank accession number: AJ291298) was used as the reference gene since it was the most closely related *AP1* homologue to *STAP1* and *CMAP1* found in this study. Sequences of all eight *PEAM4* exons were present for the *CMAP1* fragment, with partial sequences for exons 1 and 8. While the sizes of exons 5 and 6 were totally conserved between *CMAP1* and *PEAM4*, the size of exons 2, 3, 4 and 7 were also very similar between the two homologues, being 80, 65, 100 and 116bp, respectively, for *PEAM4*, and 78, 66, 102 and 115 bp, respectively, for *CMAP1*. The structure of *STAP1* was totally conserved between *STAP1* and *CMAP1* for exons 2-5.

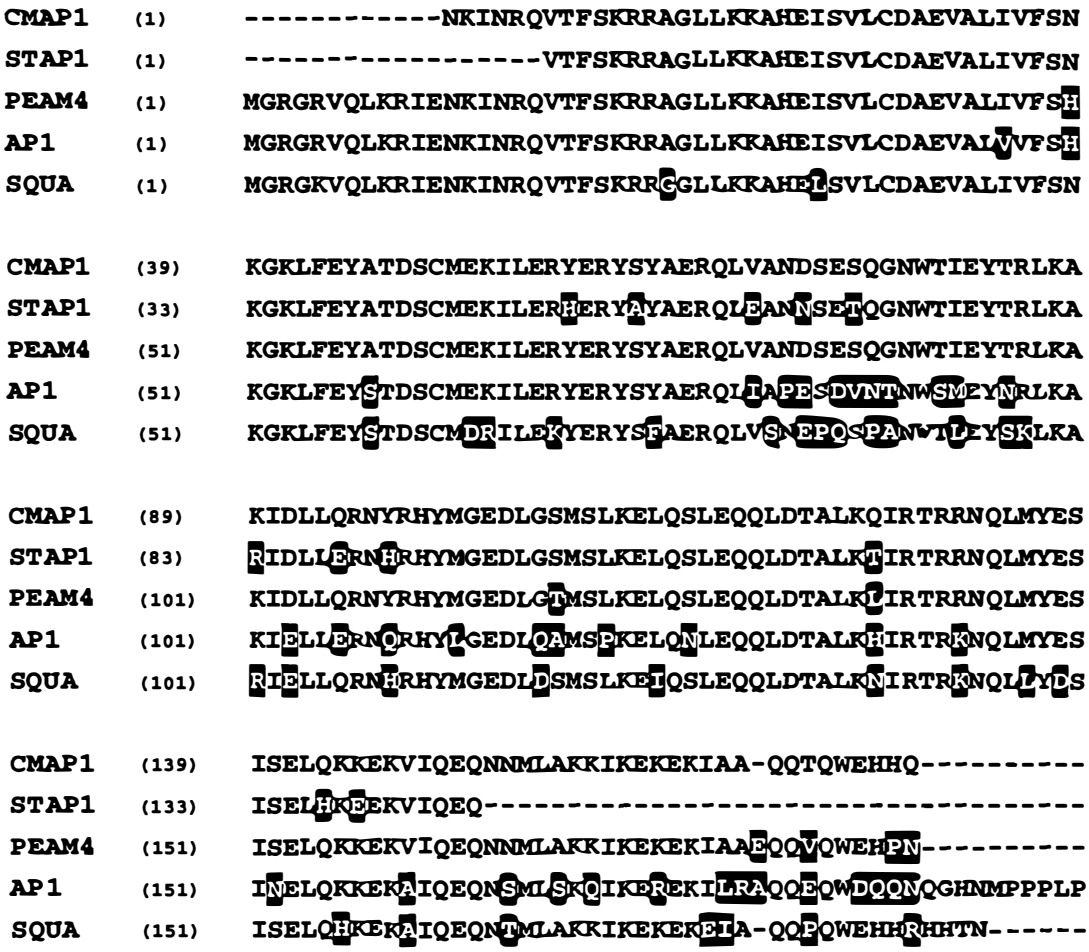

Figure 4.4 Comparison of amino acid sequences of CMAP1 and STAP1 with some AP1/SQUA-like proteins

Amino acids that differed from the CMAP1 sequence are were shaded. Sequence segments not covered by the CMAP1 fragment were not included for comparison.

Table 4.2 Sequence similarities between *CMAP1*, *STAP1* and selected *API/SQUA* homologue genes

		STAP1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2	*CMAP1*	93																		
3	*PEAM4*	91	98																	
4	*AP1*	81	83	84																
5	*DcMADS1*	79	83	81	77															
6	*SQUA*	79	82	81	77	82														
7	*BOAP1*	78	81	81	97	75	75													
8	*PTAP1-1*	77	80	81	79	75	77	79												
9	*PTAP1-2*	75	79	79	79	77	76	79	91											
10	*CsAP1*	75	79	79	79	79	73	77	75	74										
11	*MdMADS2*	75	79	78	76	80	76	75	74	74	75									
12	*VvMADS6*	72	75	75	75	75	75	73	72	72	71	77								
13	*NAP1*	72	75	74	74	77	74	73	72	72	72	86	77							
14	*EAP1*	72	75	75	74	78	74	72	71	71	72	84	74	83						
15	*LpMADS1*	69	73	72	70	70	68	68	66	67	70	73	70	74	72					
16	*RAP1B*	68	71	70	71	69	67	71	66	68	70	71	68	69	68	91				
17	*MEAP1*	60	60	59	56	60	57	55	58	58	54	62	56	62	61	56	58			
18	*AAP1*	53	56	56	54	52	52	54	57	57	52	51	50	52	51	48	50	72		
19	*AG*	40	42	41	41	44	43	40	38	40	38	42	43	41	42	42	43	36	32	
20	*PI*	35	37	36	36	37	36	36	34	34	35	38	35	34	35	37	40	34	30	33

Similarities were calculated based on the sequence fragments of 146 amino acids that covered *STAP1* and *CMAP1* sequences for all genes other than *MEAP1* and *AAP1*, for which only shorter sequences were available in the GenBank database.

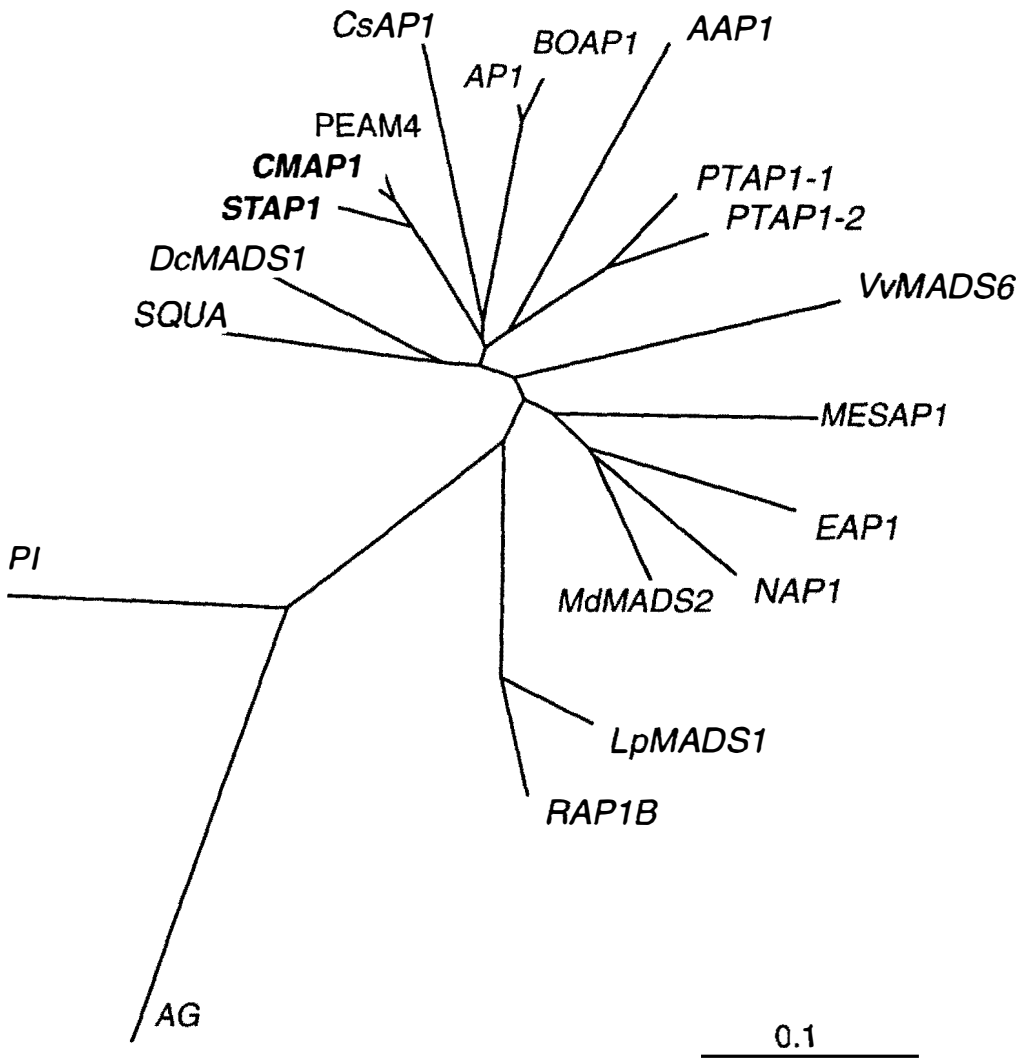

Figure 4.5 Phylogenetic relationship of some *AP1* homologues and of *PI* and *AG* from a wide range of angiosperm species

Bar represents 10% amino acid substitution along protein fragments of around 150 amino acid residues. Gene codes and species names are described in Section 4.2.3.3.

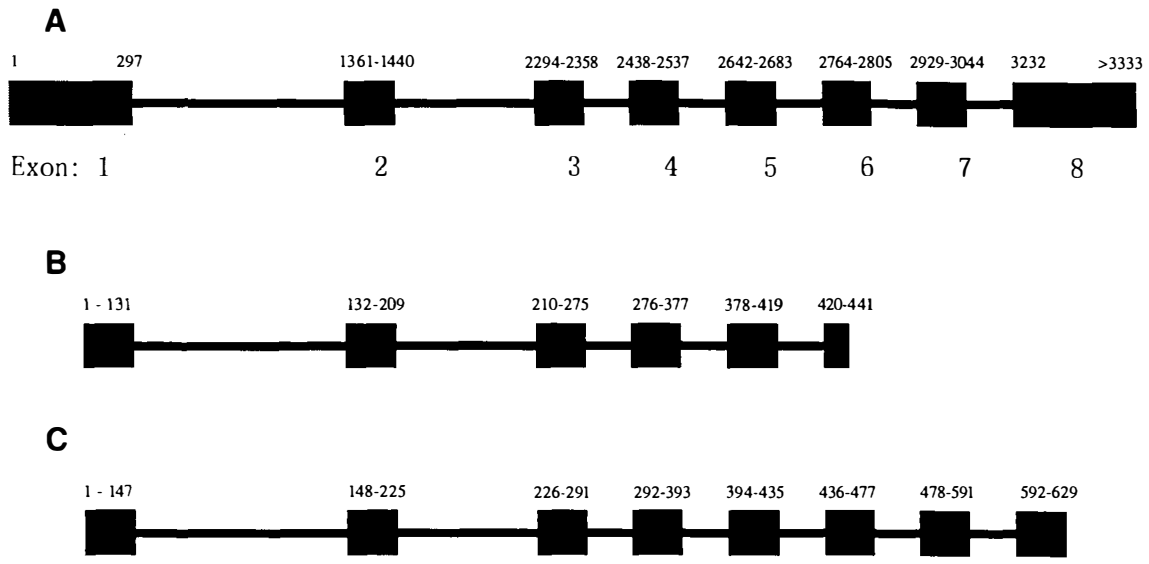

Figure 4.6 Deduced gene structure of *STAP1* and *CMAP1*

A: *Pisum sativum AP1* homologue *PEAM4*, full length sequence with eight exons (GenBank access No: AJ291298); B: *Sophora* putative *AP1* homologue *STAP1*; C: *Clianthus* putative *AP1* homologue *CMAP1*.

4.3.3 Analysis of *PISTILLATA* sequences, *CMPI* and *STPI*

A 323 bp putative *PI* homologues for *Sophora* was obtained through RT-PCR and direct DNA sequencing, and named *STPI* (Appendix 3). The confirmed cDNA sequence of this fragment was as follows:

ATGGGAAGAGGCAAGATTGAGATCAAGAGGATTGAGAACACAAGCAACAGGCAAGTTAC
CTACTCAAAGAGGAAGAATGGGATCTTAAAGAAGGCAAAGGAAATCACTGTTCTATGTGAT
GCTCAAGTTTCCCTCATCATCTTTGGTTCCTCTGGAAAGATGCATGAATACATCAGCCCCTC
CACCACGTTGATTGACATCCTGGACAGATACCATAAAGCCTCTGGGAAGAGGCTCTGGGAC
GCCAAGCATGAGAACCTCAGCAATGAAATTGATAGAGTGAAAAAGGAGAATGACAGCATG
CAAATTGAGCTCAGGCACCT

The deduced amino acid sequence was:

MGRGKIEIKRIENTSNRQVTYSKRKNGILKKAKEITVLCDAQVSLIIFGSSGKMHEYISPSTTLID
ILDRYHKASGKRLWDAKHENLSNEIDRVKKENDSMQIELRH

A 329 bp putative *PI* homologues for *Clianthus* was obtained through RT-PCR and direct sequencing, and was named *CMPI* (Appendix 3). The confirmed cDNA sequence was

as follows:

ATGGGAAGAGGCAAGATTGAGATCAAGAGGATTGAGAACACAAGCAACAGGCAAGTTAC
CTATTCAAAGAGAAAGAATGGGATCCTTAAGAAAGCAAAGGAAATTAGTGTTCTATGTGAT
GCTCAACTTTCCCTTATCATCTTTGGTGCCTCTGGAAAGATGCATGAATATATCAGTCCCTCA
ACCACGTTGATTGATATCCTGGACAGATACCAGAGAGCTTCTGGGAAAACGATCTGGGATA
CTAAGCATGAGAACCTCAGCAATGAAATTGATAGAATCAAAAAAGAGAATGACAGCATGC
AAATTGAGCTCAGGCACCTGAAAGG

The deduced amino acid sequence was:

MGRGKIEIKRIENTSNRQVTYSKRKNGILKKAKEISVLCDAQLSLIIFGASGKMHEYISPSTTLID
ILDRYQRASGKTIWDTKHENLSNEIDRIKKENDSMQIELRHLK

The sequences of *STPI* and *CMPI* shared 90.2% identity at the nucleotide level and 91.6% identity at the amino acid level. BLAST search for similar sequences in the GenBank amino acid database with both *STPI* and *CMPI* amino acid sequences yielded only *PI/GLO* homologues. The highest similar sequence to *CMPI* was for the PI/GLO-like protein PEAM1 for *Pisum sativum*, with 93.58% similarity (102/109). This was followed by HaPI for *Helianthus annuus* and GGLO1 for *Gerbera*, both with the similarity to *CMPI* of 82.6 (90/109). Blast search with STPI revealed a similar result, with the similarity of 90% for PEAM4 and 82.6 for HaPI and GGLO1 (Appendix 6).

The result of multiple alignment with these homologue proteins (Fig. 4.7) showed that the amino acid sequences of STPI and CMPI aligned well with PI/GLO homologues but not with the AP3 sequence. In fact, AP3 differed from all the aligned PI/GLO homologues along the whole aligned sequence region, but the difference along the second half was much greater. STPI, CMPI and PEAM1 sequences remained the most similar of the aligned members. The majority of residue differences among these three sequences sat within the ranges 36-50 and 72-80. However, the difference of these sequences to GLO and PI were more randomly positioned along the overlapping region.

Sequence comparison in more *PI/GLO* homologues from species of a broader taxonomic range (Table 4.3) revealed a similar result, with the highest sequence similarities observed among *STPI*, *CMPI*, *PEAM1*, *HaPI*, *GLO*, and *NGL9* for *Medicago sativa*. In general, the sequences of *STPI* and *CMPI* were more similar to higher eudicot species than to lower dicot species and monocot species. Both *STPI* and *CMPI* had remarkably higher sequence similarity to *GLO* than to *PI*. The similarity of *STPI* and *CMPI* to *DEF* and *AP3* remained

the lowest, being around 50% only. The similarity between *PI* and *GLO* was 73%.

The result of phylogenetic analysis (Fig. 4.8) confirmed that of the direct sequence comparison, showing that *STPI* and *CMPI* were closely related to *PI/GLO* homologues for higher eudicot species, especially to those for *Pisum*, *Medicago* and *Helianthus*.

The possible structures of the *STPI* and *CMPI* fragments were proposed after comparing their cDNA and amino acid sequences with those of *MdPI* for *Malus domestica* (GenBank accession number: AJ291491) (Fig. 4.9). Both *STPI* and *CMPI* fragments consisted of the entire sequences for exons 1-3 and partial sequences for exon 4. The sizes of all comparable exons were conserved, being 189bp, 66bp and 63bp for exons 1, 2 and 3, respectively.

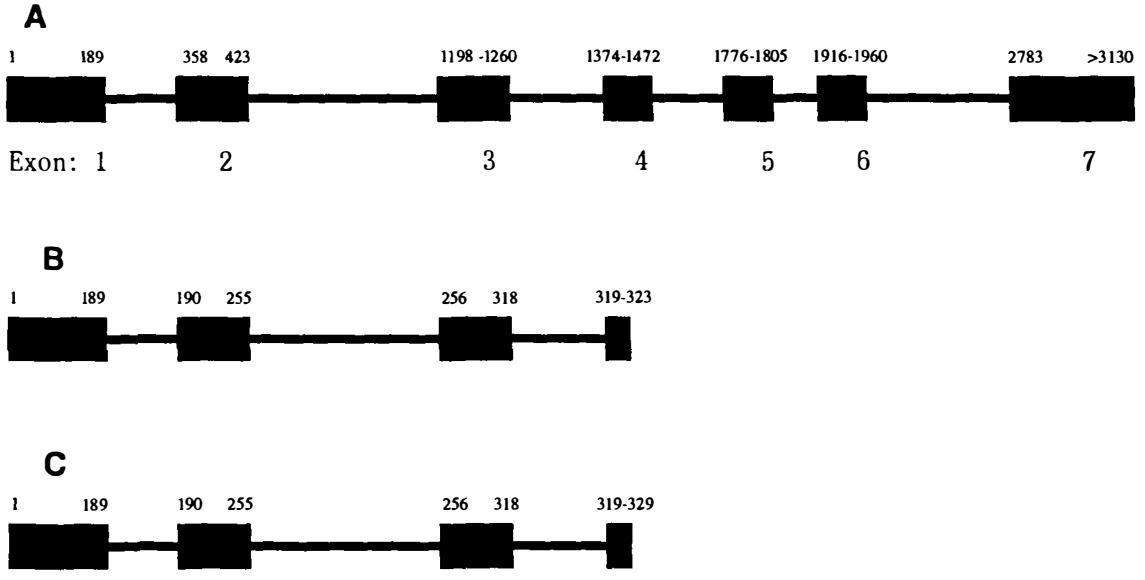

Figure 4.9 Deduced gene structure of *STPI* and *CMPI*

A: *Malus domestica PI* homologue, full length gene sequence with seven exons (GenBank access No: AJ291491); B: *Sophora* putative *PI* homologue *STPI*; C: *Clianthus* putative *PI* homologue *CMPI*.

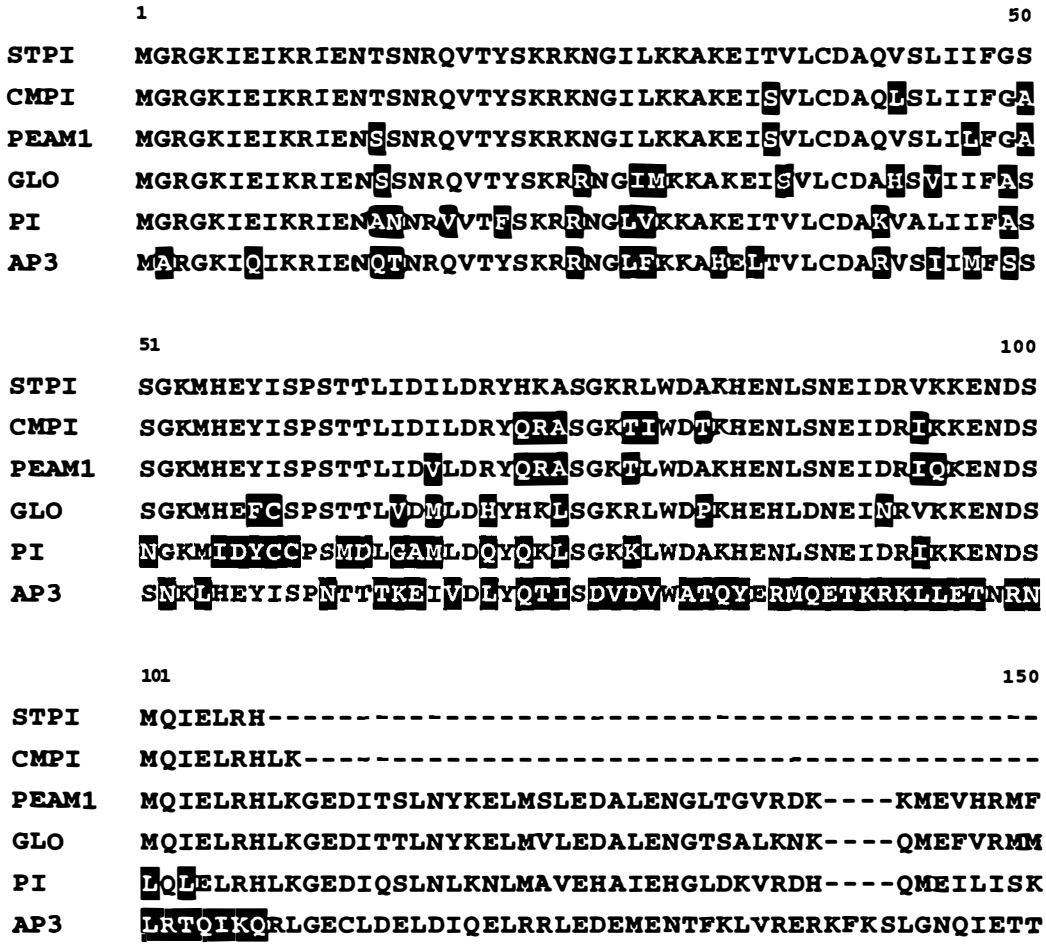

Figure 4.7 Comparison of amino acid sequences of STPI and CMPI with some PI-like proteins and AP3 protein

Amino acids that differed from the STPI sequence are shaded. Sequence segments not covered by STPI were not included for comparison.

Table 4.3 Sequence similarities between some *PI/GLO* homologue and orthologue genes

	STPI	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2 *CMPI*	92															
3 *PEAM1*	91	94														
4 *HaPI*	84	82	82													
5 *GLO*	84	79	80	77												
6 *NGL9*	83	80	81	75	77											
7 *MdPI*	83	77	78	79	79	69										
8 *FBP1*	79	72	73	70	84	72	73									
9 *PI*	75	71	72	74	73	66	74	66								
10 *SvPI*	68	64	65	65	76	61	63	66	63							
11 *DaPI*	64	61	62	62	59	56	61	59	55	73						
12 *RbPI*	53	50	50	55	51	46	54	54	52	66	70					
13 *LrGLO*	62	63	61	62	62	56	62	56	57	76	75	68				
14 *OrcPI*	62	61	62	62	58	58	61	51	58	74	71	64	84			
15 *MADS4*	57	55	54	54	56	52	56	50	54	67	70	60	78	79		
16 *DEF*	50	48	50	47	49	49	48	49	47	39	39	32	37	36	32	
17 *AP3*	51	50	49	47	48	49	50	47	44	38	40	30	38	35	34	75

Similarities were calculated based on the sequence fragments of around 107 amino acids that covered *STPI* and *CMPI* sequences

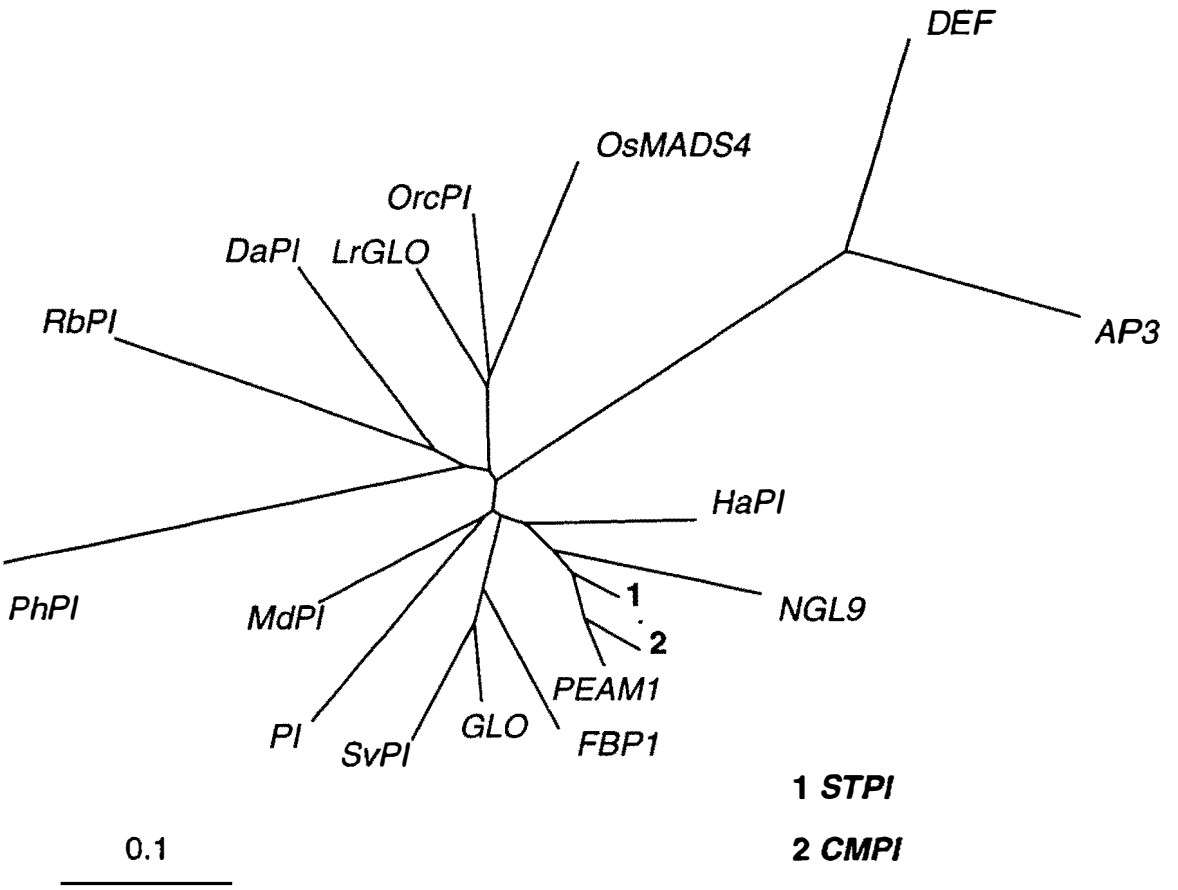

Figure 4.8 Phylogenetic relationship of some *PI* homologues and paralogues from wide range of angiosperm species

Bar represents 10% amino acid substitution along around 110 amino acid residues. Gene codes and species names are described in Section 4.2.3.3.

4.3.4 Analysis of *AGAMOUS* sequences, *CMAG* and *STAG*

A 343 bp putative *AG* homologues for *Sophora* was obtained through RT-PCR (Appendix 3) and direct DNA sequencing, and named *STAG*. The confirmed cDNA sequence of this fragment was as follows:

TTGCTCTCATTGTCTTCTCTAGCCGTGGTCGCCTCTATGAATATGCTAATAACAGTGTCAAAG
CAACTATTGAGAGGTACAAGAAAGCATGCTCAGATTCATCTGGTGCTGGATCTGCTTCTGA
GGCTAATGCTCAGTTTTACCAGCAAGAAGCAGACAAACTGCGTGTGCAAATTAGTAACCTG
CAAAATAACAACAAGCAAATGATGGGTGAGCATTTGGGCAGTATGAATGCCAAGGATCTCA
AAAACCTGGAGGGTAAATTAGAAAAGGGAATTAGCAAGATTCGTTCCAAAAAGAATGAGC
TGCTATTTGCTGAAATTGAGTACATGCAAAAAAGGGA

The deduced amino acid sequence was:

ALIVFSSRGRLYEYANNSVKATIERYKKACSDSSGAGSASEANAQFYQQEADKLRVQISNLQN
NNKQMMGEHLGSMNAKDLKNLEGKLEKGISKIRSKKNELLFAEIEYMQKR

Similarly, a 297 bp putative *AG* homologues for *Clianthus* was obtained through RT-PCR (Appendix 3) and direct DNA sequencing, and named *CMAG*. The confirmed cDNA sequence of this fragment was as follows:

ATTGTCTTCTCTAGCCGTGGTCGCCTCTATGAATATGCTAATAACAGTGTCAAAGCAACTATT
GAGAGGTACAAGAAAGCATGCTCAGATTCATCTGGTGCTGGATCTGCTTTTGAGGCTAATG
CTCAGTTTTACCAGCAAGAAGCAGACAAACTGCGTGTGCAAATTAGTAACCTGCAAAATA
ACAACAAGCAAATGATGGGTGAGCATTTGGGCAGTATGAATGCCAAGGATCTCAAAAACC
TGGAGGGTAAATTAGAAAAGGGAATTAGCAAGATTCGTTCCAAAAAGAATGAG

The deduced amino acid sequence was:

IVFSSRGRLYEYANNSVKATIERYKKACSDSSGAGSAFEANAQFYQQEADKLRVQISNLQNNN
KQMMGEHLGSMNAKDLKNLEGKLEKGISKIRSKKNE

Along the overlapped sequences, the *STAG* and *CMAG* fragments shared 99.7% identity at the nucleotide level and 99.0% identity at the amino acid level. All of the sequences found by BLAST searching of CMAG aa sequence against the GenBank amino acid database were AG/PLE homologue proteins (Appendix 7). The most similar sequence to CMAG was AG/PLE-like protein CMB1 for *Cucumis sativus*, with sequence similarity of 79.8% (79/99). This was followed by NAG1 for *Nicotiana tabacum* and TAG1 for *Lycopersicon esculentum*, with sequence similarities to CMAG of 78.8% (78/99) and

76.8% (76/99), respectively. The Blast search with STAG yielded a similar result.

Multiple alignment of *STAG* and *CMAG* with some *AG/PLE* homologue proteins (Fig. 4.10) showed very high sequence similarity. *STAG* and *CMAG* had only one residue difference at position 40. However, the differences of *STAG* and *CMAG* to other aligned sequences were quite high, and these differences dispersed through the whole aligned sequence region. Furthermore, sequences other than *STAG* and *CMAG* also differed remarkably among themselves.

A similar result was obtained when comparing *STAG* and *CMAG* with more *AG/PLE* homologues from a broad range of eudicot and monocot species (Table 4.4). In general, the sequence similarities of *STAG* and *CMAG* to other *AG/PLE* homologues were quite low, ranging from 70-80% to those of eudicot species, and 61-67% to those of selected monocot species. However, these similarities were still remarkably high compared to other subfamily genes *AP1* and *PI*, which were only around 30%. The sequence similarity between *AG* and *PLE* was as low as 64%.

In the neighbour-joining phylogenetic tree constructed using the above sequence data (Fig. 4.11), both *STAG* and *CMAG* were grouped well into the *AG/PLE* gene subfamily, being closely grouped together with homologues from eudicot species. Both *AP1* and *PI* from the other subfamily genes were positioned far away from *AG/PLE* homologues.

The possible structure of the *STAG* and *CMAG* gene fragments were deduced by comparing their cDNA and amino acid sequences with those of the *PTAG1* gene for *Populus trichocarpa* (GenBank accession number: AF052570), which consisted of nine exons (Fig. 4.12). *STAG* and *CMAG* fragments consisted of five (2-6) and four (2-5) exons, respectively. While exon 5 of both *STAG* and *CMAG* fragments were 102 bp in size instead of 99 bp for *PTAG1*, the size of exons 3, 4 and 6 were conserved among the three homologues.

Figure 4.10 Comparison of amino acid sequences of STAG and CMAG with some AG/PLE-like proteins

Amino acids that differed from the STAG sequence are shaded. Sequence segments not covered by STAG were not included for comparison.

Table 4.4 Sequence similarities between some *AG/PLE* homologues

		STAG	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	*CMAG*	93																	
3	*NAG1*	78	81																
4	*TAG1*	75	78	87															
5	*PTAG2*	76	78	83	77														
6	*PTAG1*	74	76	80	79	92													
7	*HaAG*	75	69	70	68	71	66												
8	*MADS15*	73	75	77	76	78	81	67											
9	*LAG*	73	74	74	72	76	79	65	71										
10	*AG*	72	72	69	70	73	76	66	71	73									
11	*PLE*	70	72	72	68	74	73	61	73	69	64								
12	*RAG1*	69	71	72	73	79	78	65	79	68	70	74							
13	*BAG1*	70	70	67	68	71	74	65	69	71	97	63	68						
14	*HvAG1*	68	67	69	67	66	66	60	62	66	70	63	62	68					
15	*WAG*	65	65	64	62	64	64	59	61	61	69	54	61	70	78				
16	*MADS13*	61	61	61	58	64	65	54	57	61	61	56	58	59	70	64			
17	*ZAG1*	60	61	60	56	62	60	60	61	55	65	53	59	66	66	76	59		
18	*AP1*	33	34	36	36	36	38	30	36	34	34	34	36	36	30	33	34	34	
19	*PI*	29	29	29	26	26	29	21	24	27	26	25	23	25	23	23	28	22	25

Similarities were calculated based on the sequence fragments of 99 amino acids that covered *STAG* and *CMAG* sequences

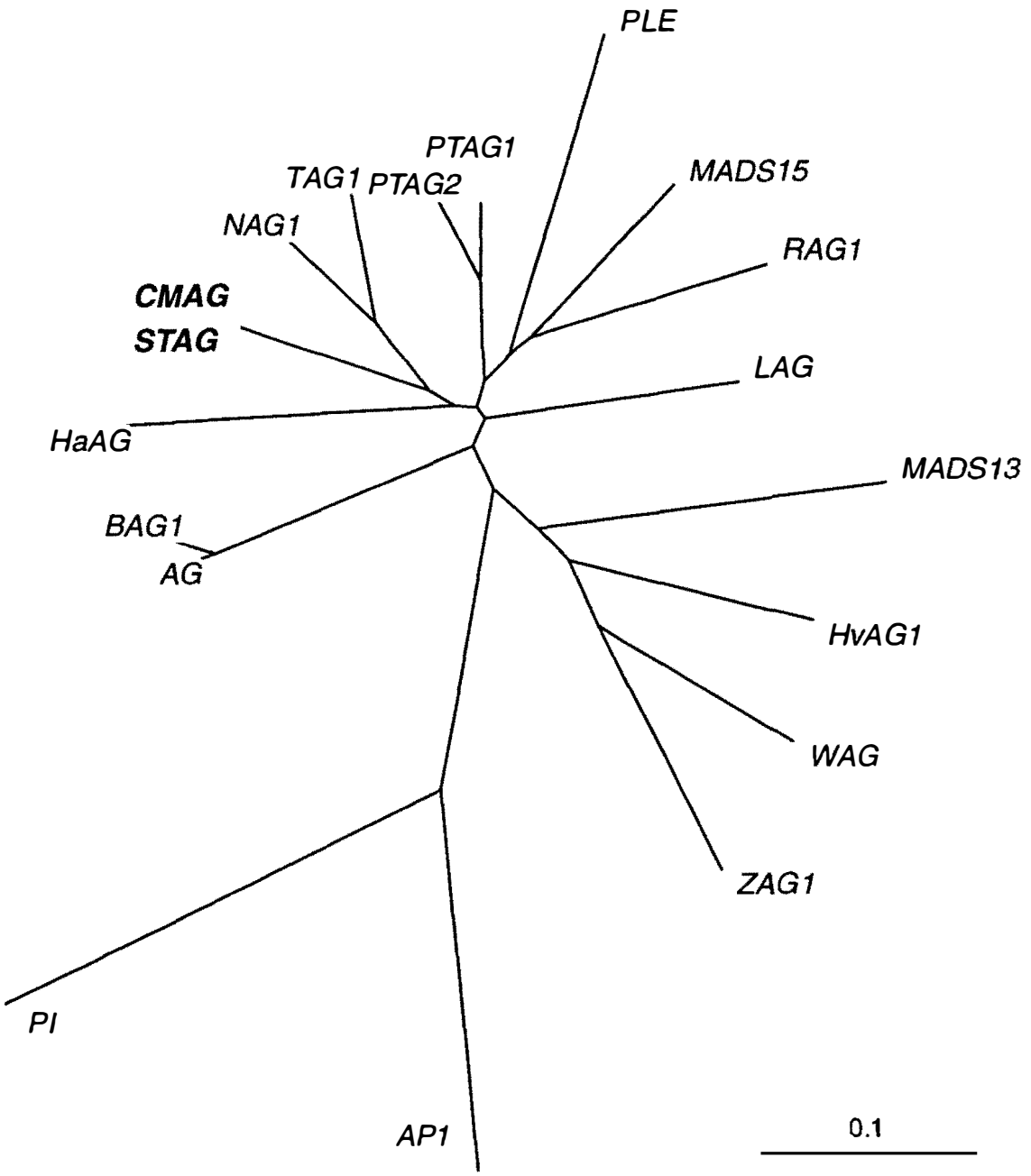

Figure 4.11 Phylogenetic relationship of some *AG* homologues, and of *PI* and *AP1*, from a wide range of angiosperm species

Putative genes *STAG* and *CMAG* were indicated in bold letters. *PI* and *AP1* served as outgroup genes. Bar represents 10% amino acid substitution along the protein fragments of around 100 amino acid residues. Gene codes and species names are described in Section 4.2.3.3.

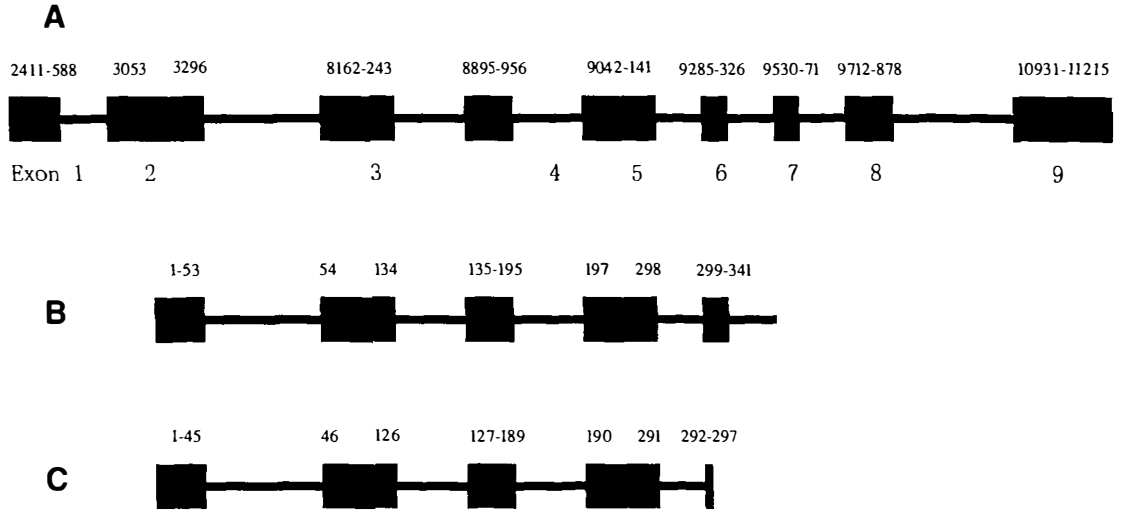

Figure 4.12 Deduced gene structure of *STAG* and *CMAG*

A: *Populus trichocarpa AG* homologue *PTAG1*, full length gene sequence with nine exons (GenBank access No: AF052570); B: *Sophora* putative *AG* homologue *STAG*; C: *Clianthus* putative *AG* homologue *CMAG*.

4.3.5 Isolation and verification of housekeeping gene sequences used as internal controls for gene expression studies

Putative homologue sequences of 18S rRNA, β-actin and GAPDH for *Sophora* and *Clianthus* were isolated using the same strategy as for that of the other target genes, and named *ST18S*, *CM18S*, *STACT*, *CMACT*, *STGAP* and *CMGAP*, respectively (Appendix 3). Each sequence was confirmed by BLAST searching in the GenBank database using deduced amino acid sequence. The confirmed housekeeping gene sequences were used for further gene expression studies as the internal controls.

4.4 Discussion

In the present study, floral identity gene homologues *STLFY*, *STAP1*, *STPI* and *STAG* for *Sophora*, and *CMLFY*, *CMAP1*, *CMPI* and *CMAG* for *Clianthus* were isolated and characterized. The combined evidences provided by BLAST search against the GenBank, sequence comparison and phylogenetic analysis strongly suggested that these genes are most probably homologues of *LFY/FLO*, *AP1/SQUA*, *PI/GLO* and *AG/PLE* for *Arabidopsis* and *Antirrhinum*, respectively. Thus, the hypothesis that there would be homologues present of the herbaceous floral identity genes in the reproductive organs of both *Sophora* and *Clianthus* was supported.

Although more than one homologue gene can often be found in the same species, such as *LFY* homologues *AFL1* and *AFL2* from *Malus* (Wada et al., 2002), *AP1* homologues *EAP1* and *EAP2* from *Eucalyptus* (Kyozuka et al., 1997), *PI* homologues *RbPI-1* and *RbPI-2* from *Ranunculus* (Kramer et al., 1998) and *AG* homologues *PTAG1* and *PTAG2* from *Populus* (Brunner et al., 2000), only one putative gene sequence was identified for each floral identity gene group in the present study. However, it cannot be said with certainty that there exists only a single copy of each floral identity genes for *Sophora* and *Clianthus*, since no Southern hybridization assay was carried out, and only PCR products of appropriate size were sequenced. However, it was beyond the aim of this study to confirm the copy numbers of these genes. The objective to isolate one true homologue gene for *LFY* and each of the ABC class genes for further gene expression study was fulfilled.

As genetic cloning from New Zealand native species is strictly regulated, all target gene sequences in the present study were determined through direct sequencing of RT-PCR product using tissue specific cDNA as PCR template. Degenerate primers were used for PCR amplification and sequencing because there was no previous molecular information for floral genes and housekeeping genes available for these two species, and only a little such information was available for leguminous species and woody species to date. The major disadvantage of using degenerate primers was that more than one PCR product could be produced and all of these products with appropriate size could be sequenced. Furthermore, the sequencing efficiency is much lower when using degenerate primers than specific primers. To overcome these problems, and to efficiently determine the final sequence of the target gene, at least two separate sequencing reactions for both DNA

strands were conducted in the present study.

In order to safely confirm the identity of the newly isolated putative fragments, at least 15 reported homologues from a broad taxonomic range of plant species, consisting of dicot, monocot angiosperms and even gymnosperms, were selected and included in the present study for direct sequence comparison and phylogenetic analysis of each separate gene group. Whenever possible, the representative gene sequences of paralogues or genes from other sub-families were included for direct comparison of each putative group of gene homologues. In all cases, the similarities of putative gene sequences, at the amino acid level, were remarkably lower to paralogues or other sub-family gene sequences than to their homologues from species across wide taxonomic ranges.

The isolated putative *LFY* homologue fragments *STLFY* and *CMLFY* showed high sequence similarity to other reported *LFY/FLO* homologues from a broad range of plant species across angiosperm and gymnosperm. Since these fragments had 93-95% sequence similarity to *LFY* and *FLO*, were well placed in the middle of the eudicot *LFY/FLO* gene group, and there was no sequences from any other gene family found by the BLAST search against the GenBank, it was sound to consider that *STLFY* and *CMLFY* are partial *LFY/FLO* homologues for *Sophora* and *Clianthus*, respectively. It is evident that *STLFY* and *CMLFY* had the highest homology (95-97%) at the amino acid level to FLO/LFY-like protein (BLFL) for *Brownea leucantha* (Archambault and Bruneau, 2004) and UNIFOLIATA (UNI) protein for *Pisum sativum* (Hofer and Ellis, 1998) which was similar to that between *STLFY* and *CMLFY* (97%). This was consistent with their taxonomic relationship since they all belong to the family Fabaceae.

It is evident that *STAP1* and *CMAP1* had the highest sequence similarity at the amino acid level (91% and 98%) to *PEAM4* (Berbel et al., 2001), the *AP1* homologue from *Pisum sativum*, supporting a model for *AP1*-like genes controlling both floral meristem and floral organ identity. Given that *STAP1* and *CMAP1* shared much more sequence similarity to *AP1* and *SQUA* (79-83%) than to *AG* and *PI* (35-42%), it could be safely concluded that *STAP1* and *CMAP1* are partial *AP1/SQUA* homologues for *Sophora* and *Clianthus*, respectively.

The fact that *STPI* and *CMPI* had over 70% sequence similarity to *PI* and *GLO*, and 91% and 94% sequence similarity to the functional *PI* homologue *PEAM1* from *Pisum sativum* (Ferrández and Beltran, 2004), and that only *PI/GLO* homologues were found from the

Genbank, strongly support the interpretation that they are indeed fragments of the *PI/GLO* gene homologues for *Sophora* and *Clianthus*. Since gene sequences from the *PI/GLO* paralogues from the other sub-class of B-class genes, *AP3* and *DEF*, were included for direct sequence comparison and phylogenetic analysis, and *STPI* and *CMPI* were grouped far away from these paralogues, there was little chance for *STPI* and *CMPI* to belong to this paralogue class.

Although relatively low amino acid sequence similarities were observed among *AG/PLE* homologues, the BLAST search in the GenBank still yielded only *AG/PLE* homologues. Together with the fact that the sequence similarities of *STAG* and *CMAG* to all *AG/PLE* homologues (61-80%) was remarkably higher than to *AP1* and *PI* homologues (29-34%), this evidence strongly indicates that *STAG* and *CMAG* are partial homologues of *Sophora* and *Clianthus*.

In comparison between different gene groups or subfamilies, the sequence similarity of the putative genes to their homologues varied considerably. The similarity of *STLFY* and *CMLFY* to *LFY/FLO* were the highest and those of *STAG* and *CMAG* to *AG/PLE* were the lowest, being 93-95% and 71-72%, respectively. This pattern of variation was also the case between other homologues from each gene group. For instance, the similarity between *LFY* and *FLO*, *AG1* and *SQUA*, *PI* and *GLO*, *AG* and *PLE*, were 91%, 77%, 73% and 64%, respectively. This suggests that gene sequences for *LFY/FLO* homologues were the most conserved and those for *AG/PLE* were the least conserved, at least within the region covered by the putative gene sequences. However, this might not reflect the actual variation among these gene groups at the whole gene level since the putative sequences covered different regions of the genes, which are more or less conserved in general.

Results from the present study also revealed that the structures (exon-intron boundaries, exon number and size) of the floral identity genes were very conserved across a broad taxonomic range. For instance, even though the amino acid sequences of *STAG* and *CMAG* differed considerably from that of the *Populus AG* homologue *PTAG1* (Brunner et al., 2000), with sequence similarities of only 76% and 75%, the position and size of all comparable exons were identical or very similar among these species. The same was true for *STPI* and *CMPI* to the *Malus PI* homologue *MdPI* (Yao et al., 2001). This result is consistent with the fact that the MADS box gene structure is conserved across angiosperm and gymnosperm species (Huang et al., 1995; Montag et al., 1995; Sundstroem et al.,

1999). This conservation of gene structures has been observed even in MADS box genes from non-flowering plant species. In comparing the exon-intron structures of the genomic loci of a moss (*Physcomitrella patens*) MADS box genes with that of *SQUA* from the model plant *Antirrhinum majus*, Henschel et al. (2002) observed highly conserved or even identical exon-intron structures among all tested entries, and stated that this conservation in some regions might exist for the whole MADS box gene family. However, future work is required to definitely determine the intron / exon structure of the putative gene fragments isolated in the current study. This could be achieved by comparing the genomic sequences with the cDNA sequences.

In the present study, two different nucleotide signals presented at the same nucleotide site in several cases. This might be caused by either site mutation at different tissue cells or by allelic variation and the heterozygous nature of the individual plant. To solve this problem, separate samples were taken from several plants, and the higher probability of nucleotide was used for the final sequence.

It was noticed, during the multiple sequence comparison and phylogenetic analysis process in the present study, that the sequence similarity between two entry genes would be notably different if the length of the two sequences were different or covered different regions of the homologue genes. This could also affect their positions in the phylogenetic tree. Therefore, the result of phylogenetic analysis would presumably be somewhat biased if the reported sequences were included in the analysis without considering their differences in length and covering regions of the genes in question. For this reason, all sequences, either isolated in the present study or obtained from the GenBank database, were cut to the same length and covered the same region of the homologue gene before being used for sequence comparison and phylogenetic analysis. In the case when a sequence in the GenBank had particular significance but was shorter than the putative gene sequences isolated in the present study, it was included in the analysis for reference only and was not used for direct comparison. This was especially the case of *MEL* and *MESAP1* for *Metrosideros excelsa* (Sreekantan et al., 2004) and of *ALF* and *AAP1* for *Actinidia deliciosa* (Walton et al., 2001). Also for the same reason, the results of sequence similarities and phylogenetic position obtained in the present study for a specific gene could be different from that in other studies.

The isolated fragments of floral identity genes *STLFY*, *CMLFY*, *STAP1*, *CMAP1*, *STPI*,

It also provided useful information for molecular studies of other leguminous species, especially woody legumes. However, further investigations toward their functions and expression patterns in different organ tissues and across the different developmental stages should be carried out to confirm unequivocally whether they have the same roles as their homologues in *Arabidopsis* and other plant species. While function studies of these homologues were beyond the reach of the current project, studies of the detailed expression characteristics of each of these genes were subsequently conducted and are presented in Chapter 5.

Chapter 5 Temporal and developmental expression of floral identity genes in *Clianthus* and *Sophora* using real-time RT-PCR

5.1 Introduction

Genes controlling the specification of floral organ identity and development have been isolated and characterized in a variety of plant species. Intensive studies and analyses of identity mutations in *Arabidopsis* and *Antirrhinum* have led to the ABC model of floral organ identity specification (Bowman et al., 1991; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Although analyses of genes encoding the ABC functions have largely substantiated the applicability of this model, a number of refinements have been made (Zik and Irish, 2003). For instance, homologues of the A function genes *AP1* and *AP2* play a minor role in the specification of sepals and petals in a number of species, such as *Antirrhinum*, *Petunia*, maize and apple, although they have a role in floral meristem identity (Chuck et al., 1998; Maes et al., 2001; Muller et al., 2001).

Expression of these floral identity genes have also been studied in a number of woody perennial species, including *Eucalyptus* (Kyozuka et al., 1997), *Populus* (Rottmann et al., 2000), *Actinidia* (Walton et al., 2001), *Malus* (Wada et al., 2002), *Vitis* (Carmona et al., 2002) and the New Zealand genus *Metrosideros* (Sreekantan et al., 2004). However, most of these studies focused on the initiation of the floral meristem and floral organs. Little is known about the detailed expression profiles of floral identity genes during different developmental stages, and in different floral organs. Study of the expression profile of all three classes of the floral organ identity genes and their upstream controller genes for the same species and at the same time has not been reported to date, even for model herbaceous species.

Partial homologues of all three classes (A, B and C) of *Arabidopsis* floral identity genes, *APETALA1*, *PISTALLATA* and *AGAMOUS*, and their up-stream controller gene *LEAFY* were successfully isolated from *Sophora tetraptera* and *Clianthus maximus* (Chapter 4). These partial homologues were named *STAP1, STPI, STAG* and *STLFY* for *Sophora,* and *CMAP1, CMPI, CMAG* and *CMLFY,* respectively, for *Clianthus.* The high sequence similarity of these genes to their *Arabidopsis* homologues lent support to the hypothesis

that the *Arabidopsis* based floral gene model would be applicable to these two woody perennial leguminous species. To test this hypothesis, real-time RT-PCR was used to quantify the temporal and developmental expression of these genes.

Real-time quantitative RT-PCR is an extremely powerful and preferred method for gene expression studies, which can generate reliable, reproducible, and biologically meaningful results (Bustin and Nolan, 2004). The method is based on the detection of a fluorescent signal produced and monitored during the amplification process and can quantitate the initial amount of template in a dynamic range of up to 10^7-fold without post-PCR processing of PCR products (Dorak, 2004). The incorporation of SYBR Green I into real-time RT-PCR makes this technique a relatively economical method for the quantification of gene expression (Ramos-Payan et al., 2003). The non-specific DNA detecting feature of SYBR Green I can be overcome by analyzing the melting-curve of PCR product and/or by using the intron spanning primer designing strategy. Accurate normalization of experimental data is an absolute prerequisite for correct measurement of gene expression using real-time RT-PCR. The most commonly used normalization strategy involves standardization to a constitutively expressed housekeeping gene as the internal control (Aerts et al., 2004; Andersen et al., 2004; Dheda et al., 2004). However, such housekeeping genes are usually used without a thorough investigation of how invariant their mRNA levels are under the experimental conditions being investigated. If housekeeping genes are to be used, they must be validated for the specific experiment (Bustin and Nolan, 2004)

In this chapter, the detailed spatial (developmental) and temporal expression profiles of *LEAFY, APETALA1*, *PISTILLATA* and *AGAMOUS* homologues in *Sophora* and *Clianthus* were investigated, using real-time PCR in combination with the SYBR Green I detection system, and multiple housekeeping genes.

5.2 Materials and methods

5.2.1 Plant materials

Three year old *Clianthus maximus* Kaka King® and *Sophora tetraptera* plants were grown in the Palmerston North site nursery of the New Zealand Institute for Crop and Food Research Limited, and used for RNA samples. These were the same plants used for

microscopic analysis and growth monitoring studies described in Chapter 3.

For *S. tetraptera,* inflorescences and floral buds at different developmental stages, from the emergence of inflorescence until senescence of flowers, were harvested randomly at 2-4 weekly intervals from October 2002 to October 2003. Vegetative shoot tips and leaves were harvested in January 2003 well after the initiation period of inflorescences, but before the new vegetative growth period terminated so that no inflorescence primordia could possibly be included in the shoot tip samples (Table 5.1).

For *C. maximus*, inflorescences and floral buds at different developmental stages, from the emergence of inflorescence until senescence of flowers, were harvested at 2-4 weekly intervals from November 2002 to October 2003 (Table 5.2). At the time of harvest, samples were immediately frozen in liquid nitrogen and then stored at –80°C until use.

5.2.2 RNA isolation

For the majority of tissue samples used for gene expression studies, total RNA was extracted using the mini-preparation protocol adapted from the TRI Reagent method as described in Section 4.2. For early stage tissue samples, such as inflorescences and floral buds, when the availability of samples was limited, the newly developed RNA micro-preparation protocol was used.

For RNA micro-preparation, 5-10 mg of fresh or –80°C stored tissue sample was ground in a 1.5 ml centrifuge tube with a plastic mini pestle in the presence of liquid nitrogen. TRI reagent (200 ml) was added and vortexed for 20-30 s. The mixed sample was transferred to a 0.5 ml centrifuge tube and centrifuged for 5 min at 10000g at 4°C in a benchtop Eppendorf centrifuge. The supernatant was transferred to a new 0.5 ml tube and stored at room temperature for 5 min. Chloroform (200 µl, isoamyalcohol-free) was added, shaken vigorously for 15 s and stored for 10 min before being centrifuged for 15 min at 10000g at 4°C. The aqueous phase was transferred to a new tube. Isopropanol (100 µl) was added, well mixed, stored for 10 min and then centrifuged at 10000g for 8 min at 4°C. The RNA pellet was washed with 400 µl 75% ethanol and centrifuged at 4000g for 5 min at 4°C. Ethanol was removed and the pellet air-dried for 3-5 min. RNA was dissolved in 5-10 µl TE buffer or RNAse-free water with 1x RNAsecure™ (Ambion Inc) and heated to 65°C for 15 min to activate its RNA protection ability.

Table 5.1 ***Sophora tetraptera*** **tissue samples for RNA preparation (2002-2003)**

Sample types	Sample description	Sampling date
Reproductive	Shoot tips with inflorescence primordia	06/10/2002
structures	Inflorescences (1 mm)	17/10/2002
	Inflorescences (2-3 mm)	03/11/2002
	Inflorescences (10-12 mm)	19/12/2002
	Inflorescences (<15 mm) with floral primordia	19/12/2002
	Inflorescences (20-30 mm) with flower bud (c.1 mm)	08/01/2003
	Flower buds (1-1.5 mm)	28/01/2003
	Flower buds (1-1.5 mm)	16/02/2003
	Flower buds (1-1.5 mm)	01/03/2003
	Flower buds (1-1.5 mm)	04/04/2003
	Flower buds (1-1.5 mm)	01/05/2003
	Flower buds (1-1.5 mm)	16/05/2003
	Flower buds (1.5-2 mm)	26/06/2003
	Flower buds (2-3 mm)	24/07/2003
	Flower buds (5-6 mm)	15/08/2003
	Flower buds (10-12 mm)	29/08/2003
	Flower buds (18-20 mm), showing petal tip	18/09/2003
		26/09/2003
	Sepals from flower buds showing petal tip	26/09/2003
	Petals from flower buds showing petal tip	26/09/2003
	Stamens from flower buds showing petal tip	26/09/2003
	Carpels from flower buds showing petal tip	26/09/2003
	Mature flower buds before opening	26/09/2003
		07/10/2003
	Fully opened flowers before senescence	26/09/2003
		07/10/2003
	Young legumes 2 weeks after flower senescence	20/10/2003
Vegetative	Vegetative shoot tips	08/01/2003
tissue	Fully expanded leaves	08/01/2003

Table 5.2 ***Clianthus maximus*** **Kaka King® tissue samples for RNA preparation**

Sample types	Sample description	Sampling date
Vegetative tissue +/- reproductive structures	Shoot tips with/without inflorescence primordia	08/11/2002
	Shoot tips with/without inflorescence primordia	08/01/2003
	Shoot tips with/without inflorescence primordia	24/02/2003
	Shoot tips with/without inflorescence primordia	04/04/2003
	Shoot tips without inflorescence primordia	01/05/2003
	Shoot tips with inflorescence primordia	01/05/2003
	Fully expanded leaves	16/05/2003
	Shoot tips with/without inflorescence primordia	04/07/2003
	Shoot tips with/without inflorescence primordia	17/09/2003
Reproductive structures	Inflorescences (1-2 mm)	01/05/2003
	Inflorescences (15-20 mm)	28/01/2003
	Inflorescences (15-20 mm)	24/02/2003
	Inflorescences (15-20 mm)	04/04/2003
	Inflorescences (15-20 mm)	30/04/2003
	Inflorescences (15-20 mm)	11/06/2003
	Inflorescences (25-35 mm) with flower buds around 1 mm	11/06/2003
	Flower buds (2-3 mm)	11/06/2003
		24/07/2003
	Flower buds (5-7 mm)	11/06/2003
	Flower buds (10-12 mm) showing petal tip	15/08/2003
		26/09/2003
	Sepals from flower buds showing petal tip	26/09/2003
	Petals from flower buds showing petal tip	26/09/2003
	Stamens from flower buds showing petal tip	26/09/2003
	Carpels from flower buds showing petal tip	26/09/2003
	Mature flower buds before open	18/09/2003
		26/09/2003
	Fully opened flowers before senescence	26/09/2003
	Young seed pods 2 weeks after flower senescence	07/10/2003

For both mini-preparation and micro-preparation, two independent RNA extractions from each tissue sample were mixed before the quantity and quality were measured using a NanoDrop spectrophotometer (Nyxor, ND-100). Its integrity was checked by visualization of ethidium bromide-stained RNA separated on 2% (w/v) agarose gel containing 2% (v/v) formaldehyde. Only RNA without detectable degradation of 18S and 28S rRNA was used for subsequent cDNA synthesis either immediately or following storage at –20°C. No DNAse treatment was needed to prevent genomic DNA amplification in these experiments, because all primer pairs spanned at least one intron to detect genomic DNA contamination in PCR.

5.2.3 cDNA synthesis

For RT-PCR assays, the reverse transcription reaction can be initiated using random primers, oligo-dT, or target-specific primers. Each of the three methods differs significantly with respect to cDNA yield, variety and specificity, and can cause marked variation in calculated mRNA copy numbers (Zhang and Byrne, 1999).

Random primers produce more than one nonspecific cDNA transcript per original target and yield the most cDNA. It also generates the least bias in the resulting cDNA (Ginzinger, 2002). However, the majority of cDNA synthesized from total RNA will be ribosomal RNA-derived. This could create problems if the target of interest is present at low levels, as it may not be primed effectively by random primers and its amplification may not be quantitative (Deprez et al., 2002). Nevertheless, this random priming of cDNA can yield reliable and reproducible results if it is carried out in a careful, competent manner (Bustin and Nolan, 2004).

cDNA synthesis using oligo-dT is more specific to mRNA than random priming, as it will not transcribe rRNA. However, oligo-dT priming requires very high-quality full length RNA since it will not prime any RNAs that lack a polyA tail. It is, therefore, not suitable for transcribing RNA that is likely to be fragmented, lacks a polyA tail, or has secondary structures (Bustin and Nolan, 2004).

DTT was reported to be inhibitory to subsequent PCR reactions, especially real-time PCR reactions. Omission of DTT could improve the real-time PCR efficiency without reducing the cDNA yield (Deprez et al., 2002).

Therefore, different combinations of oligo-$(dT)_{15}$ primers ($p(DT)_{15}$) and hexa-random primers (p(DN) with or without DTT were tested to determine the appropriate first strand cDNA synthesis method for the LightCycler real-time PCR used in current study, in which *18S rRNA* served as one of the internal control genes. The following gradients were included in the RT reactions for these combinations:

A: 50 pmoles p(DT)15 primers

B: 100 pmoles p(DN)6 random primers

C: 50 pmoles p(DT)15 + 100 pmoles p(DN)

D: 25 pmoles p(DT)15 + 50 pmoles p(DN)

E: 50 pmoles p(DT)15 - DTT

F: 100 pmoles p(DN) – DTT

G: 50 pmoles p(DT)15 + 100 pmoles p(DN) – DTT

RNA samples were quantified using the NanoDrop spectrophotometer before reverse transcription so that the same amount of total RNA was used in all RT reactions from different tissue samples. One μg of total RNA was used for each reaction following the protocol described in Section 4.2. Two separate reverse transcription assays were carried out for each tissue sample, and the resulting cDNAs were bulked to minimize error caused by variation in reverse transcription efficiency. A 20-30-fold dilution of the RT reaction was used for real-time analysis.

Target genes and *18S rRNA* were used for LightCycler™ real-time amplification with three to four replications. For more precise comparison of different RT reaction recipes, the smallest C_t value of the LightCycler™ real-time reaction within the same replication served as the calibrator, and was set to zero. All other values were subtracted by the original C_t of the calibrator to generate the new set of adjusted C_t data.

5.2.4 Real-time PCR primer design

For real-time PCR analysis, specific primers were designed for each gene according to the general requirements of real-time PCR primers (Roche Diagnostics) using the Premier Primer 5 software, based on the cDNA sequence determined for each target gene and housekeeping genes described in Section 4.3. Introns and exons were identified from the structure diagram of each gene (Fig. 4.3, 4.6, 4.9, and 4.12).

The primer target sites were carefully selected in order to span at least one intron to eliminate any PCR-amplified fragments of genomic DNA, that were a possible minor component in the RNA preparations, and were expected to be significantly longer. All the primers were spaced such that the sizes of amplified products were as similar as possible and within the range of 100-350 nucleotides.

To add specificity for gene quantification, the primers were designed to work only with the selected specific genes and not with other homologues, e.g., other members of the MADS box or floral identity gene families. Generally, two to four primer pairs were designed for each target gene, and screened for gene specificity and PCR efficiency to select the best one for further real-time analysis. The specificity of each primer pair was confirmed, in the first instance, by absence of significant homology with other known DNA sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) of the GenBank database (National Resource for Molecular Biology Information), and then by electrophoresis and melting-curve analysis of PCR products using the LightCycler™ instrument and the cDNA samples under investigation.

5.2.5 Real-time PCR assay

Real-time PCR amplifications were carried out in glass capillaries in a total volume of 15 µl in a LightCycler™ 2.0 instrument (Roche Diagnostics Corporation). Each reaction mixture contained 1.5 µl of 20-30 fold diluted cDNA template from different tissue samples, 1.5-2.1 µl of 25 mM $MgCl_2$ at the final concentration of 3.5-4.5 mM, 1.0 µl of each primer (15 µM), 1.5 µl of FastStart DNA Master SYBR® Green I (Roche Diagnostics), and sterile distilled water making the total reaction volume of 15 µl. The thermal cycling conditions were an initial denaturation at 95°C for 10 min followed by 45 cycles at 95°C for 10 s, 52°C for 20 s, 72°C for 20 s, and 80-84°C for 1 s, in order to detect and quantify the fluorescence at a temperature above the denaturation of primer dimers, using a slope of 20°C s^{-1}. A melting-curve temperature profile was obtained by programming the LightCycler™ for one cycle at 95°C for 0 s, and 50°C for 40 s using a slope of 20°C s^{-1}, followed by 95°C for 0 s, using a slope of 0.1°C s^{-1}. After standardizing the baseline, relative template abundance was quantified using the second derivative maximum method of the LightCycler™ version 3.5 software (Roche Diagnostics) to determine the threshold cycle (C_t) at which each PCR reached exponential amplification, a requirement for quantitative PCR.

For each experiment, a no-template reaction containing the same reaction mixture with 1.5 μl of sterile water replacing the cDNA template, was included as a negative control.

The lack of primer-dimer or non-specific product accumulation was first checked by melting-curve analysis and then confirmed by 1.5% agarose gel electrophoresis using 1× TAE buffer. A 1 kb plus DNA ladder (Invitrogen) was used as molecular size standard.

5.2.6 Determination of optimal $MgCl_2$ concentration

$MgCl_2$ plays a very important role in the PCR amplification process and can greatly affect the PCR efficiency. The appropriate $MgCl_2$ concentration could vary significantly for different primer pairs that have different binding ability to the templates and produce different lengths and sequences of PCR amplicons. Therefore, the appropriate $MgCl_2$ concentration should be determined for each gene and each primer combination of the same gene.

The optimal $MgCl_2$ concentration for real-time PCR amplification was determined individually for the selected primer pair of each of the target and housekeeping genes for further expression investigation. Since the FastStart DNA Master SYBR® Green I (Roche Diagnostics) contains 1 mM of $MgCl_2$, additional 0, 0.6, 1.2, 1.8, 2.4, and 3.0 μl of 25 mM $MgCl_2$ solution was added to obtain the final $MgCl_2$ concentration of 1, 2, 3, 4, 5, and 6 mM , respectively, in a 15 μl LightCycler™ PCR reaction.

5.2.7 PCR amplification efficiency determination

A serial dilution of 10, 100, 1000, 10,000 and 100,000-fold of the same first strand cDNA was used as template for LightCycler™ reactions to determinate the amplification efficiency of each of the target genes and housekeeping genes. A standard curve was obtained by plotting the C_t value versus the logarithm of the concentration. Two to three replicates were carried out for each gene. The PCR efficiency (E) was calculated according to the following formula described by Pfaffl (2001):

$$E=10^{(-1/slope)}-1$$

5.2.8 Controlling sample variation

For accurate quantification of gene expression, it is essential to control the variation between samples introduced at a number of stages throughout the experimental protocol, such as sample input, RNA extraction, and reverse transcription. Various strategies have been applied to normalize these variations, including good-quality RNA extraction under controlled conditions, RNA mass quantity control, and the use internal control genes.

To verify the variations derived from different sources, experiments were conducted involving in RNA isolation, cDNA synthesis, PCR runs, and PCR reactions. Firstly, three total RNAs were extracted from a single floral tissue sample of *Sophora* followed by separate cDNA synthesis. Secondly, three cDNAs were synthesized using the same amount of total RNA for which the concentration was predetermined using the NanoDrop instrument. And finally, two sets of triplicate PCR reactions were run separately in the LightCycler™ instrument.

5.2.9 Determination of appropriate internal controls

Housekeeping genes are believed to be constitutively expressed and minimally regulated in different tissues and developmental stages of an organism, and have been widely used as internal controls for quantitative analysis in real-time RT-PCR assays. However, a number of recent studies have shown that housekeeping gene expression can vary considerably (Tricarico et al., 2002; Andersen et al., 2004; Dheda et al., 2004). These unstable control genes cannot completely remove nonspecific variation, and can even add more variation, resulting in larger so-called gene-specific variations for the tested control genes and in missed small differences between genes of interest.

To address the issue of choosing optimal control genes for real-time RT-PCR, *GAPDH, β-actin*, and *18S rRNA*, the most frequently used housekeeping genes for gene expression studies using conventional and real-time PCR, were selected in the current study. They were firstly compared for their expression stability over different RNA isolations, reverse transcriptions and PCR runs in the same tissue sample, using one of the most stable of the expressed target genes, *STAP1*, as expression control. And then, their expression levels were compared over a series of tissue samples in different developmental stages, with three and four replicates. The stability of each housekeeping gene was evaluated using

geNorm version 3.3 software (http://allserv.ugent.be/~jvdesomp/genorm/index.html). To minimize the possible errors derived from using only one housekeeping gene for normalization, the geometric mean of these three housekeeping genes was calculated according to the method described by Vandesompele et al. (2002), and its correlation to the control target gene were compared to that of each single gene. The most suitable housekeeping gene or genes were selected for gene expression studies.

5.2.10 Gene expression quantification

Template quantification was based on the threshold cycle (C_t) of each sample, depending on the number of PCR cycles required for the threshold detection of the fluorescence signal. The relative quantification method (percentage) was used in the current study. The gene expression levels of a series of samples for the same gene reflected by difference of mRNA or cDNA concentrations in the initial template were compared. To minimize experimental error, PCR reactions containing cDNAs from a series of experimental samples were generally included in a single LightCycler™ run, with genes of interest and housekeeping genes sharing the same cDNA template in each of the investigated samples.

For the genes of interest, the C_t value of each sample was adjusted according to the normalization factor or the geometric mean of the housekeeping genes. The normalization factor of the internal controls was calculated using geNorm version 3.3 software. The geometric mean was calculated based on the method described by Vandesompele et al. (2002). The adjusted C_t values were then transformed to relative quantities. The highest relative quantity for each gene of interest served as the calibrator and was set to 100%. Each of the normalised values for the same gene of interest was divided by the calibrator to generate the relative expression value, ranging 0-100%.

To compare the expression levels of samples that could not be included in the same PCR run, separate runs were carried out using the same PCR master mix prepared for both run. In this case, at least two overlapping samples were included in each PCR run for both the gene of interest and the housekeeping genes so that the C_t values of one run were adjusted based on the relative C_t values of these overlapping samples. To compare the expression levels between different genes in the same tissue sample, the C_t value of each gene was first corrected by its amplification efficiency.

5.2.11 Data analysis

Experimental data were analysed as a randomised block experiment by analysis of variance, where PCR-run was the block factor, the stage of development was the treatment factor, and relative transcript abundance was the response variable. Using a block factor reduced the effect of variation between PCR runs on the response.

5.3 Results

5.3.1 Establishment and optimisation of real-time RT-PCR methodology for expression analysis of floral identity genes in *Clianthus* and *Sophora*

5.3.1.1 RNA Isolation

RNA quality is one of the most important determinants of the reproducibility and biological relevance of subsequent qRT-PCR results. Total RNA isolated using the mini-preparation method generally gave very good quality, with a 260/280 ratio of 1.8-1.9 in distilled water. This could be directly used for successful cDNA synthesis without any pretreatment. The yield of total RNA from 100 mg of fresh tissue sample was between 120 and 150 µg (Table 5.3), sufficient for over 100 reverse transcription reactions and thousands of PCR reactions. The integrity of RNA assessed using gel electrophoresis was also very good, with the two major 18S and 28S ribosomal RNA bands, and many minor, but detectable mRNA bands below these major bands (Fig. 5.1).

During the early developmental stages of inflorescence and floral buds, the individual tissues to be sampled were small (often less than 1 mg in weight) and a large number of buds were, therefore, needed for a normal RNA isolation protocol. This was very limiting and even impossible in many cases in terms of time and availability of the biological resources. Therefore, further attempts were made to decrease the quantity of sample required. A simple and efficient micro-preparation total RNA isolation protocol from the previously developed mini-preparation protocol based on the TRI Reagent method as described in Section 5.2.2 was developed. Using this protocol, only 5-10 mg of fresh tissue sample was needed to obtain enough total RNA for 10 cDNA synthesis and

hundreds of real-time PCR reactions. This protocol yielded 1.5-2 fold of total RNA and was 20-30-fold more cost effective compared to the protocol using a commercial RNA isolation kit (Table 5.3), with adequate RNA quality and integrity for cDNA synthesis and gene expression quantification using real-time PCR.

Table 5.3 Comparison of RNA isolation methods using *Sophora* flower buds

Method	Replicate	Sample weight (mg)	RNA yield (μg/mg)	A260/A280
Mini-preparation	I	150	1.61	1.920
	II	150	1.34	1.852
	III	100	1.23	1.950
	M	133.3	1.39	1.907
	SE	/	0.113	0.029
Micro-preparation	I	6	1.72	1.864
	II	5.7	1.68	1.848
	III	6	1.85	1.838
	Mean	5.9	1.75	1.850
	SE	/	0.07	0.008
RNeasy mini-kit	I	6.5	0.87	2.025
	II	6.0	1.21	2.015
	III	8.0	0.77	2.005
	Mean	6.83	0.95	2.015
	SE	/	0.13	0.006

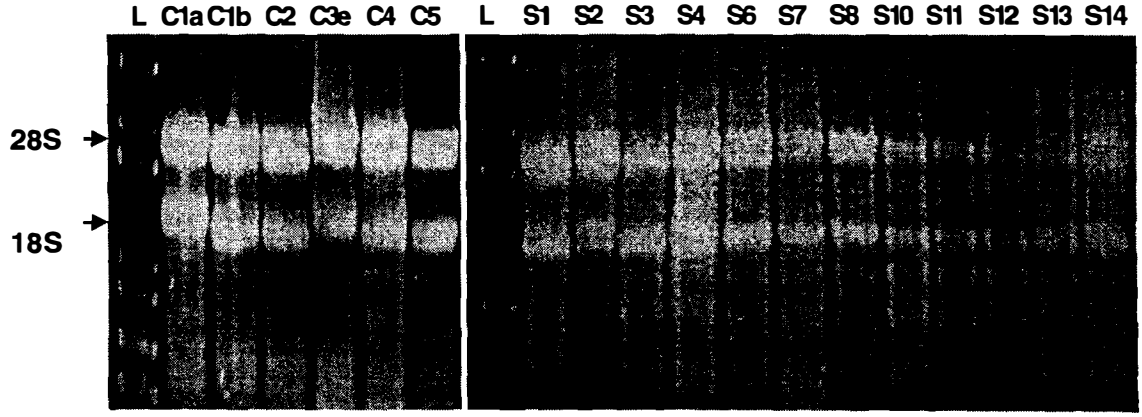

Figure 5.1 Total RNAs isolated using the mini-preparation protocol

Lanes C1a-C5: *Clianthus maximus;* Lanes S1-S14: *Sophora tetraptera*; L: 1kb plus DNA size marker

5.3.1.2 cDNA synthesis methods

Real-time PCR quantification curves indicated that cDNAs synthesized from all primer combinations, with or without DTT resulted in normal PCR reactions represented by typical PCR product accumulation profiles (Fig. 5.2), although the cDNA yields varied as indicated by the significant differences in the C_t values.

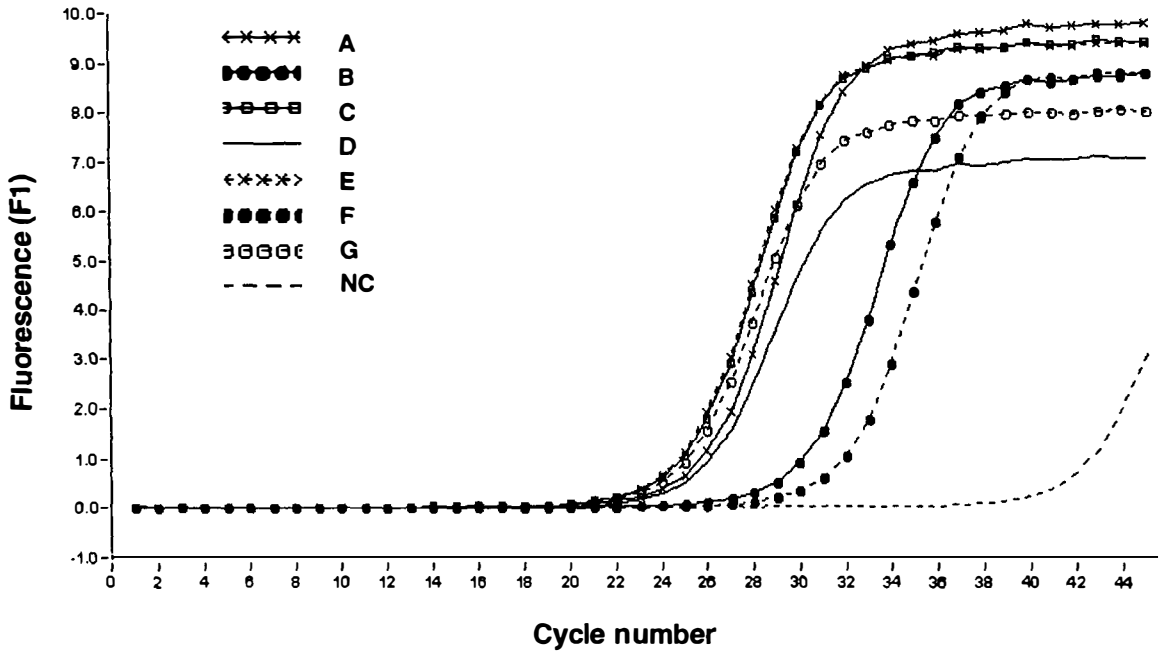

Figure 5.2 LightCycler™ quantification curves of *STAP1* for different cDNA synthesis ingredients

A: 50 pmoles of p(DT)15 primers; B: 100 pmoles of p(DN)6 random primers; C: 50 pmoles of p(DT)15 + 100 pmoles p(DN); D: 25 pmoles of p(DT)15 + 50 pmoles p(DN); E: 50 pmoles of p(DT)15 – DTT; F: 100 pmoles of p(DN) – DTT; G: 50 pmoles of p(DT)15 + 100 pmoles of p(DN) – DTT; NC: negative control without template.

Relative C_t values (Fig. 5.3) indicated that real-time PCR efficiency was dramatically affected by primer, with a variation of expression level of 40-50-fold for *STAP1* (a C_t value difference of c. 7.47), and 4-5-fold variation for *18S rRNA*. For *STAP1*, poly-T primers had a 15-fold higher expression level compared to that of random primers. The combination of oligo-$(dT)_{15}$ and hexa-random primer (treatment C) had a 2-fold expression level compared to oligo-$(dT)_{15}$ primers alone (treatment A). For 18 S rRNA, hexa-random primers had 4-5-fold higher expression levels than oligo-$(dT)_{15}$ primers, but had only a non-significantly higher expression level ($P < 0.05$) compared to the mixture

of two primers. Removing DTT from the RT reaction did improve the real-time PCR efficiency for 18 S rRNA, while having a mixed result for *STAP1*. This result suggested that the mixture of poly-dT and random primers was the best choice for cDNA synthesis with or without DTT, whether or not the 18 S rRNA was used as an internal control.

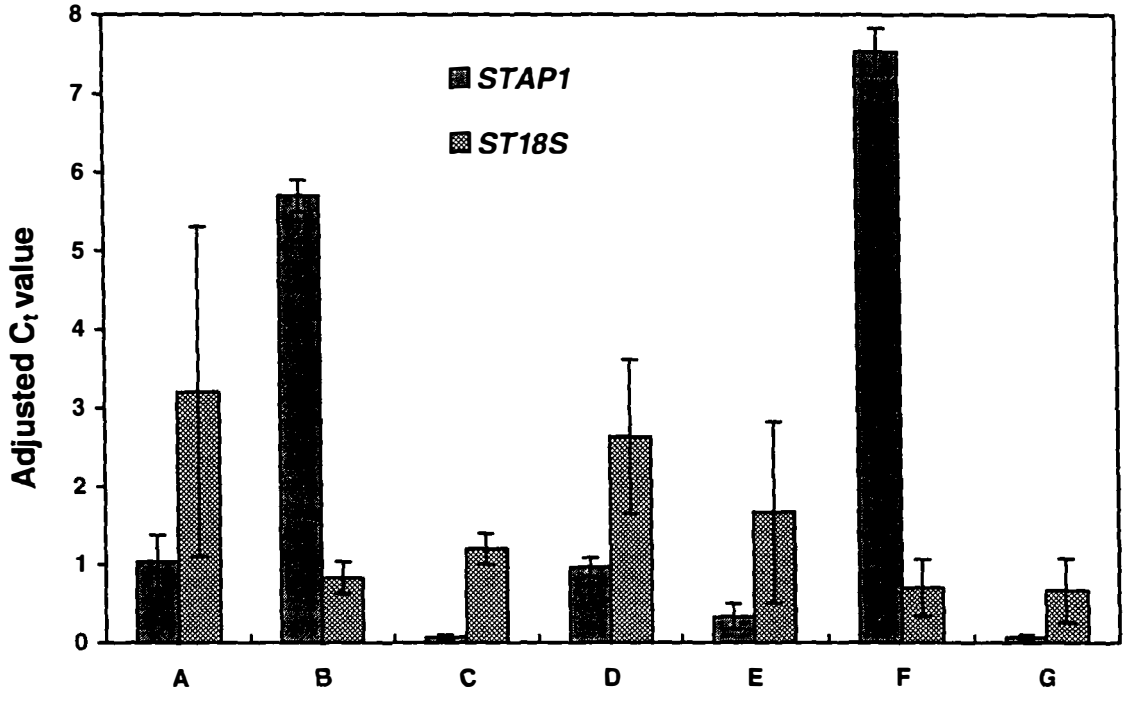

Figure 5.3 Comparison of the C_t values of cDNAs synthesized using different primers and DTT combinations

A: 50 pmoles of p(DT)15 primers; B: 100 pmoles of p(DN)6 random primers; C: 50 pmoles of p(DT)15 + 100 pmoles p(DN); D: 25 pmoles of p(DT)15 + 50 pmoles p(DN); E: 50 pmoles of p(DT)15 – DTT; F: 100 pmoles of p(DN) – DTT; G: 50 pmoles of p(DT)15 + 100 pmoles of p(DN) – DTT. Values were the mean (± SE) relative Ct values of four replicates.

5.3.1.3 cDNA dilution

Many ingredients for the reverse transcription reaction remaining in the resulting cDNA could be inhibitory to the real-time PCR reaction if directly used as template. Appropriate dilution of cDNA was expected to reduce to some extent the deleterious effects of these substances. Initial real-time PCR assays using 5-fold diluted cDNA as template, which was routinely used for conventional RT-PCR, resulted in very unstable PCR results accompanying abnormal amplicon accumulation curves. Therefore, a series of 10-fold cDNA dilutions as LightCycler™ PCR template for the target genes and housekeeping genes selected for gene expression studies was tested.

The results showed that the undiluted cDNA was very inhibitory to real-time PCR reactions for all the tested genes, resulting in an abnormal PCR accumulation curve, a shorter exponential phase, a lower plateau value, and then much lower PCR products (Fig. 5.4). A 10-fold dilution increased the PCR efficiency. With a cDNA dilution of 100-fold or over, the inhibitory effects were removed in most cases, and PCR efficiency greatly improved.

However, the increased cDNA dilution factor could make the quantification of expression difficult. This would be especially the case for not highly expressed genes, which is the case for many genes of interest in biological studies. Considering that there were distinct differences in terms of the quantification curves and melting peaks (Fig. 5.4) between the 10-fold dilution and the 100-fold dilution, more cDNA dilutions between 10- and 100-fold were tested. In most cases, a 20- to 50-fold dilution was satisfactory to remove most of the inhibitory effects and to have the PCR result comparable to that of the 100-fold cDNA dilution (Fig. 5.5).

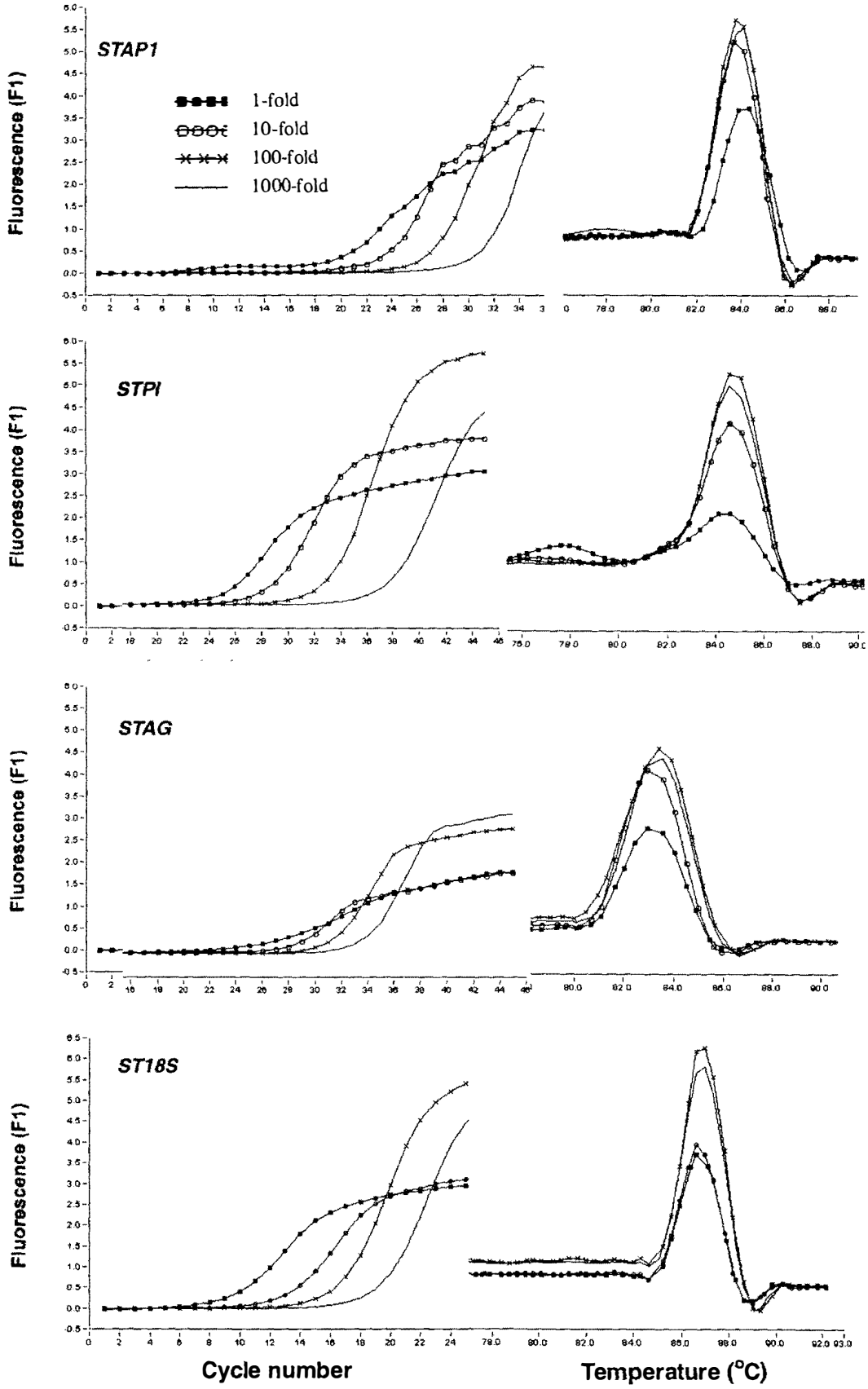

Figure 5.4 Effect of cDNA dilution on real-time PCR performance of *Sophora* floral identity genes

Left panels: LightCycler™ PCR quantification curves; Right panels: melting curves.

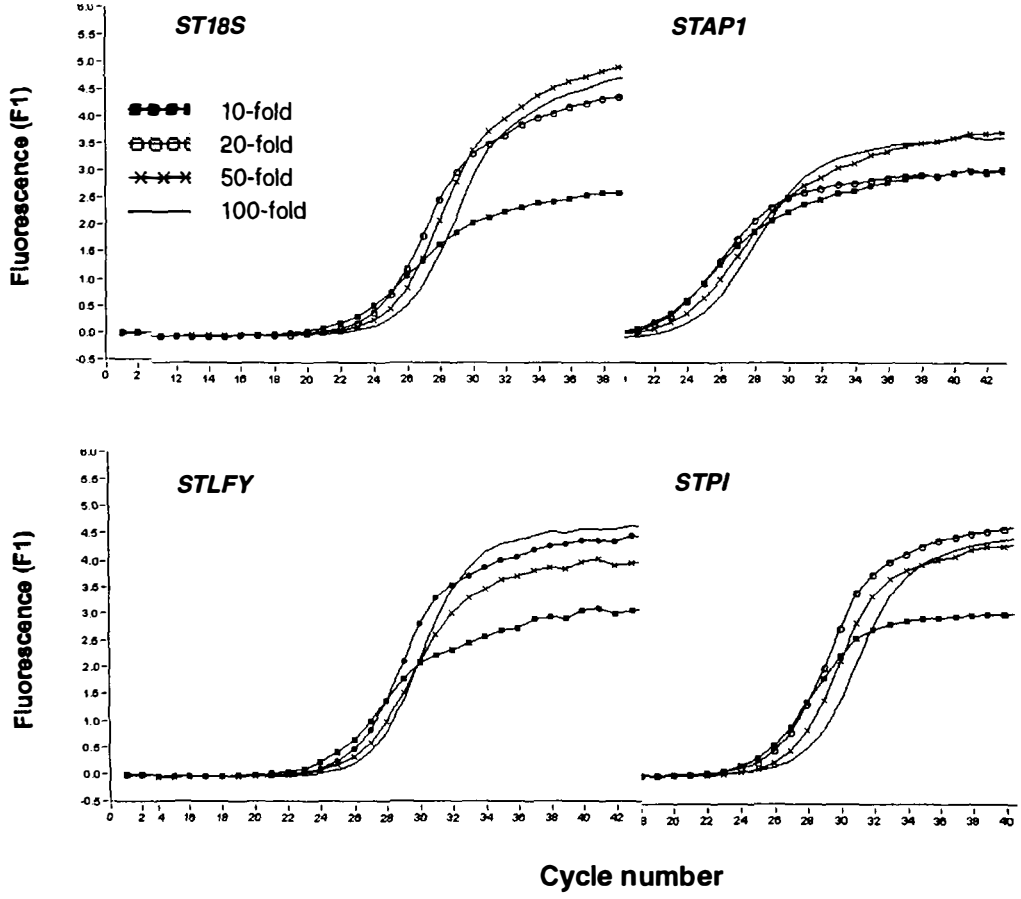

Figure 5.5 Effect of cDNA dilution (10- to 100-fold) on real-time PCR performance of *Sophora* floral identity genes

5.3.1.4 $MgCl_2$ concentration determination

Results obtained in this study showed that $MgCl_2$ was a key element for successful PCR amplification, and that the concentration of $MgCl_2$ critically affected the PCR efficiency. No PCR product was detected in PCR reactions containing 1 mM $MgCl_2$ for all the tested genes, and very low PCR amplicon was detected, or the C_t appeared much later, in reactions containing 2 mM $MgCl_2$. PCR efficiency was greatly improved when the $MgCl_2$ concentration was higher than 3 mM, reaching the highest expression levels in reactions containing 4 mM $MgCl_2$ for 18 S rRNA and 5 mM $MgCl_2$ for all other genes as reflected by C_t values (Table 5.4, Fig. 5.6 and Fig. 5.7). PCR efficiency was inhibited at higher $MgCl_2$ concentrations. It was evident that the sensitivity to $MgCl_2$ concentration for successful amplifications varied significantly among different genes, with *CMAG, 18S, and β-actin* being much less sensitive than other investigated genes.

Table 5.4 Effect of $MgCl_2$ concentration on real-time PCR efficiency of *C. maximus* floral identity genes and selected housekeeping genes

$MgCl_2$ (mM)	1.0	2.0	3.0	4.0	5.0	6.0
CMAP1	0	0.29 (0)	17.94 (9.04)	81.42 (9.77)	99.31 (0.69)	nt
CMPI	0	0.70 (0.46)	22.31 (8.97)	89.18 (6.57)	100 (0)	nt
CMAG	0	24.02 (7.41)	79.88 (7.18)	96.62 (2.01)	100 (0)	60.29 (10.0)
CMLFY	0	0	15.23 (10.12)	54.51 (15.62)	95.09 (4.91)	85.11 (14.89)
CM18S	0	7.54	79.55	100	90.13	nt
CMGAP	0	0	18.95	73.56	100	nt
CMAC	0	14.97	13.49	80.11	100	nt

Relative expressed values represented by calculated percentage of the best reaction from the same template included in the same PCR run. Values were means of 3-4 replicates for target genes with the standard error in parenthess. nt: not tested.

However, the relative expression level calculated using the C_t value did not completely correctly evaluate the performance of PCR reactions with different $MgCl_2$ concentrations. For *CMPI*, for instance, the relative expression level calculated using the C_t value were 89.18% and 100%, respectively, for 4 mM and 5 mM $MgCl_2$ (Table 5.4), while the real-time amplicon accumulation curves (Fig. 5.6) showed that the reaction with 5 mM $MgCl_2$ had a very short exponential phase and a low plateau level compared to that of the reaction containing 4 mM $MgCl_2$. This indicated that 5 mM $MgCl_2$ was inhibitory to the PCR reaction. Similar situations occurred for *CMAP1* and *CMAG*.

Therefore, considering the data for both the relative expression level and the real-time amplicon curve, the appropriate $MgCl_2$ concentrations were 3-4 mM for *CMPI* and *CMAG*, 4 mM for *CMAP1* and *CM18S* rRNA, 4-5 mM for *β-actin*, and 5 mM for *CMLFY* and *GAPDH*.

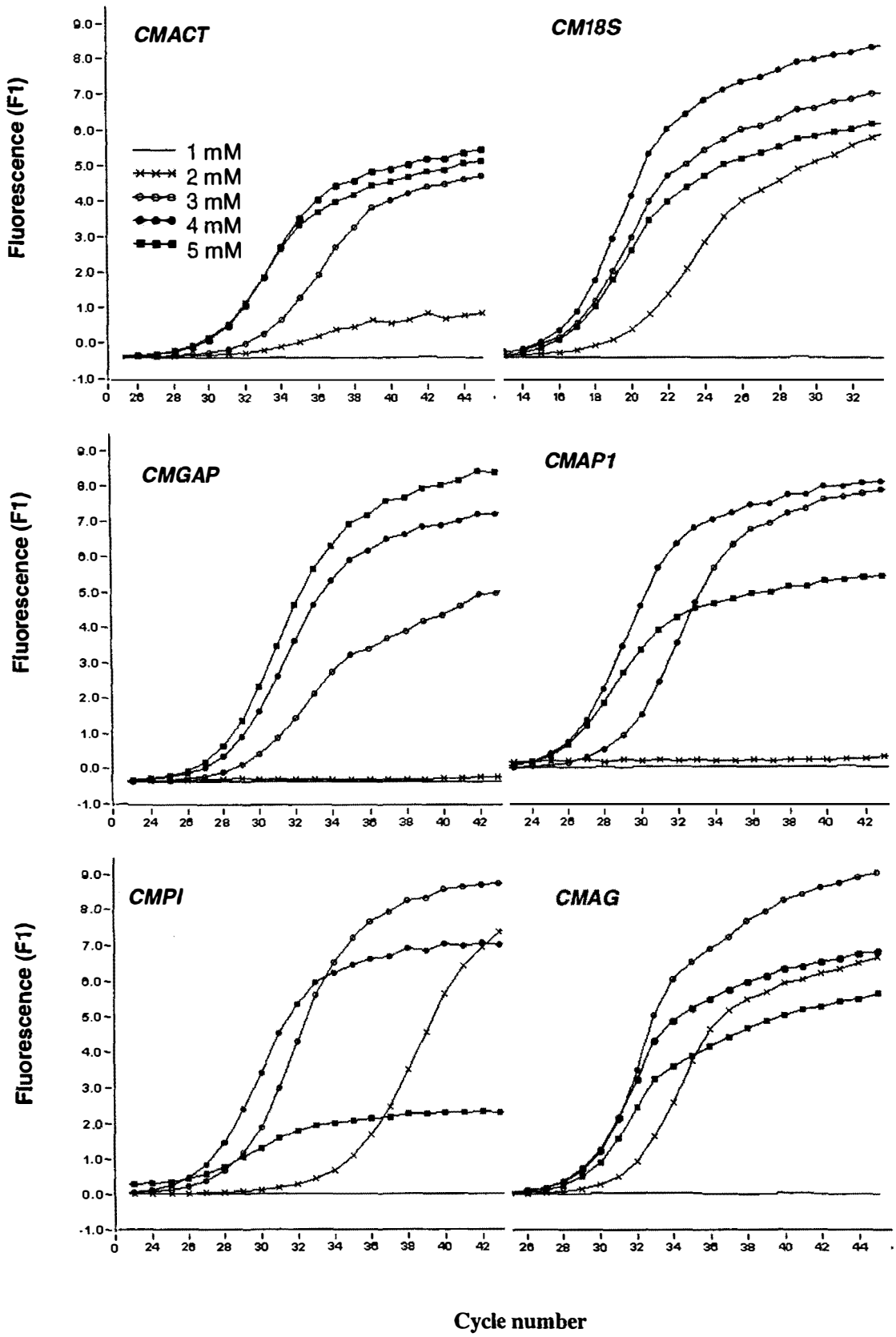

Figure 5.6 Effect of $MgCl_2$ concentration on PCR efficiency of *Clianthus* floral identity genes and housekeeping genes

An example of LightCylcer™ run for each gene showing the real time accumulation profiles of PCR products.

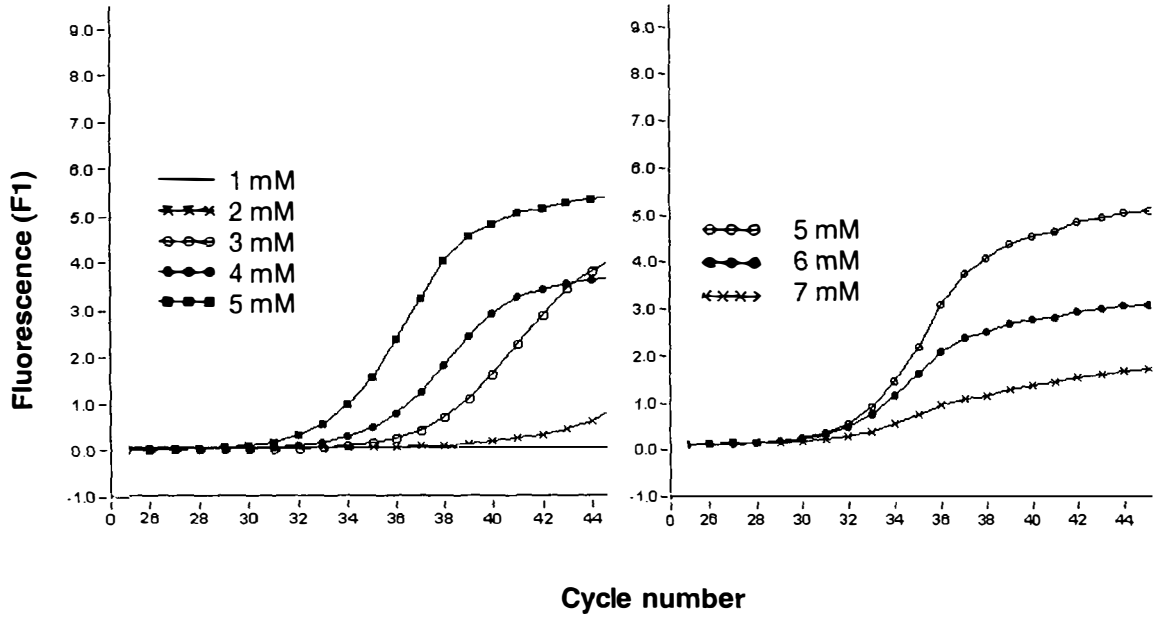

Figure 5.7 Effect of $MgCl_2$ concentration on LightCycler real-time PCR efficiency of the *CMLFY* gene

5.3.1.5 Primer pair selection for *Sophora* and *Clianthus* floral identity genes and selected housekeeping genes

The PCR efficiency of different primer combinations for the same gene can vary because of the difference in amplicon length, sequence, and annealing temperature of the primer pair. This can be reflected by both C_t value and end point amount of PCR product. For instance, different primer pairs for the *STLFY* gene (Fig. 5.8) showed that PCR performance could be drastically changed even if only one of the primers was replaced. Therefore, the primer pairs with the highest amplification efficiency should be the best choice for an individual gene. It should be noted that all housekeeping genes and the target gene included in the same experiment should have as similar a PCR efficiency as possible to facilitate the accurate comparison of their relative expression levels.

Therefore, the C_t values and the length and slope of the exponential phase of the amplicon accumulation curve were compared for several primer combinations of each gene, and the best, but also the most comparable primer pair was selected for further gene expression studies. They were *STLFY* F2/R3, *STAP1* F1/R1, *STP1* F1/R3, *STAG* F2/R3, *STAC* F3/R4, *STGAP* F3/R3, and *ST18S* F1/R1 for *Sophora*; and *CMLFY* F2/R3, *CMAP1* F1/R1, *CMP1*

F1/R3, *CMAG* F2/R3, *CMAC* F3/R4, *CMGAP F3/R3,* and *CM18S* F1/R1 for *Clianthus* (Table 5.5).

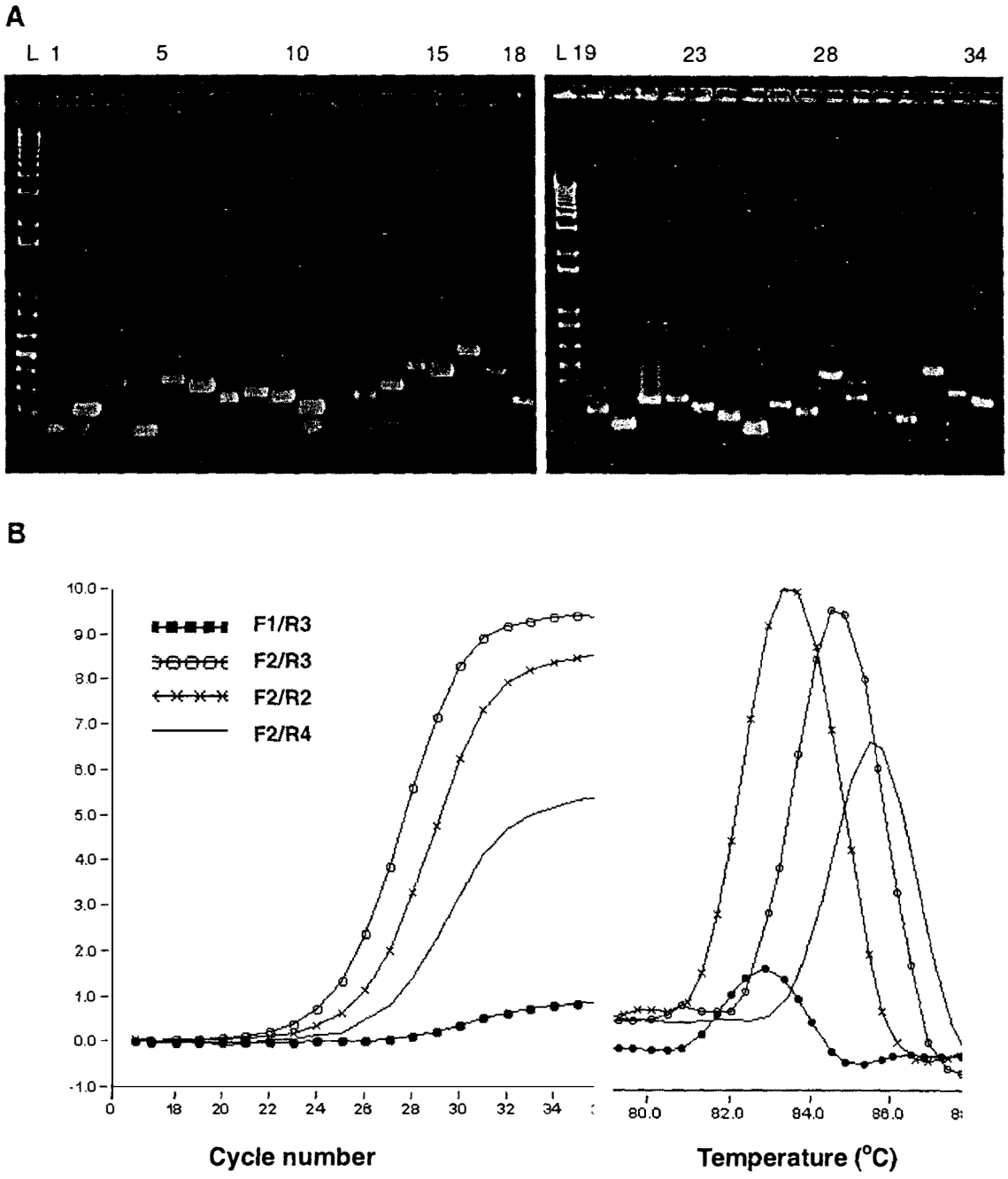

Figure 5.8 Real-time PCR performance of floral identity genes in *Sophora* and *Clianthus*

A: PCR products of different primer pairs of each floral identity genes. 1-4: *STLFY*; 5-6: *STAP1*; 7-8: *STPI*; 9-11: *STAG*; 12-14: *STACT*; 15-16: *STGAP*; 17-18: *ST18S*; 19-21:*CMLFY*; 22-23: *CMAP1*; 24-25: *CMAG*; 26-27: *CMPI*; 28-31: *CM18S*; 32: *CMGAP*; 33-34: *CMACT*.

B: PCR performance for different primer pairs of the *STLFY* gene (lanes 1-4 of part A). Lane 1: F1/R3; Lane 2: F2/R3; Lane 3: F2/R2; Lane 4: F2/R4. Left panels: LightCycler™ quantification curves showing the Ct values; Right panels: Melting curve peaks showing nature and amount of PCR products.

Table 5.5 Sequences of the selected real-time PCR primers for expression analysis of *Sophora* and *Clianthus* floral identity genes and selected housekeeping genes

Gene	Species	Forward (F) / reverse (R) primer sequences
LEAFY	*Sophora*	F2: 5' GTG AGT TCT TGA TTC AGG TT 3'
		R3: 5' CAG TGC ACG TAG TGT CGC ATT 3'
	Clianthus	F2: 5' ACG ATC AAT GCC GTG AGT TC 3'
		R3: 5' CAG TGC ACG TAG TGT CGC ATT 3'
APETALA1	*Sophora*	F1: 5' ATG CTG AGG TCG CTT TGA TTG 3'
		R1: 5' TCT AAC TGC TGC TCC AGA CTT TG 3'
	Clianthus	F1: 5' ATG CTG AGG TTG CTT TGA TTG 3'
		R1: 5' TCT AAC TGC TGT TCC AGA CTT TG 3'
PISTALLATA	*Sophora*	F1: 5' GTT CTA TGT GAT GCT CAA GTT TC 3'
		R3: 5'GTG CCT GAG CTC AAT TTG CAT 3'
	Clianthus	F1: 5' AGAACA CAA GCA ACA GGC AAG T 3'
		R3: 5'GTG CCT GAG CTC AAT TTG CAT 3'
AGAMOUS	*Sophora/Clianthus*	F2: 5' TGG TCG CCT CTA TGA ATA TGC TA 3'
		R3: 5'GTT TGT CTG CTT CTT GCT GGT AA 3'
β-Actin	*Sophora*	F3: 5' GAG CTA TGA GTT ACC TGA TGG ACA 3'
		R4: 5' GTA ATC TCC TTG CTC ATC CTA TCA 3'
	Clianthus	F3: 5' GAG CTA TGA GCT GCC TGA TGG ACA 3'
		R4: 5' GTA ATC TCC TTG CTC ATC CTA TCA 3'
GAPDH	*Sophora/Clianthus*	F3: 5' ATG ACA GRT TTG GMA TTG TTG A 3'
		R3: 5' TGC CCT CAG AYT CYT CCT TGA 3'
18S rRNA	*Sophora/Clianthus*	F1: 5' TAC CGT CCT AGT CTC AAC CAT AA 3'
		R1: 5' AGA ACA TCT AAG GGC ATC ACA 3'

5.3.1.6 PCR amplification efficiency determination for selected genes

A number of variables can affect the efficiency of the real-time PCR. These factors include length of the amplicon, secondary structure, and primer quality. Therefore, for comparison of expression levels between different genes of interest, experiments, and LightCycler™ runs, the PCR efficiency for each gene should be determined based on the standard curve by serial dilution of templates.

In the current study, a serial dilution of the same first strand cDNA was used as template for LightCycler™ reactions to determinate the amplification efficiency of each of the target genes and housekeeping genes. Only the replicates with acceptable assay accuracy and error level ($r > 0.99$, error<0.1) were used for efficient calculation. All the selected primer pairs for the target and housekeeping genes for further expression investigation had an PCR efficiency of 0.85 and over, ranging from 0.865 for *STLFY* and 0.973 for *ST18S* rRNA (Table 5.6).

Table 5.6 PCR efficiencies of floral identity genes and selected housekeeping genes

Gene	Slope	Coefficient	Intercept	Error	Efficiency
STAP1	-3.664	-1.00	30.90	0.0939	0.875
CMAP1	-3.646	-1.00	29.10	0.0635	0.8805
STPI	-3.624	-1.00	27.32	0.0237	0.888
CMPI	-3.540	-0.99	26.38	0.033	0.916
STAG/CMAG	-3.497	-1.00	25.15	0.050	0.932
STLFY	-3.696	-0.99	22.49	0.090	0.865
CMLFY	-3.662	-1.00	27.25	0.072	0.875
ST18S/CM18S	-3.389	-1.00	10.09	0.078	0.973
GAPDH	-3.663	-1.00	24.46	0.0127	0.875
STAC	-3.543	-0.99	23.32	0.052	0.915
CMAC	-3.566	-1.00	24.38	0.040	0.907

A serial dilution of 10-, 100-, 1000-, 10,000- and 100,000- fold of the same first strand cDNA was used as template for LightCycler™ reactions of each gene.

5.3.1.7 Controlling sample variation for gene expression quantification

Experimental results showed that non-genetic variation of gene expression could be introduced by errors during RNA isolation, cDNA synthesis, PCR runs, and PCR reactions. A 2.5-fold variation of RNA yield was observed among the same batch of extractions from the same tissue sample. The quality of this RNA also varied. A final variation of 44% in terms of relative cDNA concentration was revealed by real-time PCR, although the same amount of total RNA was used for cDNA synthesis. However, different cDNAs synthesized from the same RNA gave more homogenous results, with relative variation no greater than 20%. Furthermore, in two sets of triplicate PCR reactions, the variation remained less than 10% in the same PCR run, and between two PCR runs (Fig. 5.9, Fig. 5.10). This result suggests that the quality of RNA plays the most important role in introducing sample variations, while the variations produced during the reverse transcription could be easily controlled by adding the same amount of total RNA. Also, variations among PCR runs and PCR reactions are much less important compared to that of RNA samples.

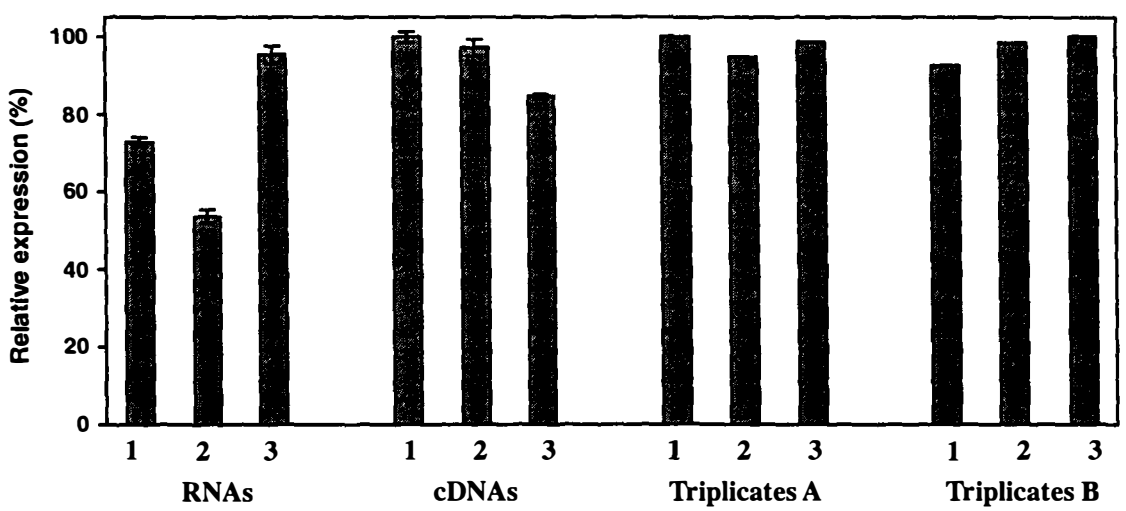

Figure 5.9 Comparison of variation during different steps of gene quantification

Three RNAs isolated from the same floral tissue sample of *Sophora*. Three cDNA synthesized using the same amount of RNA determined with the NanoDrop instrument. Two sets A and B) of triplicate PCR reactions run separately in the LightCycler™ instrument.

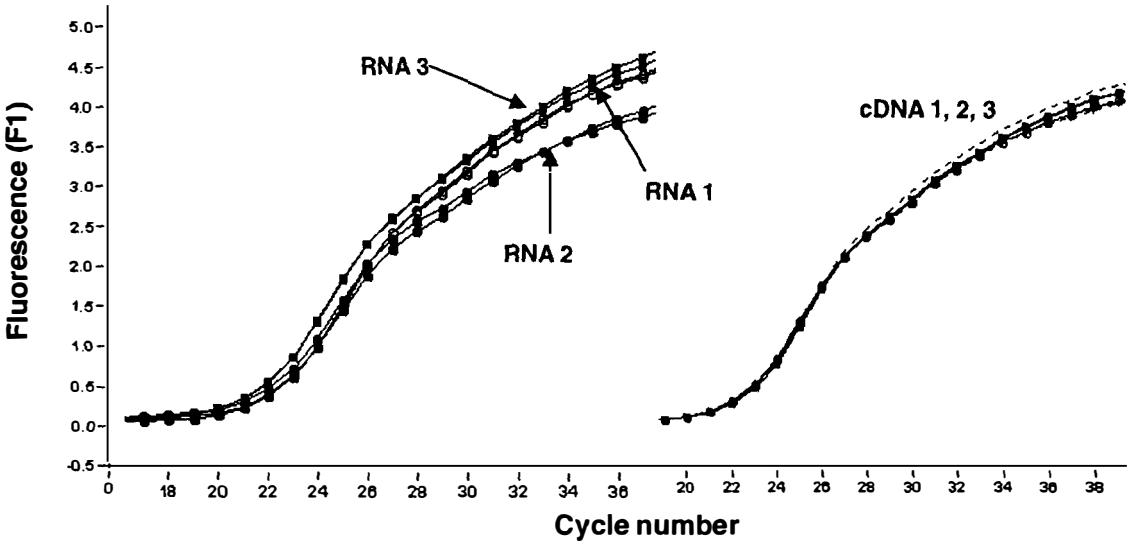

Figure 5.10 Variation of LightCycler quantification curves resulted from three different RNA extractions (left, with duplicate PCR reactions) and three cDNA synthesis reactions (right) included in the same PCR run

5.3.2 Determination of appropriate housekeeping genes as internal controls

To choose the appropriate internal control genes for normalizing target gene expression using real-time RT-PCR, the expression variability of the most commonly used housekeeping genes, *18S rRNA*, *β-actin*, and *GAPDH* homologues was evaluated. Two of the four target genes, *LFY* and *AG* homologues, were themselves chosen as "controls" for housekeeping gene expression since they were generally highly expressed (mainly during early and late developmental stages, respectively) across a broad spectrum of developmental stages. Homologues of these genes for both *Sophora* and *Clianthus* were isolated as described in Chapter 4.

5.3.2.1 Stability of selected housekeeping genes in different developmental stages of *Sophora* floral tissues

In order to test the expression stability of housekeeping genes *ST18S*, *STACT*, and *STGAP*, *Sophora* floral tissues at three development stages that reflected different levels of target gene expression were used. These were inflorescences 10-15 mm in length bearing floral

primordia, early stage flower buds 2-3 mm in length, and mid-stage flower buds 18-20 mm in length showing petal tips.

Results based on the C_t values (Table 5.7) showed that the expression level of the target control gene *STAG* varied significantly among different developmental stages ($P < 10^{-6}$). The mean C_t values were 27.38, 25.25, and 22.12 for inflorescences, early stage flower buds and mid-stage flower buds, respectively, representing a 38.3-fold difference in expression level. The expression level of all three tested housekeeping genes also varied significantly among different developmental stages, with $P < 10^{-5}$ for *ST18S* and *STGAP*, and $<10^{-6}$ for *STACT*. The difference of C_t values among different developmental stages reflected a 24.8-fold, 17.5-fold and 16.2-fold difference of expression level for *ST18S*, *STACT*, and *STGAP*, respectively.

Variation between replicates across three developmental stages was not significant for *ST18S* and *STGAP* but was significant for *STAG* and *STACT* ($P < 0.05$ and 0.01, respectively) (Table 5.7). In all cases, the variation between replicates in C_t value was much smaller than that between developmental stages. However, even when the differences in C_t value were not statistically significant, the variation of expression level between replicates was in most cases a 2- to 4-fold difference for all developmental stages (Table 5.7, Fig. 5.11). For instance, the relative expression of *ST18S* among four replicates was 50.35-100% for inflorescences, 28.32-100% for early-stage flower buds, and 38.42-100% for mid-stage flower buds. In general, no obvious difference of expression stability between the three housekeeping genes was observed. Although the variation of *STGAP* was smaller than that of *ST18S* and *STACT* for inflorescences and early-stage flower buds (Fig. 5.11, A, B), it varied the most for mid-stage flower buds (Fig. 5.11, C). In terms of variation between replicates, there was not a remarked difference between housekeeping genes and the target gene *STAG*.

If the mRNA concentration of the target gene *STAG* at the stage of mid-stage flower buds was designated as 100%, the expression levels relative to it were 2.42% and 10.05% for inflorescences and early stage flower buds, respectively (Fig. 5.12). In general, the expression of the three housekeeping genes showed a similar pattern to that of the target gene, with some variation both within and among developmental stages. When the expression of the three housekeeping genes was adjusted using the normalization factors (GMNF) calculated based on their geometric mean (Table 5.8) and then combined, the variation between replicates within the same developmental stage was reduced. However,

variation between developmental stages was not greatly changed (Fig. 5.12).

When *STAG* expression was normalized by each individual housekeeping gene, none of these genes was capable of eliminating the expression variation in expression of *STAG*, either within the same developmental stage or among developmental stages (Fig. 5.13, B-D). This could even enlarge the within developmental stage variation (shown by the size of error bars representing standard error) and among developmental stage variations. In some cases, even the expression order of replicates could be altered. However, when *STAG* expression was normalized by the normalization factor (GMNF, Table 5.8) based on the geometric mean of the three housekeeping genes, a much better result than the use of any of the housekeeping gene alone was obtained. The result was clearly smaller within stage variation and relative variation among different developmental stages (Fig. 5.13, E).

In summary, the variation ranges of housekeeping gene expression were comparable to that of the target gene *STAG* among replicates within the same developmental stage. However, the variation of housekeeping gene expression was much smaller than that of the target gene across different developmental stages. The geometric mean of three housekeeping genes could reduce variation between replicates within the same developmental stage. While no completely satisfactory result was obtained to remove expression variation of the target gene using individual housekeeping genes as normalizer, the normalization factor based on the geometric mean of three housekeeping genes did reduce the expression variation of *STAG*.

Table 5.7 Comparison of gene expression (C_t) stability within the same tissue sample for *Sophora* flowers at different developmental stages

Genes	Samples	Replicates				Mean	SE	P value
		r1	r2	r3	r4			
ST18S	I	20.21	20.26	19.27	19.90	19.91	0.228	$6.83*10^{-5}$
	EF	23.60	23.62	22.32	21.80	22.84	0.460	
	MF	18.31	18.30	17.42	18.80	18.21	0.287	
	Mean	20.71	20.73	19.67	20.17			
	SE	1.547	1.553	1.429	0.876			
	P value	0.162						
STACT	I	26.15	26.12	25.81	24.90	25.75	0.292	$4.09*10^{-6}$
	EF	25.42	25.14	25.56	23.80	24.98	0.403	
	MF	22.03	22.01	21.34	21.10	21.62	0.236	
	Mean	24.53	24.42	24.24	23.27			
	SE	1.269	1.239	1.450	1.129			
	P value	0.010						
STGAP	I	21.68	21.60	21.27	22.00	21.64	0.150	$2.14*10^{-5}$
	EF	20.61	20.56	20.49	20.50	20.54	0.028	
	MF	17.44	17.53	16.80	18.70	17.62	0.396	
	Mean	19.91	19.90	19.52	20.40			
	SE	1.273	1.221	1.379	0.954			
	P value	0.169						
STAG	I	28.03	27.90	26.80	26.80	27.38	0.340	$4.52*10^{-7}$
	EF	25.62	25.80	24.80	24.90	25.28	0.250	
	MF	22.07	22.50	21.90	22.00	22.12	0.132	
	Mean	25.23	25.40	24.50	24.57			
	SE	1.713	1.572	1.422	1.396			
	P value	0.013						

I: inflorescences; EF: early stage flower buds 2-3 mm in length, organogenesis complete; MF: mid-stage flower buds 18-20 mm in length showing petal tip; SE: standard error of the mean based on four replicates.

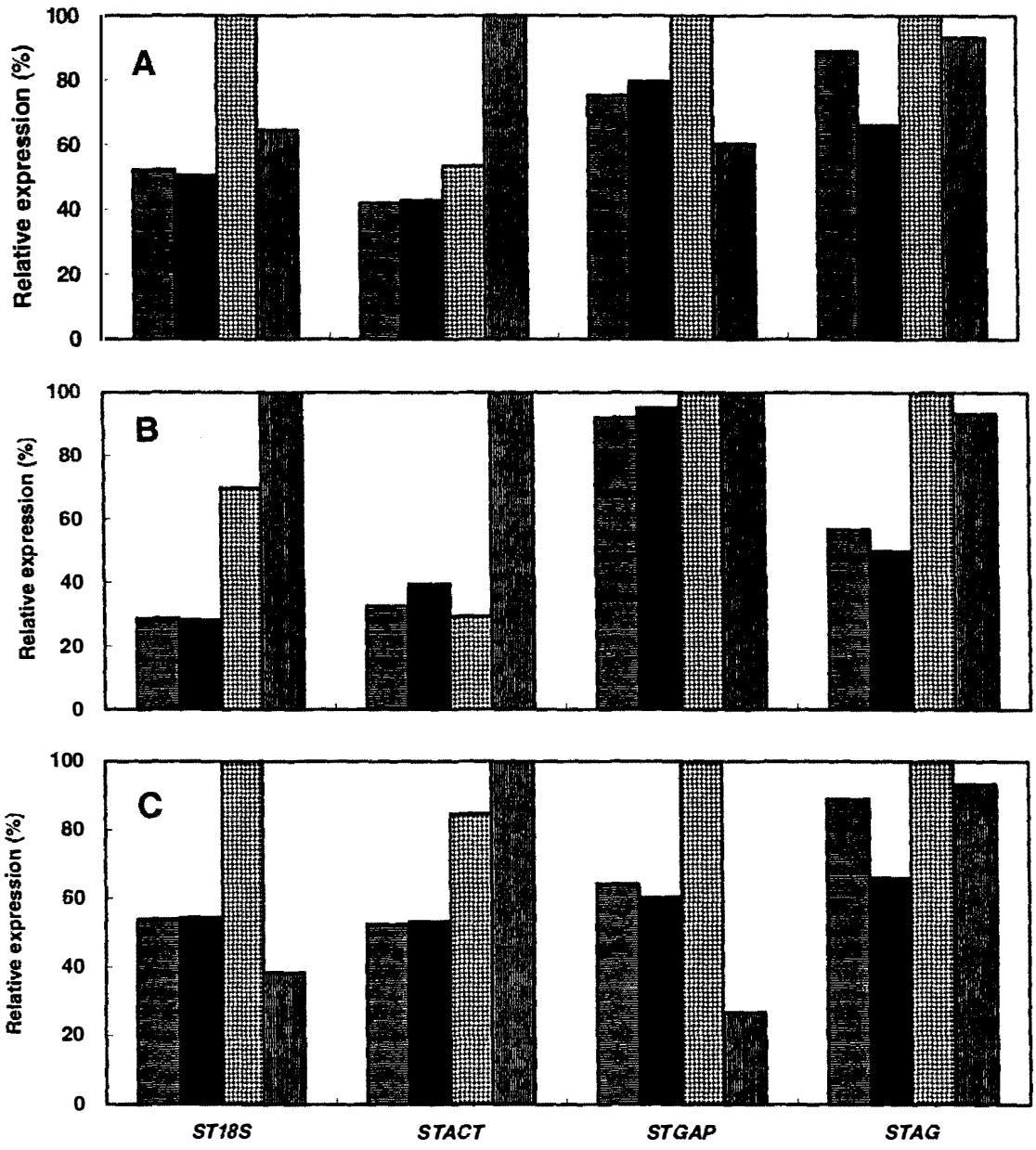

Figure 5.11 Variation in relative expression of three housekeeping genes and one target gene within the same tissue sample for *Sophora* flowers at different developmental stages

A: Inflorescence with flower primordia; B: Early stage flower bud 2-3 mm in length; C: Mid-stage flower buds showing petal tip. The same cDNA template for each tissue type was used for all genes with four replicates (columns within the same gene).

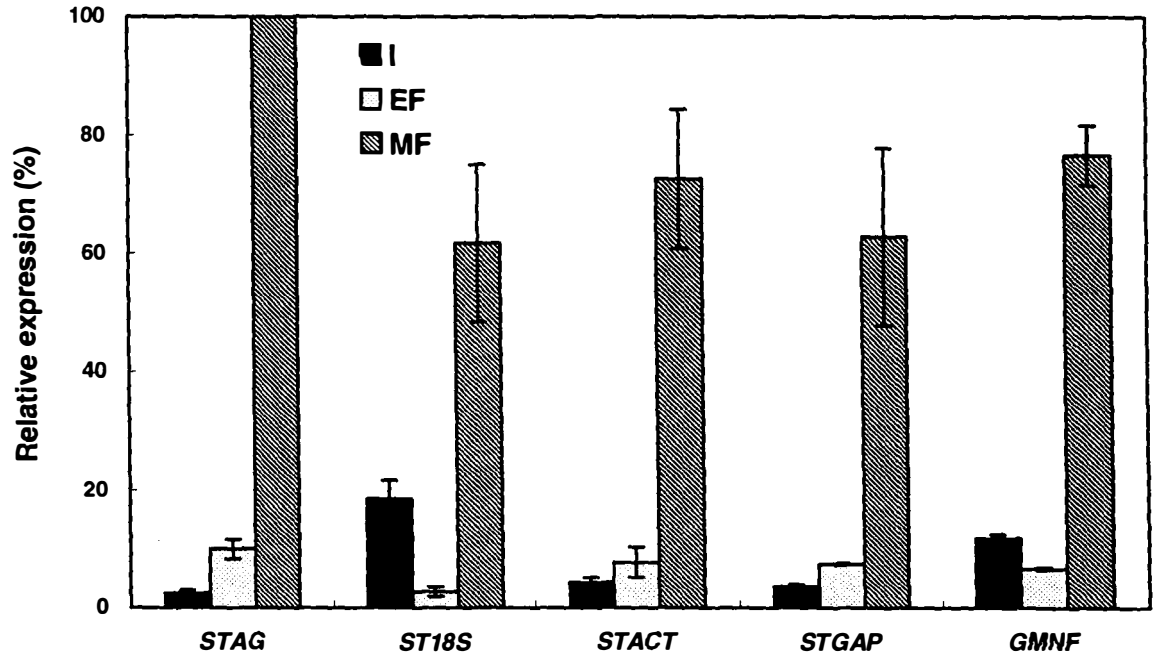

Figure 5.12 Expression variation of *Sophora* housekeeping genes at different developmental stages

I: Inflorescences; EF: Early stage flower buds 2-3 mm in length; MF: Mid-stage flower buds showing petal tip. GMNF: Geometric mean normalized expression of three housekeeping genes; Error bars were standard errors based on four replicates.

Table 5.8 Normalization factors for *Sophora* and *Clianthus* tissue samples calculated based on geometric mean of housekeeping genes 18S, actin and GAPDH

Replicates	*Sophora*			*Clianthus*		
	I	EF	MF	I	EF	MF
r1	1.011	1.018	1.005	1.010	1.025	1.012
r2	1.010	1.014	1.006	1.022	0.999	1.003
r3	0.985	1.000	0.966	0.968	0.977	0.986
r4	0.994	0.969	1.023			

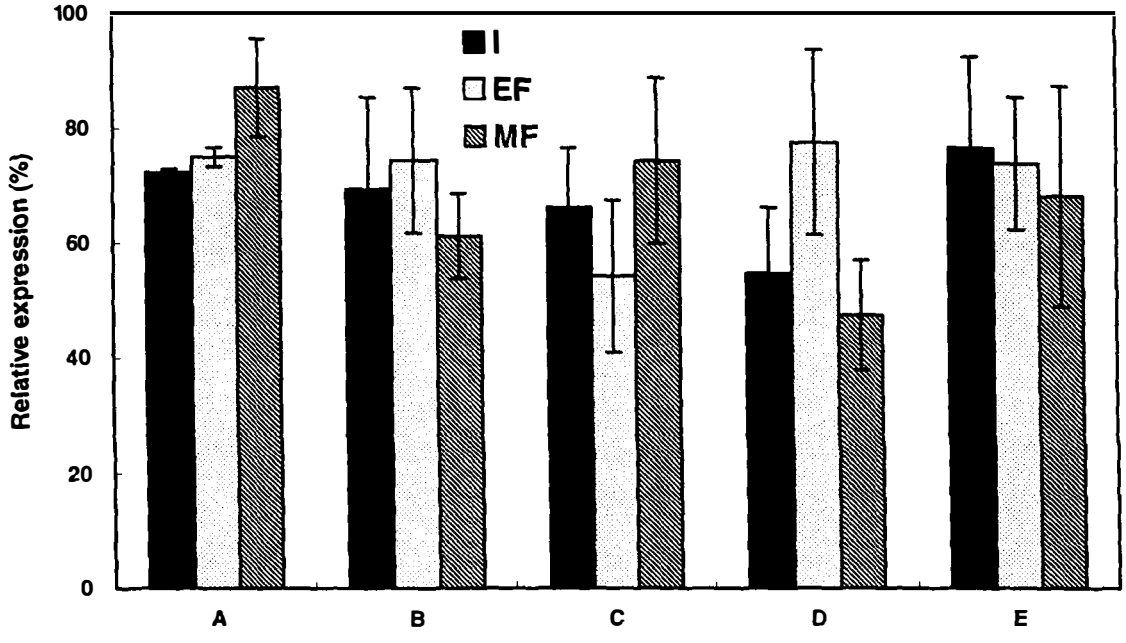

Figure 5.13 Normalization effect on *STAG* expression using different housekeeping genes for *Sophora* of different developmental stages

I: Inflorescences; EF: Early stage flower buds 2-3 mm in length; MF: Mid-stage flower buds showing petal tips. A: No normalized; B: Normalized using *ST18S*; C: Normalized using *STACT*; D: Normalized using *STGAP*; E: Normalized using geometric average of three housekeeping genes; Error bars were standard errors based on four replicates.

5.3.2.2 Stability of selected housekeeping genes in different developmental stages of *Clianthus* floral tissues

Similar to those for *Sophora*, samples representing different expression levels of the target gene *CMLFY*, including inflorescences 1-2 mm in length, early-stage flower buds 2-3 mm in length, and mid-stage flower buds 10-12 mm in length showing petal tips, were used for *Clianthus* to evaluate the expression stability of the housekeeping genes *CM18S, CMACT,* and *CMGAP,* and their efficiency in normalizing the expression of target genes in a relatively broad developmental range.

The mean C_t values of the target gene *CMLFY* among different developmental stages were 25.77, 25.20, and 29.93 for inflorescences, early-stage flower buds and mid-stage flower buds, respectively, representing a 26.6-fold difference in expression level. These variations were highly significant ($P < 10^{-11}$). The expression level of all three tested housekeeping genes also varied significantly among different developmental stages, with $P < 0.05$, 0.01 and 10^{-4} for *CM18S, CMACT,* and *CMGAP*, respectively. The difference in

C_t values among different developmental stages reflected 4.6-, 6.4- and 2.7-fold expression changes for *CM18S*, *CMACT*, and *CMGAP*, respectively (Table 5.9).

Table 5.9 Comparison of gene expression stability (C_t) within the same tissue sample for *Clianthus* at different developmental stages

Genes	Samples	Replicates			Mean	SE	P value
		r1	r2	r3			
CM18S	I	16.29	16.49	14.93	15.90	0.503	0.015
	EF	18.03	16.45	15.82	16.77	0.649	
	MF	18.45	18.32	17.48	18.10	0.306	
	Mean	17.60	17.10	16.07			
	SE	0.666	0.600	0.762			
	P value	0.050					
CMACT	I	23.51	23.55	21.67	22.93	0.617	0.004
	EF	22.40	22.24	21.73	22.10	0.208	
	MF	24.90	25.08	24.32	24.77	0.240	
	Mean	23.60	23.63	22.57			
	SE	0.723	0.837	0.867			
	P value	0.060					
CMGAP	I	19.52	19.50	19.23	19.40	0.100	$7*10^{-5}$
	EF	18.82	18.78	18.50	18.70	0.100	
	MF	20.30	20.30	19.77	20.13	0.167	
	Mean	19.53	19.53	19.17			
	SE	0.433	0.433	0.376			
	P value	0.008					
CMLFY	I	25.35	26.20	25.70	25.77	0.233	$2*10^{-12}$
	EF	25.32	25.30	25.00	25.20	0.100	
	MF	30.26	29.20	30.30	29.93	0.367	
	Mean	27.00	26.90	27.00			
	SE	1.650	1.179	1.662			
	P value	0.749					

SE: Standard error based on four replicates. I: Inflorescences; EF: Early stage flower buds 2-3 mm in length; MF: Mid-stage flower buds showing petal tips.

While variation among replicates across three developmental stages was significant for *CMGAP*, no significant variation was observed for *CMLFY*, *CM18S*, and *CMACT* (Table 5.9). However, the actual difference was still remarkable despite the lack of statistical significance, both across developmental stages and among replicates within the same

stage (Table 5.9, Fig. 5.14). For *CM18S,* for instance, the relative expression levels among the three replicates were 34-100% for inflorescences, 22-100% for early-stage flower buds, and 51-100% for mid-stage flower buds. Similar variation levels were observed for *CMACT.* However, the variation was smaller for *CMGAP,* with the relative expression levels among the three replicates being 80-100% for inflorescences and early-stage flower buds, and around 70-100% for mid-stage flower buds. The expression variation for the target gene *CMLFY* was not evidently different from those of housekeeping genes (Fig. 5.14).

The highest mRNA concentration of the target gene *CMLFY* was detected at the early-stage flower buds, while those for inflorescences and mid-stage flower buds were about 70% and 4% of the highest level, respectively (Fig. 5.15). Similar patterns of expression trend of three housekeeping genes were observed, with remarkable variation both within and among developmental stages. However, when the expression of the three housekeeping genes was adjusted using the normalization factors (GMNF) calculated based on their geometric mean (Table 5.8) and combined, the variation between replicates within the same developmental stage was greatly reduced.

Normalization of *CMLFY* expression by individual housekeeping genes could not remove its variation, either within the same developmental stage or among developmental stages (Fig. 5.16, B-D). In fact, they even enlarged the variation within the same developmental stage (showed by the length of error bars representing the standard errors) and, in most case, among developmental stages. Normalization of *CMLFY* expression using the normalization factor GMNF (Table 5.8) provided a better result than when a single housekeeping gene was used (Fig. 5.16, E).

In summary, the variation in expression of housekeeping genes was similar to that of the target gene among replicates within the same developmental stage, while the variation in expression of housekeeping genes across different developmental stages were much smaller than that of the target gene *CMLFY*. Although no completely satisfactory result was obtained for removing the expression variation of the target gene by normalizing an individual or a combination of three housekeeping genes, the geometric mean of three housekeeping genes could reduce the variation of housekeeping genes themselves between replicates within the same developmental stage.

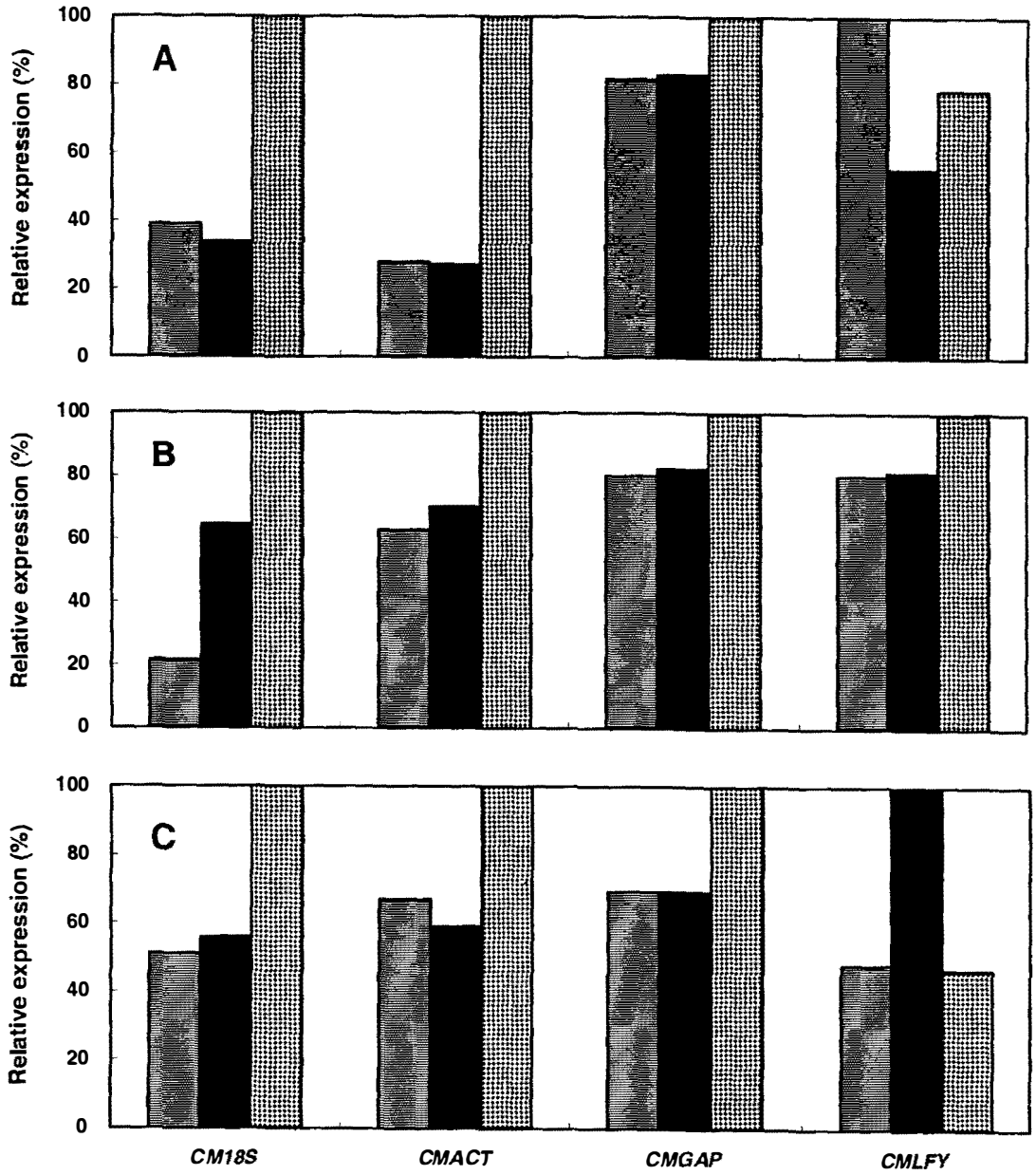

Figure 5.14 Variation in relative expression of three housekeeping genes and one target gene within the same tissue sample for *Clianthus* at different developmental stages

A: Inflorescences 1-2 mm in length; B: Early stage flower buds 2-3 mm in length; C: Mid-stage flower buds showing petal tips. The same cDNA template for each tissue type was used for all genes with three replicates (columns within the same gene).

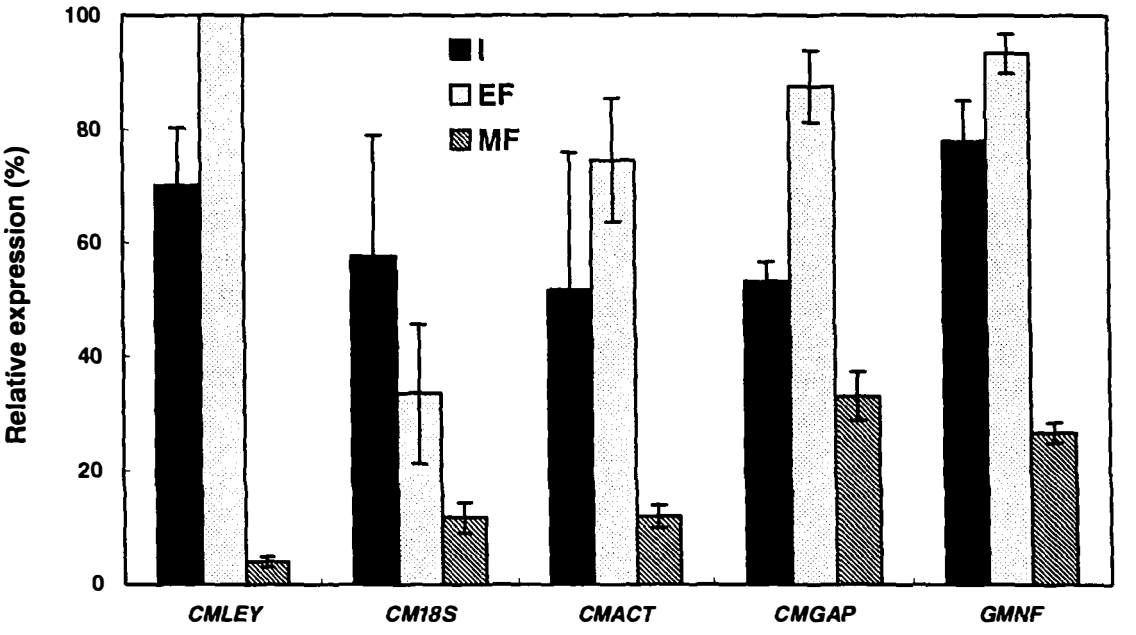

Figure 5.15 Expression variation of *Clianthus* housekeeping genes at different developmental stages

I: Inflorescences; EF: Early stage flower buds 2-3 mm in length; MF: Mid-stage flower buds showing petal tips. GMNF: Geometric mean normalized expression of three housekeeping genes; Error bars were standard errors based on three replicates.

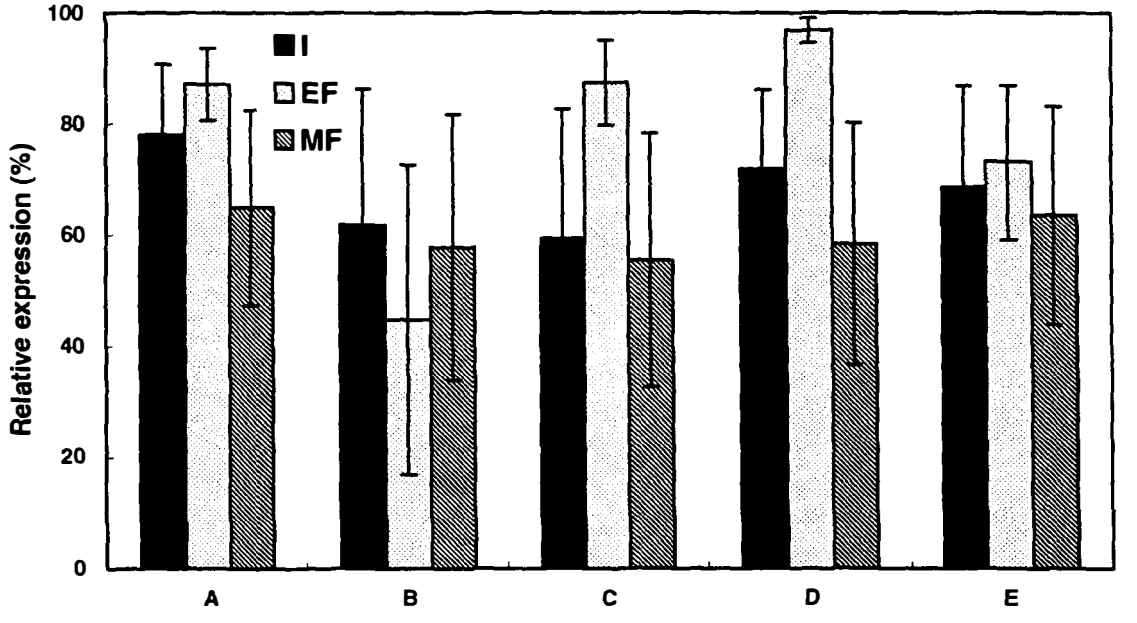

Figure 5.16 Normalization effect on *CMLFY* expression using different housekeeping genes for *Clianthus* of different developmental stages

I: Inflorescences; EF: Early stage flower buds 2-3 mm in length; MF: Mid-stage flower buds showing petal tips. A: No normalized; B: Normalized using *CM18S*; C: Normalized using *CMACT*; D: Normalized using *CMGAP*; E: Normalized using geometric average of three housekeeping genes; Error bars were standard errors based on three replicates.

5.3.3 Expression characteristics of floral identity genes in different tissue types of *Sophora* and *Clianthus*

As characterized by direct sequence comparison described in Chapter 4, the newly isolated cDNA fragments are most probably homologues of floral identity genes, *LFY, AP1, PI,* and *AG* for *Sophora* and *Clianthus*. The most interesting questions to be asked for this study were: In which part of the plant are these genes expressed? And if they express in floral tissues, do they express only in the specific floral organs defined by their role of floral identity? To answer these two questions, quantitative expression of four floral identity genes in a variety of vegetative and reproductive tissues, and then in different floral organs of *Sophora* and *Clianthus* were analysed using real-time PCR assay.

5.3.3.1 Expression of floral identity genes in different vegetative and reproductive tissues of *Sophora*

Vegetative tissues including adult leaves and shoot tips, and reproductive tissues including inflorescences 2-3 mm in length, early stage flower buds 2-3 mm in length, mid-stage flower buds 18-20 mm in length, and seed pods 2-3 weeks after flowering were used in this experiment. Inflorescences were sampled in November 2002. Early stage flower buds, mid-stage flower buds, and seed pods were harvested in July, September and October 2003, respectively. Adult leaves and shoot tips were harvested in January 2003, at least one month after the end of the inflorescence initiation period to avoid the contamination of inflorescence primordia in the shoot tips. Result showed that there was a distinct expression profile for each gene, represented by relative mRNA concentration among different tissue samples (Fig. 5.17).

STLFY activity was detected in all tested tissues. The expression level was very low in adult leaves and young seed pods, representing 0.08% and 0.07% of its peak level. Low level of expression (5.1%) was also detected in vegetative shoot tips. Compared to its highest level of expression in early stage flower buds, *STLFY* expressed at 16.2% in inflorescences, and at 0.17% in mid-stage flower buds.

In contrast to *STLFY*, no *STAP1* activity was detected in adult leaves, vegetative shoot tips, and seed pods. Similar to *STLFY, STAP1* expressed at 16.2% in inflorescences

compared to the highest expression level in early stage flower buds. Only low level (6.2%) of *STAP1* activity was detected in mid-stage flower buds.

The highest *STPI* expression was detected in mid-stage flower buds, while the expression levels in early stage flower buds and inflorescence were 38% and 0.6% of the highest expression, respectively. Similar to *STAP1,* no *STPI* activity was detected in leaves, vegetative shoot tips, and seed pods.

Similar to *STPI*, the highest *STAG* expression was detected in mid-stage flower buds. However, high *STAG* expression level (74%) was also detected in early stage flower buds while low expression (1.2%) was detected in inflorescences. At for *STAP1* and *STPI,* no *STAG* activity was detected in leaves and vegetative shoot tips. However, *STAG* also expressed in seed pods at 2.1% of its highest level, being 30-fold higher than that of *STLFY*, the only other gene expressed in seed pods.

In summary, only *STLFY* activity was detected in leaves and vegetative shoot tips, and both *STLFY* and *STAG* expressed in seed pods, all at low levels. The highest expression was detected in early stage flower buds for *STLFY* and *STAP1,* and in mid-stage flower buds for *STPI* and *STAG*. In expressed tissues types, expression levels of each gene varied 17- to 1300-fold between different tissue types.

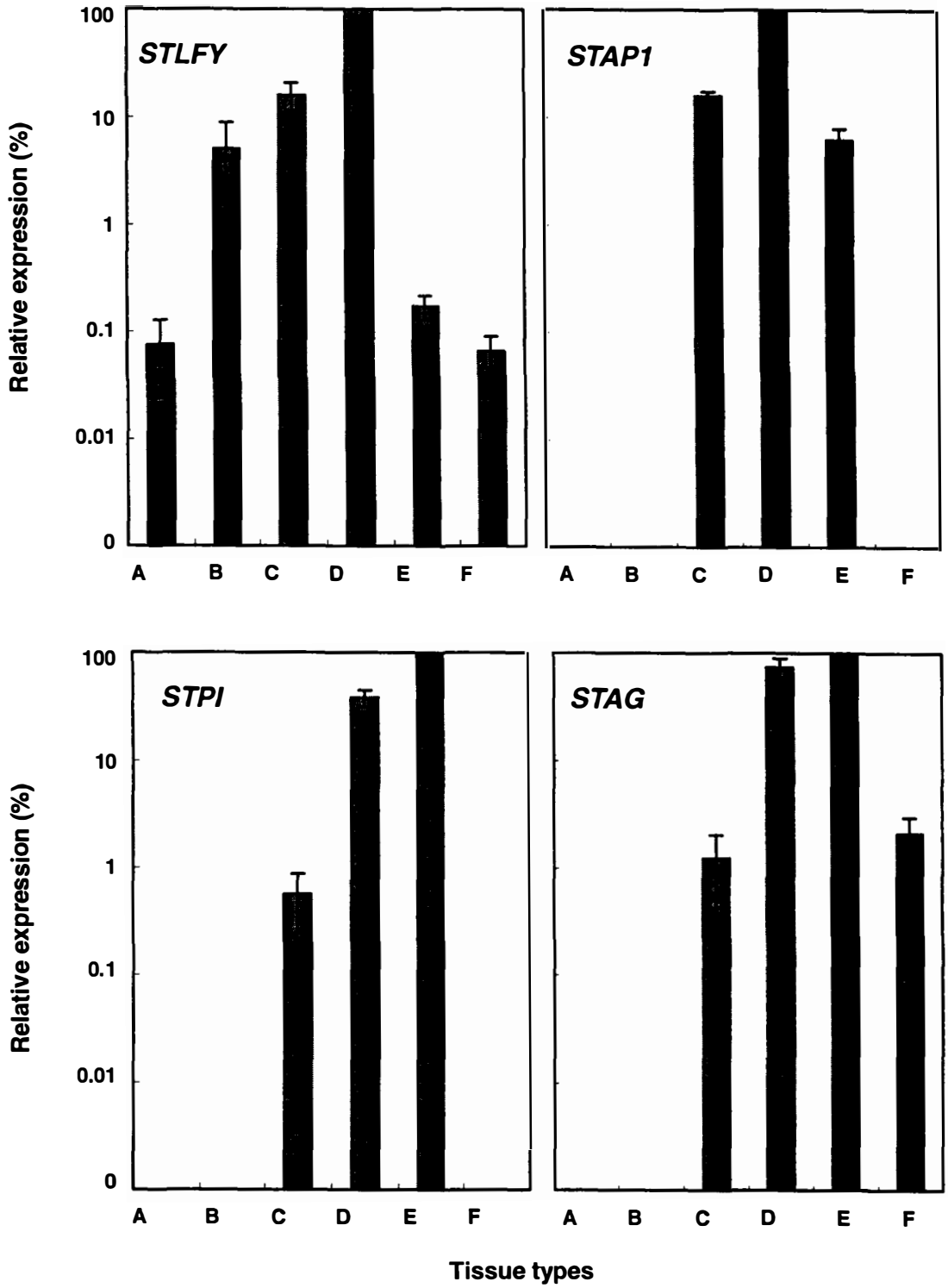

Figure 5.17 Relative expression (logarithmized scale) of four *Sophora* floral identity genes in vegetative and reproductive tissues

A: Leaves; B: Vegetative shoot tips; C: Inflorescences 2-3 mm in length; D: Early stage flower buds of 2-3 mm in length; E: Mid-stage flower buds 18-20 mm in length; F: Young legumes 2-3 weeks after flowering.

5.3.3.2 Expression of floral identity genes in different vegetative and reproductive tissues of *Clianthus*

Tissue types comparable to those of *Sophora* were tested for the four *Clianthus* floral identity genes using real-time PCR assay. Those used were shoot tips, adult leaves, and inflorescences harvested in May 2003, and early stage flower buds, mid-stage flower buds, and seed pods sampled in July, September and October 2003, respectively. As for their *Sophora* homologues, a distinct expression profile was obtained for each gene (Fig. 5.18).

All tested tissues had *CMLFY* expression at relative mRNA levels from 1.7% in seed pods, to 100% in early stage flower buds. Low *CMLFY* activity was also detected in leaves and vegetative shoot tips, representing 2-3% of its mRNA level in floral primordia. *CMLFY* also expressed at high level (45%) in inflorescences, although at a low level (7%) in mid-stage flower buds.

No *CMAP1* activity was detected in adult leaves, vegetative shoot tips, and seed pods. Apart from its highest expression level in early stage flower buds, *CMAP1* also expressed at a high level (82%) in mid-stage flower buds. A *CMAP1* mRNA level of 9% of its highest expression was detected in inflorescences.

CMPI expression was not detected in leaves, vegetative shoot tips, and seed pods. In comparison with mid-stage flower buds in which *CMPI* expressed at its highest level, only 19% and 1.5% mRNA were detected in early stage flower buds and inflorescences, respectively.

The highest *CMAG* expression was detected in mid-stage flower buds, whereas 35% and 2.4% relative expression were detected in early stage flower buds and inflorescences, respectively. No *CMAG* expression was detected in adult leaves and vegetative shoot tips. However, a *CMAG* expression as high as 35% was detected in seed pods.

In summary, only *CMLFY* expression was detected in leaves and vegetative shoot tips. While all the four genes expressed at different level in inflorescence and floral buds, the highest expression was detected in early stage flower buds for *CMLFY* and *CMAP1*, and in mid-stage flower buds for *CMPI* and *CMAG*. Both *CMLFY* and *CMAG* activity were detected in seed pods, at much higher level than those for their *Sophora* homologues.

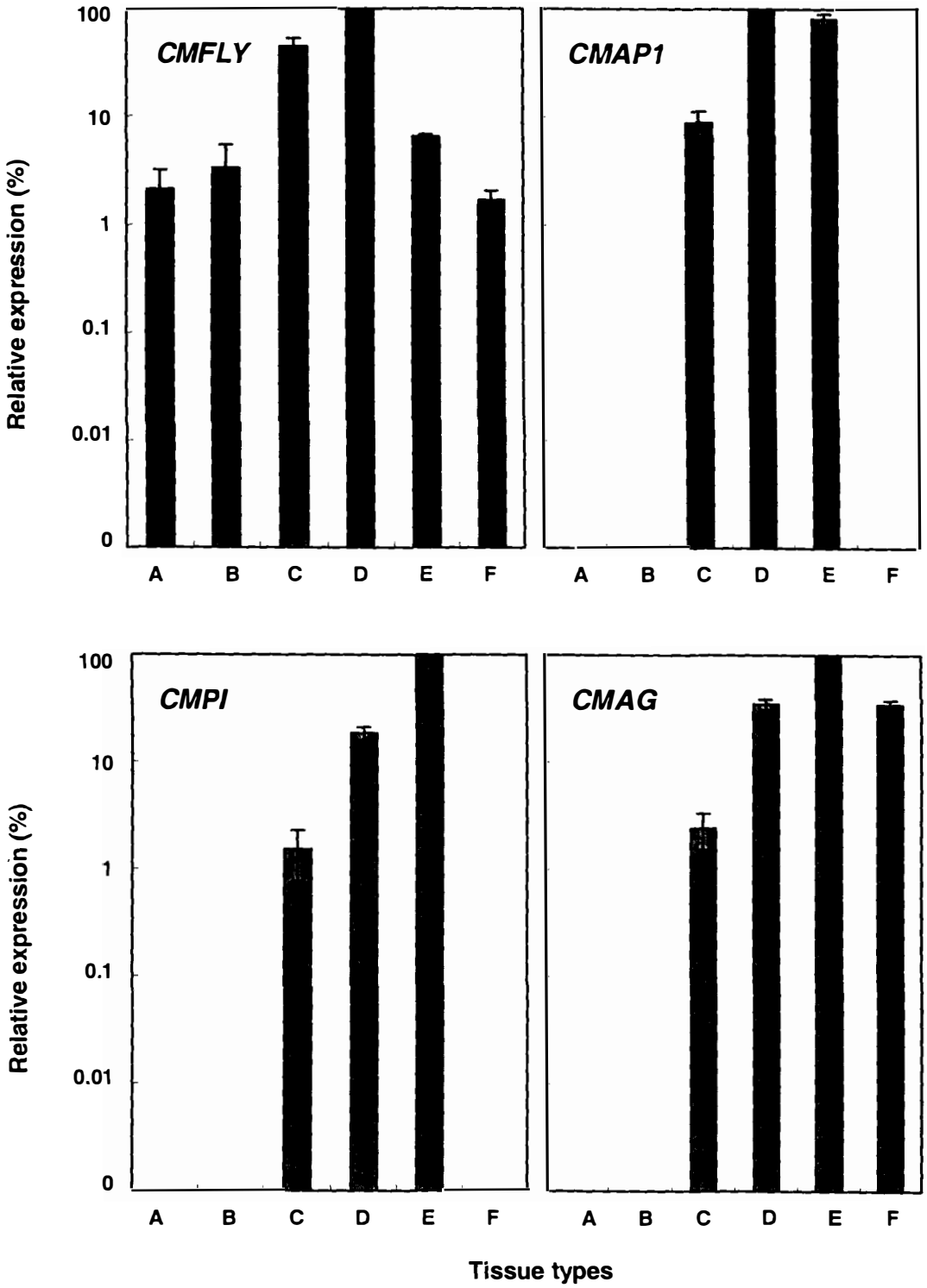

Figure 5.18 Relative expression (logarithmized scale) of four *Clianthus* floral identity genes in vegetative and reproductive tissues

A: Leaves; B: Vegetative shoot tips; C: Inflorescences 2-3 mm in length; D: Early stage flower buds of 2-3 mm in length; E: Mid-stage flower buds 10-12 mm in length; F: Young seed pods 2-3 weeks after flowering.

5.3.3.3 Expression of floral identity genes in different floral organs of *Sophora*

The objective was to test the function of *Sophora* floral identity gene homologues in specifying and identifying different flower organs, and to elucidate the applicability of the ABC model in woody leguminous species. Therefore, the expression profiles of *STAP1, STPI,* and *STAG,* the representative *Sophora* homologues of each of the ABC class genes, together with their upstream controlling gene homologue *STLFY,* and an internal control gene, *ST18S,* were determined in four whorls of flower organ tissue using real-time PCR assay. Flower buds 18-20 mm in length showing petal tip sampled in September 2003 were used in this experiment. Sepals, petals, stamens, and carpels were excised from the same flower bud. To avoid cross contamination among floral organs, only the upper half of each floral organ was used for RNA isolation. Before comparing the expression levels between tissue samples, melting curves of all PCR products were checked to confirm the nature of the amplified products.

The results showed that expression of the internal control gene *ST18S* was relatively stable across the four organ tissues, with a variation of expression level of only 40% detected between organ types (Fig. 5.19 and Fig. 5.20).

STLFY expressed in all of the four flower organs, although a near 2-fold difference was detected between different organ samples. Higher mean *STLFY* expression was detected in sepals and stamens than in carpels and petals, although this did not achieve statistical significance.

Highest *STAP1* expression was detected in sepals. Petals also had high *STAP1* mRNA concentration, representing 77% of that of sepals. No *STAP1* activity was detected in stamens, and a very low level of *STAP1* activity was detected in carpels, representing less than 1/1000 of its highest level (Fig. 5.20). The real-time quantification curves (Fig. 5.21 A) showed that PCR products accumulated normally only in sepal and petal templates. When stamen and carpel templates were used, the PCR reactions generated much delayed and abnormal accumulating curves, with shorter logarithmic phases and low plateau level. The melting curve analysis indicated that the PCR reaction from stamens generated a product with similar melting temperature to that for the no template control. However, the PCR reaction using cDNA template from carpels did generate the same PCR products as templates from sepals and petals, possessing the same melting temperature (Fig. 5.21 B).

While similar *STPI* expression level was present in stamens (100%) and petals (96%), no *STPI* activity was detected in sepals and carpels (Fig. 5.19, 5.20).

Highest *STAG* expression was detected in stamens, followed by that in carpels (76%). No *STAG* activity was detected in sepals, and a trace expression of *STAG*, representing less than 1/37000 of its highest level, was detected in petals (Fig. 5.19, 5.20).

In summary and excluding trace expressions (mRNA concentration below 0.1% of their corresponding highest expression levels), *STAP1* expressed only in sepals and petals, *STPI* expressed only in petals and stamens, and *STAG* expressed only in stamens and carpels.

Represented by the relative mRNA concentrations calculated using average values of the expression in each tissue, gene activities varied enormously among different genes at this developmental stage. *ST18S* expression was over 100,000-fold higher than that of *STLFY*. A difference of 40000-fold was detected among the four target genes. Similar amounts of gene product were detected for *STAP1* and *STAG*, which were 7- to 8-fold lower than that of *STPI*, and over 5000-fold higher than that of *STLFY* (Fig. 5.20).

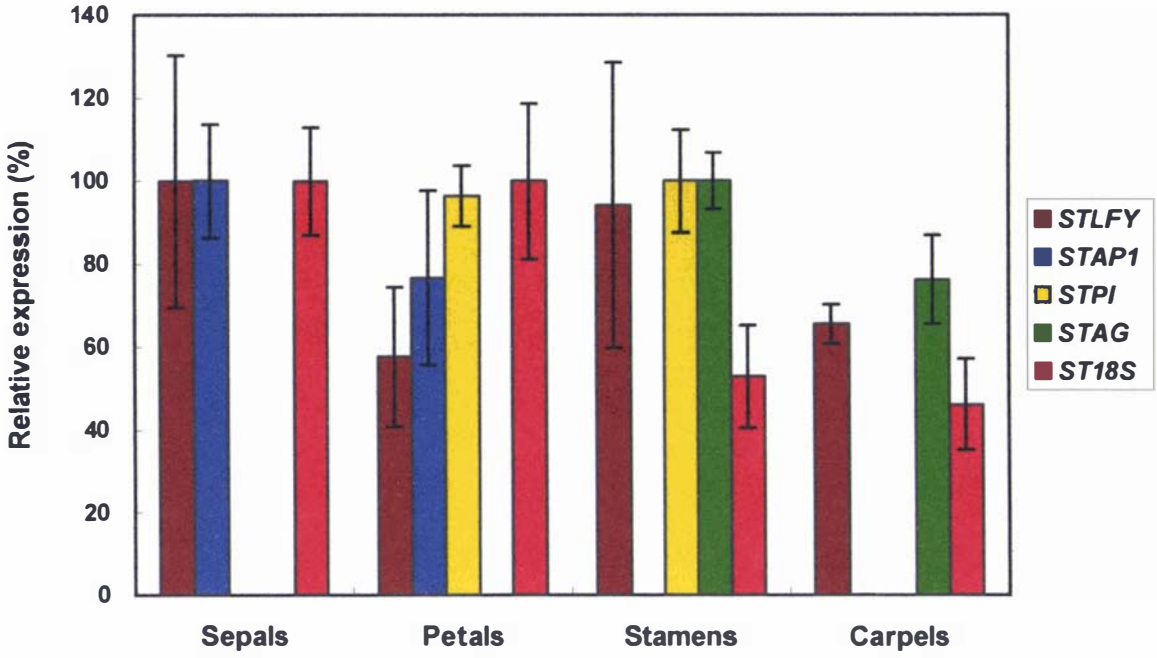

Figure 5.19 Expression of *Sophora* floral identity genes in different floral organs at the same developmental stage

Relative expression level calculated within each gene.

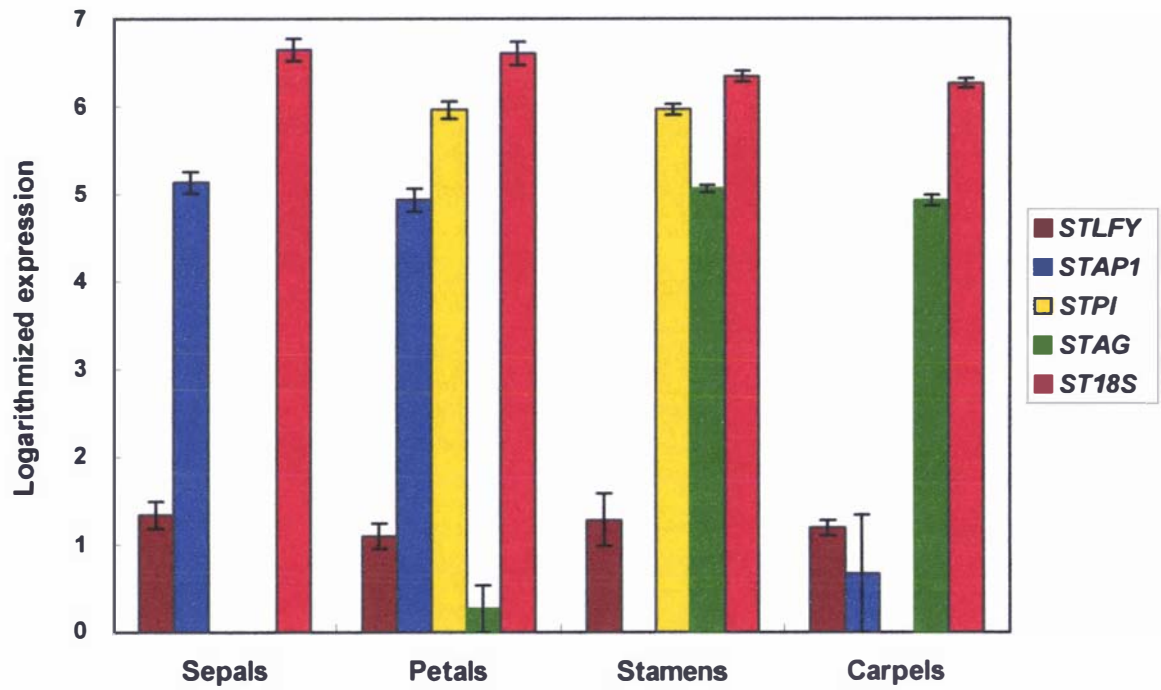

Figure 5.20 Expression (logarithmized scale) of *Sophora* floral identity genes in different floral organs at the same developmental stage

Relative expression level calculated over all tested genes.

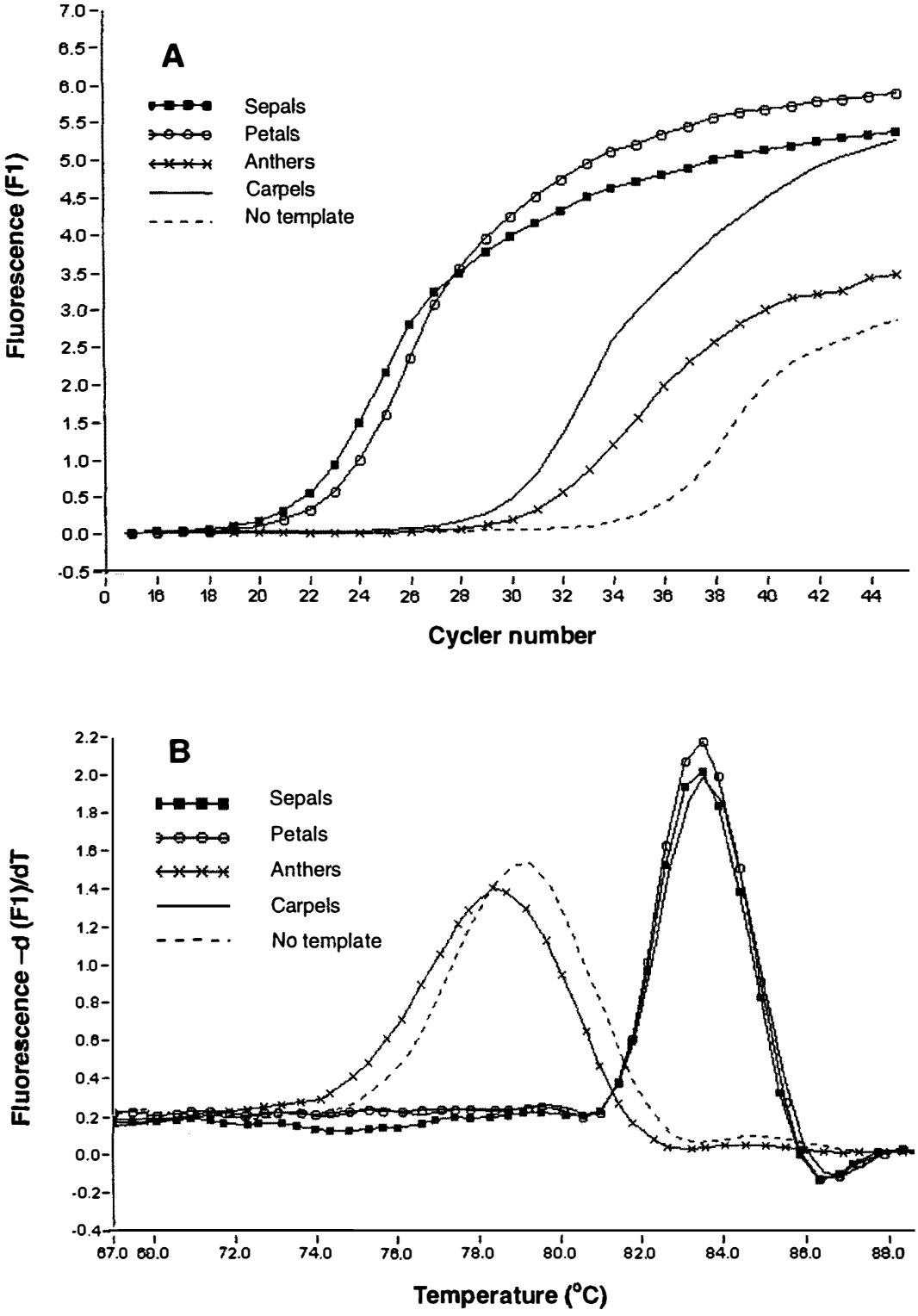

Figure 5.21 LightCycler™ quantification of the *Sophora STAP1* gene in different floral organs

A: Quantification curve analysis; B: Melting curve analysis.

5.3.3.4 Expression of floral identity genes in different floral organs of *Clianthus*

The same strategy as for *Sophora* was used to test the function of *Clianthus* floral identity gene homologues, *CMAP1, CMPI* and *CMAG*. Sepals, petals, stamens, and carpels excised from flower buds sampled in September 2003 were used for RNA isolation. The housekeeping gene homologue *CM18S* was used as the internal control gene. C*MLFY* was not tested because of its universal expression in all floral organs and low level in *Sophora.* Before comparing the expression levels between samples, melting curves of all PCR products were checked to confirm the nature of the amplified products.

The internal control gene *CM18S* expressed in all of the four floral organ tissues. The highest *CM18S* activity was detected in petals, followed by carpels and stamens, having 90% and 78% of its highest expression level. Less than 40% of *CM18S* expression was detected in sepals, being significantly lower than those in the other three floral organs (Fig. 5.22).

Highest *CMAP1* expression was detected in sepals. Petals had 58% *CMAP1* mRNA concentration compared to sepals. No *CMAP1* activity was detected in stamens and carpels (Fig. 5.22).

While the highest *CMPI* expression was detected in petals, *CMPI* expressed at 68% of its highest level in stamens. No *CMPI* activity was detected in sepals and carpels.

CMAG expressed at high level in stamens (100%) and carpels (67%). No *CMAG* activity was detected in sepals, whereas a very low expression (0.01%) was detected in petals (Fig. 5.23).

The LightCycler™ quantification curves (Fig. 5.24 A) showed that PCR reactions using templates from stamens and carpels generated normal and similar PCR product accumulation curves, with long logarithm phases and obvious plateaus. However, PCR reactions using cDNA templates from sepals and petals generated much more delayed PCR product accumulation curves, grouping together with that of the no template control. Melting curve analysis indicated that PCR reaction using cDNA template from petals did generate the same PCR products as templates from stamens and carpels, with similar melting temperatures, confirming *CMAG* activity in petals. However, PCR reaction using template from sepals generated different PCR products (Fig. 5.23).

Apart from the trace expression of *CMAG* in petals, which was 1000-fold lower than that expressed in other organs, the general expression patterns of these floral identity genes were as follows: *CMAP1* expressed in sepals and petals, *CMPI* expressed in petals and stamens, and *CMAG* expressed in stamens and carpels (Fig. 5.22).

Comparing the average overall expression of each gene in all organs defined by the ABC model, the four floral identity genes varied significantly at this developmental stage of flower buds (showing petal tips). *CM18S* expression was 20- to 240-fold higher than those of the target genes. The detected *CMPI* activity was 12-fold and 3-fold higher than that of *CMAG* and *CMAP1*.

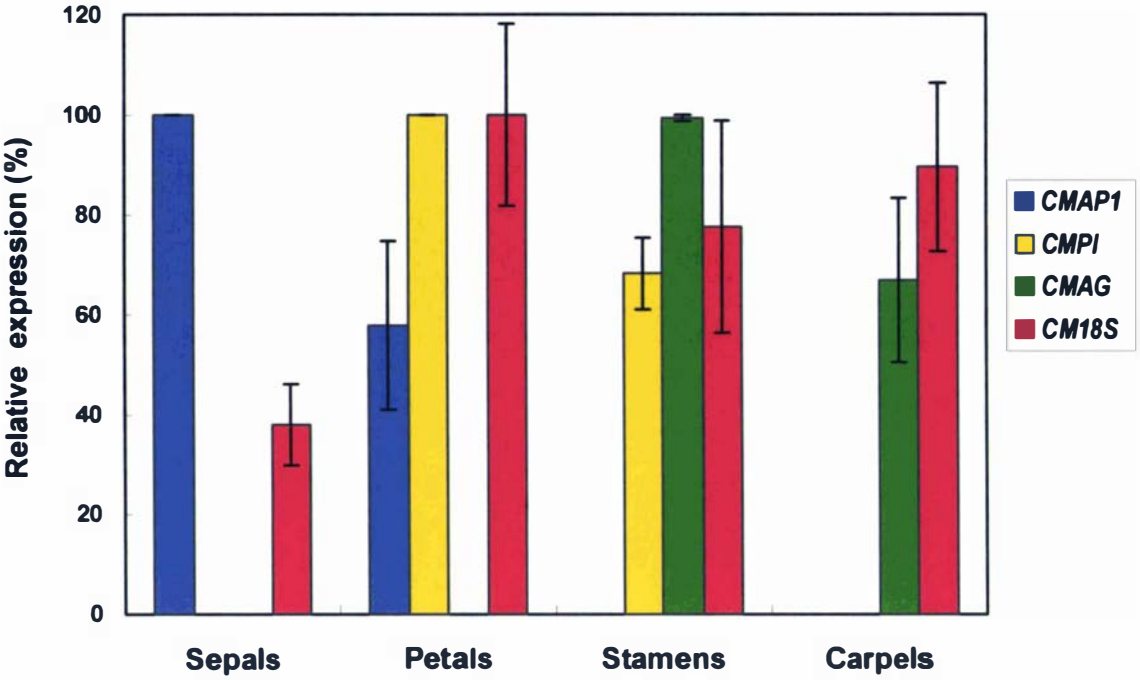

Figure 5.22 Expression of *Clianthus* floral identity genes in different floral organs at the same developmental stage

Relative expression level calculated within each gene.

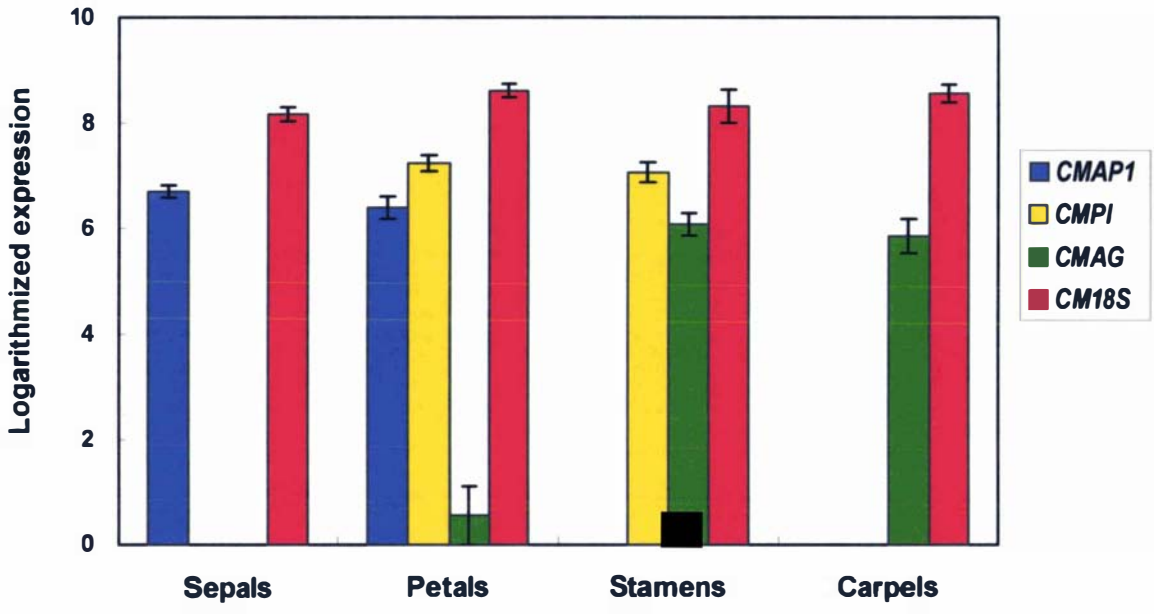

Figure 5.23 Expression (logarithmized scale) of *Clianthus* floral identity genes in different floral organs at the same developmental stage

Relative expression level calculated within each gene.

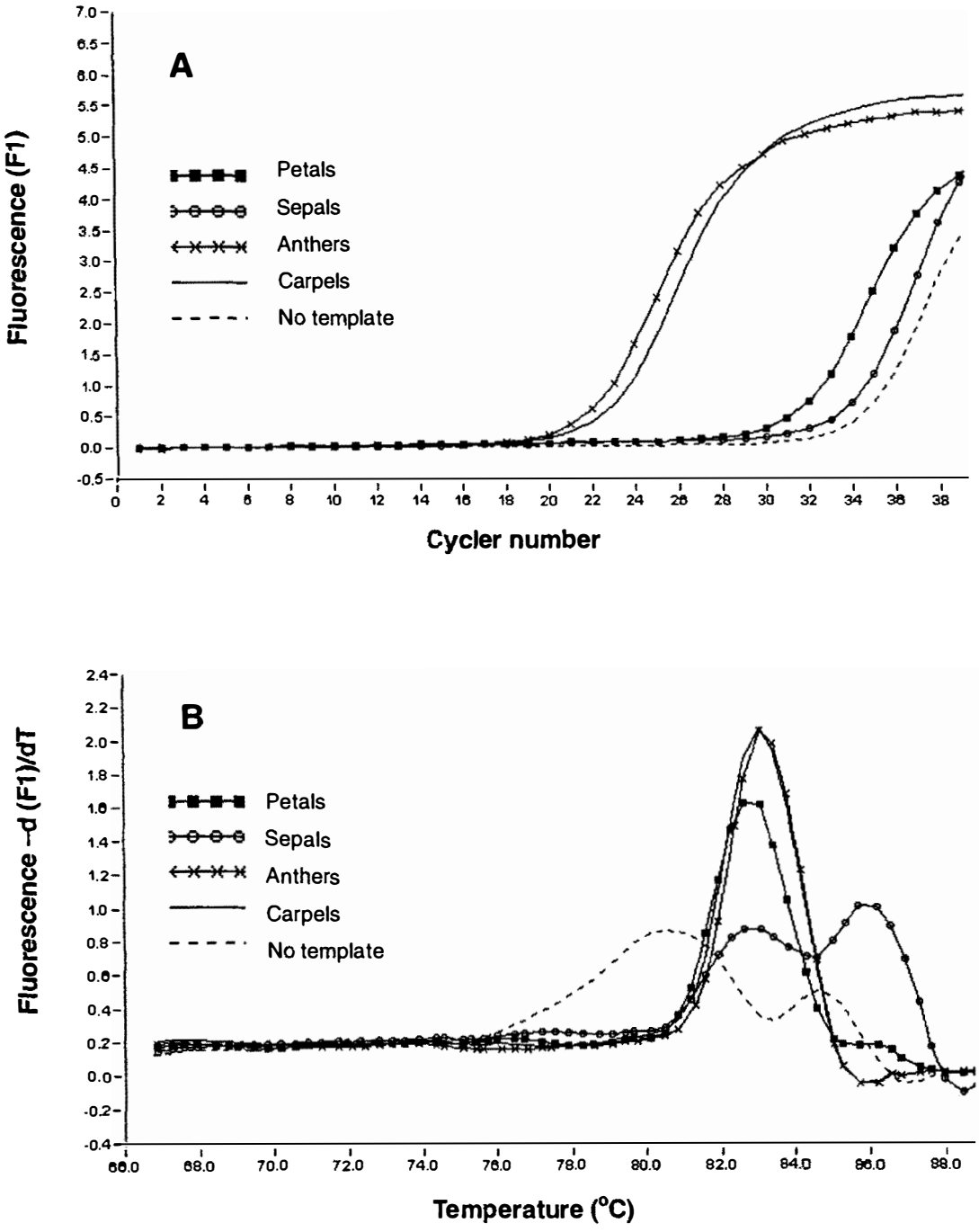

Figure 5.24 LightCycler™ quantification of the *Clianthus CMAG* gene in different floral organs

A: Quantification curve analysis; B: Melting curve analysis.

5.3.4 Temporal and developmental expression characteristics of floral identity genes in *Clianthus* and *Sophora*

Given that all target floral genes mainly expressed in inflorescences and floral buds and their expression levels varied greatly among inflorescence and the two flower stages (see Sections 5.3.3.1 and 5.3.3.2), it was decided to elucidate the detailed expression profile of these genes across a broader range of developmental stages. Also considering that the reproductive cycle is throughout the year and that there exist specific developmental events for both species, including a long dormant period of floral organogenesis for *Sophora,* and a mass abortion of *Clianthus* inflorescences during many months of the year (see Chapter 3), it was then hypothesized that the expression of these floral genes would be significantly different both temporally and developmentally. To test this hypothesis, detailed expression profiles of the four floral identity genes throughout the year, and then in a wide range of developmental stages of inflorescences and flower buds, were analysed using the real-time PCR assay for *Sophora* and *Clianthus*.

5.3.4.1 Expression profiles of floral identity genes in *Sophora* throughout the year

Sophora inflorescences and floral buds were sampled during the 2002-2003 growth cycle, from the initiation of inflorescence primordia in October (spring) to flowering time the following October, and relative expression levels of floral identity genes were detected using real-time PCR assay (Fig. 5.25).

The expression pattern of *STLFY* was characterized by a bimodal curve. In early October, during the flowering time of flower buds initiated the previous year, and when new vegetative growth and inflorescence initiation started, *STLFY* expressed at around 10% of its highest expression level. Thereafter, *STLFY* activity rapidly increased through November to December, as the inflorescences developed, floral buds emerged, and the annual vegetative growth flush ceased. *STLFY* expression reached its first peak in early January, when the inflorescences developed to their full length, and flower buds ceased to develop in size beyond 1-1.5 mm in length. It then quickly declined to c. 60% of its highest level at the end of January, and c. 20% in March, April and May (autumn), during the dormant period of the flower buds. The activity of *STLFY* increased again in June and reached its second, but lower peak (53% of the first peak) in July, when flower buds resumed their development and were 2.0-3.0 mm in length. Thereafter, *STLFY* expression

sharply decreased to 10% in August, and to 0.25% and 0.17% of its highest level in September and October (spring), respectively, before and during the flowering time.

The expression profile of *STAP1* was, to some extent, similar to that of *STLFY* except that the expression of the former was very low (under 1.5% of its peak level) in October, and that the second expression peak in spring was higher than the first one in July. Its expression also remained high (84% of its highest expression) in August, and then dropped to 43% and 6.2% of its highest expression level in September and October, respectively.

STPI and *STAG* genes shared very similar expression patterns. Unlike *STAP1* and *STLFY*, there was only one unique expression peak in August, coinciding with the fast enlargement of the floral buds. Activity of these two genes was not detected in October, and was hardly detected (<1%) in November. *STPI* and *STAG* activity was detected at a low expression level of 5-12% from December through to May. Thereafter, the expression level of *STPI* and *STAG* increased rapidly to 30-50%, and 70-80% of their peak levels in June and July, respectively. The highest *STPI* and *STAG* activities were detected by August, and the high expression levels (60-80% their peak levels) were maintained until September before dropping to 26% for *STPI,* and 6% for *STAG* in early October.

In summary, high expression level of *STLFY* and *STAP1* were detected both in summer (from December to February) and during the fast floral developmental period in winter (from June to September), with two expression peaks in January and July, respectively. However, high expression level of *STAG* and *STPI* were detected only during the fast floral developmental period in winter, with their expression peaking in August. Activity of all genes remained low in spring during the vigorous vegetative growth season, and in later autumn when initiated floral buds ceased to develop and remained in dormancy.

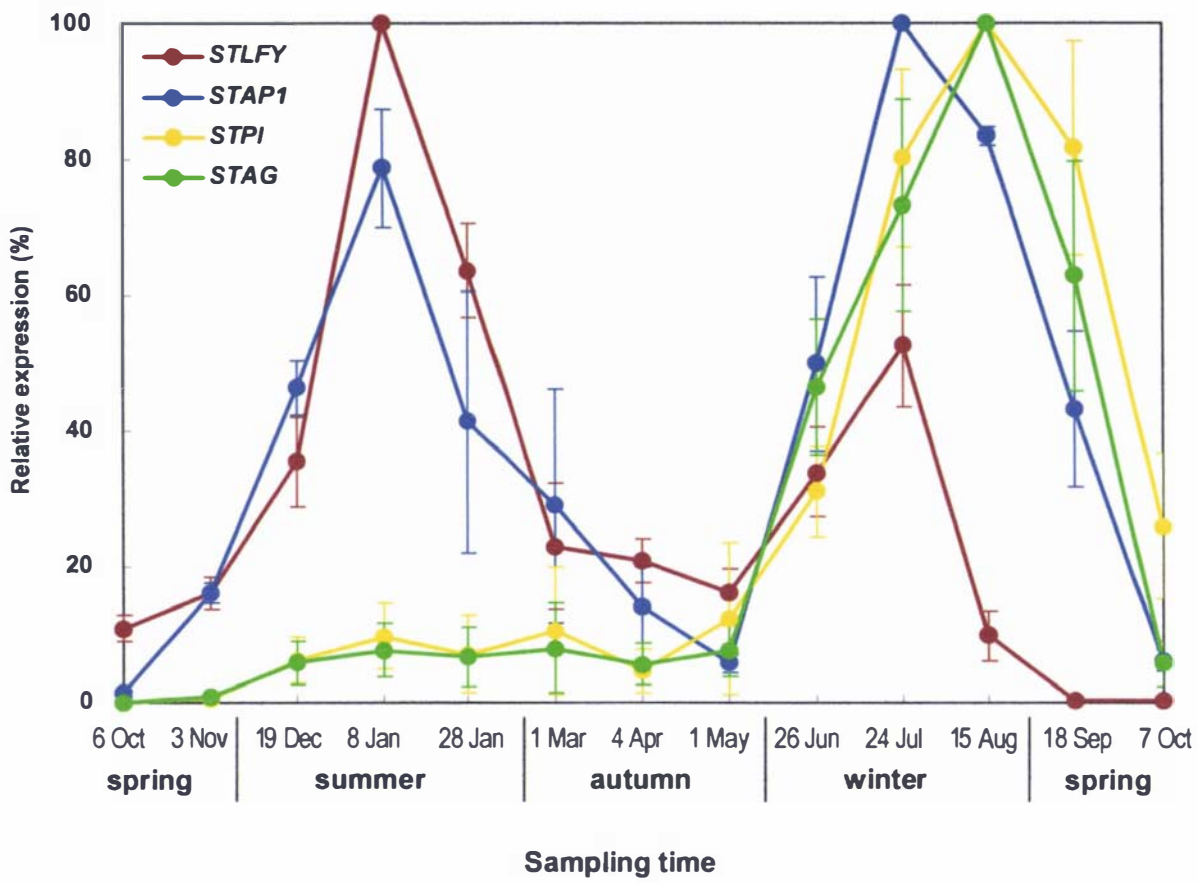

Figure 5.25 Temporal expression profiles of *Sophora* floral identity genes in inflorescences (6 October to 8 January), and individual flower buds (28 January to 7 October)

5.3.4.2 Expression profiles of floral identity genes in *Clianthus* throughout the year

According to previous observations, *Clianthus* inflorescences initiated during most of the year, and a continual mortality of inflorescence occurred before May (late autumn). To elucidate the possible mechanism of these phenomena and their correlation to the expression characteristics of floral genes, *CMLFY* expression was tested for shoot tip samples harvested throughout the year. Furthermore, all the four target genes, *CMLFY, CMAP1, CMPI,* and *CMAG,* were tested for expression in 15-20 mm long inflorescences (at similar developmental stages and vigorous status) harvested from January to June, and for the floral buds of inflorescences at an advanced developmental stage harvested from June to September (Table 5.2).

In shoot tip samples, *CMLFY* expression remained at a very low level (2-5 % compared to its corresponding highest expression in inflorescences in June) from November through to the following September, regardless of the presence or absence (at least in May) of inflorescence primordia (Fig. 5.26A).

In inflorescences and floral buds, expression of all four target genes was at a very low level from January to early April, below 6% of peak levels for *CMLFY* and *CMAP1,* and lower than 1% of highest expression levels for *CMPI* and *CMAG*. Higher expression levels of these genes were detected from April through to September. Unlike the expression patterns of their *Sophora* homologues, their expressions were characterized by a single curve occurring in June to August (Fig. 5.26B).

Expression level of *CMLFY* increased quickly in April and reached over 38% of its highest level by the end of that month, and to its highest level in June, before sharply declining in July and August. *CMLFY* activity could hardly be detected during flowering time in September, with only 0.6 % of its peak level (Fig. 5.26B).

Similar to *CMLFY*, *CMAP1* activity increased quickly from April through June, and reached its peak in July. Expression of *CMAP1* remained high (88%) in August before flowering time, and dropped to 30% of its highest expression level in September during the flowering time (Fig. 5.26B).

CMPI expression was much delayed compared to those of *CMLFY* and *CMAP1*. *CMPI* activity remained under 5% of its highest expression level until June, when *CMLFY*

expression reached its peak, and *CMAP1* expression was over 58% of its highest level. *CMPI* expression then increased to over 25% of its highest level in July, and reached its peak level by August before the flowering time. This high expression level (83%) was maintained until the flowering time in September (Fig. 5.26B).

Similar to *CMPI, CMAG* had its expression peak in August. However, the relative expression levels were 2-4 fold higher than those of *CMPI* from the end April through to July, although these were much lower than those of *CMLFY* and *CMAP1*. In contrast to *CMPI, CMAG* activity decreased drastically after its expression peak in August, and its expression level remained only 4.7% of its peak level in September, during the flowering time.

In summary, the floral identity genes showed a sequential peak in expression from May to September, with *CMLFY* in June, *CMAP1* in July, and *CMPI* and *CMAG* in August. *CMPI* expression still remained high (>80%) during flowering time in September, whereas that of other genes had declined to 0.5-30% by that time.

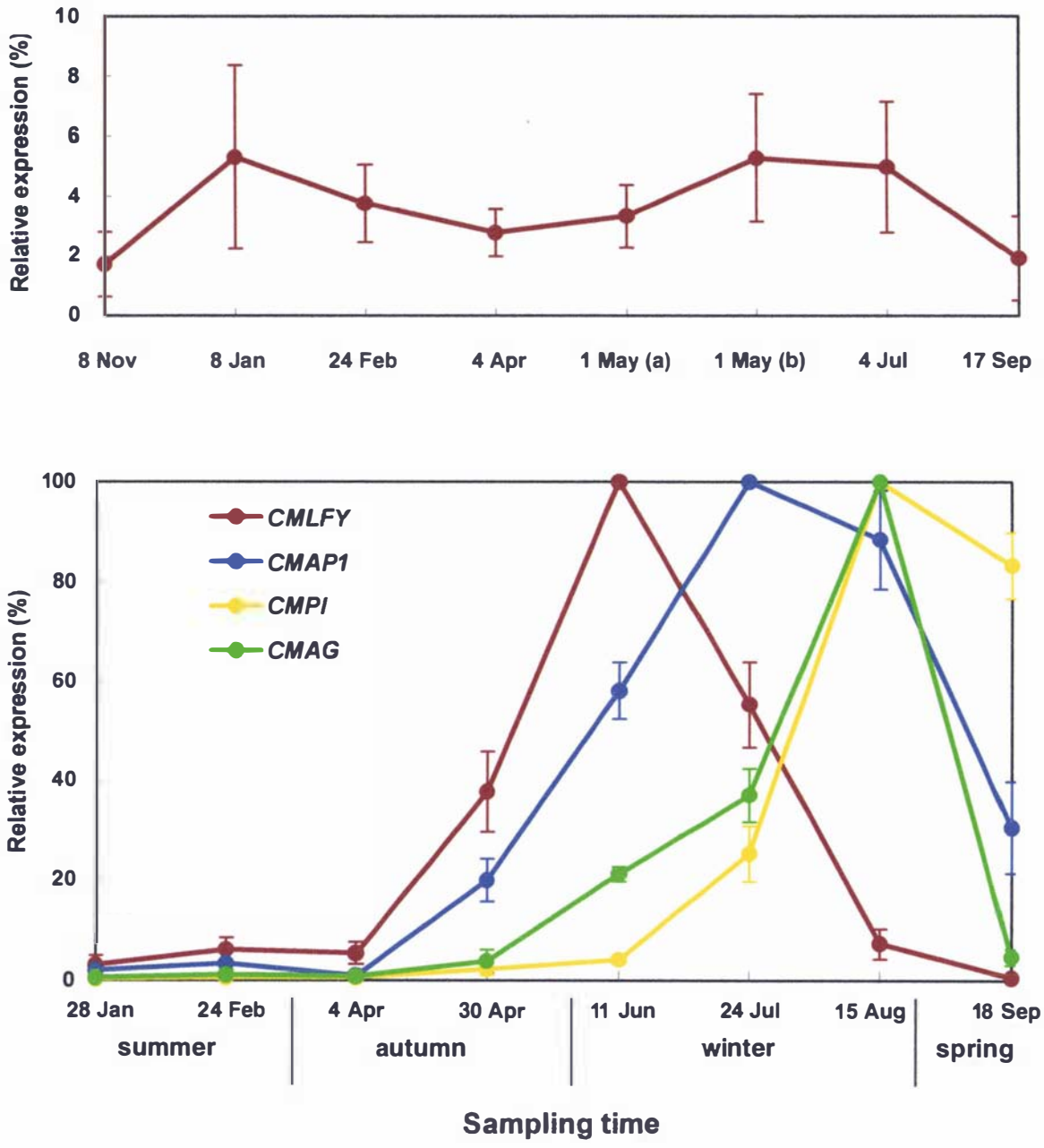

Figure 5.26 Temporal expression profiles of *Clianthus* floral identity genes throughout year

A: *CMLFY* expression in shoot tip samples. 1 May (a) and (b): Shoot tips sampled on the same day without inflorescence primordia, and with inflorescence primordia, respectively; Other samples possibly contained inflorescence primordia.

B: Floral gene expression in inflorescence (January to April) and flower bud (June to September).

5.3.4.3 Expression of *Sophora* floral genes at different developmental stages

Considering the lack of coincidence of the time course and the progress of reproductive growth in *Sophora* (see Chapter 3), the expression profile through the year might not be consistent with the developmental status of the reproductive organs. Therefore, the expression levels of these genes at a range of developmental stages were tested in this experiment. In order to minimize environmental effects, only tissue samples harvested during the fast growing period (October 2002 to January 2003 for inflorescences, and July to September 2003 for flower buds) were used. Three of the five stages of flower buds, 18-20 mm in length showing petal tip, mature before open, and fully opened before senescence, were sampled at the same time on 26 September (Table 5.1). Real-time PCR results showed that each of the four floral identity genes investigated in the current study had its own distinct expression pattern, which again varied significantly in different developmental stages of inflorescence and flower development in *Sophora* (Fig. 5.27).

STLFY activity was detected at a similar level (11-16% of its highest expression level) in vigorously growing shoot tips containing inflorescence primordia and in inflorescences 1-2 mm in length. However, its expression increased significantly as inflorescences developed, reaching its expression peak in inflorescences of 20-30 mm in length bearing floral primordia 1-1.5 mm in length, with differentiated floral organ primordia. Thereafter, its expression declined quickly as flower buds developed and bud size increased. *STLFY* activity decreased to 50% of its highest expression level in flower buds 2-3 mm in length, in which floral organs began to elongate, and to 10% in flower buds 10-12 mm in length. Only a trace mRNA of *STLFY* (0.25%) was detected in flower buds 10-12 mm in length, at the time when petal tips began to emerge from the calyx. This low level of *STLFY* expression was thereafter maintained in mature flower buds and fully opened flowers, representing 0.17% and 0.11% of its highest expression, respectively (Fig. 5.27).

Low *STAP1* expression was detected in shoot tips containing inflorescence primordia and undeveloped inflorescences (1-2 mm in length), showing 1.4% and 16% of its highest expression level, respectively. As the inflorescences developed and flower buds initiated, *STAP1* activity increased sharply. *STAP1* expression reached 46% and 79% of its highest level when inflorescences were 10-12 mm and 20-30 mm in length, respectively, bearing floral primordia. The expression peak of *STAP1* was detected when flower buds were 2-3 mm in length. In contrast to that of *STLFY*, high *STAP1* activity (84% of its highest level) was detected in flower buds 10-12 mm in length. This high expression level (43%) was

sustained until the flower buds reached a size of 18-20 mm in length, with petal tips emerged from sepals. However, *STAP1* mRNA declined to 6.2% in mature flower buds before opening, and to 0.5% in the opening flowers (Fig. 5.27).

STPI activity was undetectable in shoot tips with inflorescence primordia, and was hardly detectable (3000-fold lower than its highest level) in undeveloped inflorescences (1-2 mm in length). *STPI* expression increased slowly as the inflorescences developed and floral primordia initiated, with about 6% and 10%, respectively, of its highest expression level in 10-12 mm long inflorescences containing floral primordia and in 20-30 mm long inflorescences bearing flower buds of 1-1.5 mm in length, with differentiated floral organs. However, its expression increased abruptly as floral organs began to elongate within the flower buds, rapidly reaching 80% of its peak level in flower buds 2-3 mm in length, and reaching its peak when flower buds were 10-12 mm in length. *STPI* expression remained high (around 82% of its peak level) until flower buds reached a size of 18-20 mm in length, with petal tips emerged from the sepals. Thereafter, *STPI* activity dropped progressively as the flower buds further developed towards their mature stage. Around 26% and 2.3% of its peak expression was detected in mature flower buds before and after opening, respectively (Fig. 5.27).

The expression pattern of *STAG* was very similar to that of *STPI,* with high mRNA levels (>60%) detected only when flower buds developed to a size of 2-3 mm to 18-20 mm in length. No *STAG* activity was detected in shoot tips containing inflorescence primordia. Expression levels were around 0.3% in undeveloped inflorescences (1-2 mm in length), and 4-8% in other inflorescence and flower bud developmental stages (Fig. 5.27).

The four floral identity genes expressed sequentially in different developmental stages of *Sophora* inflorescences and flowers. *STLFY* expressed firstly but mainly during inflorescence development and at early stages of floral developmental. Its activity was hardly detected in flower buds after their mid-developmental stages. *STAP1* expression came immediately after *STLFY,* and remained high during a broad range of inflorescence and flower developmental stages. *STPI* and *STAG* were expressed later and mainly in a short period during early and mid-floral developmental stages, with no or hardly detectable activities before early inflorescence developmental stages.

All genes expressed at a very low level in fully opened flowers, with *STLFY* and *STAP1* below 0.5% of their peak level, and *STPI* and *STAG* below 4% of their peak level.

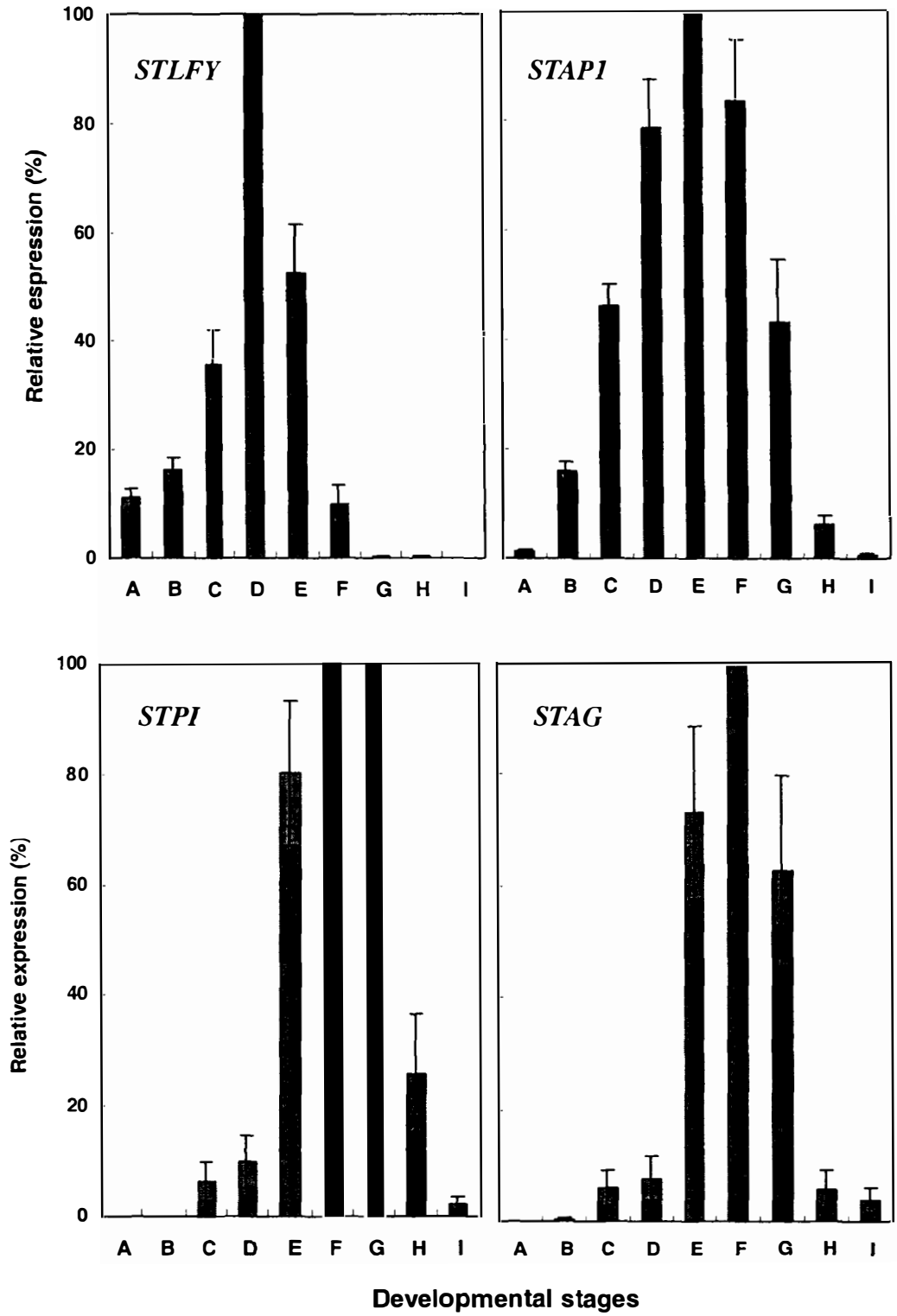

Figure 5.27 Floral identity gene expression in *Sophora* tissues at different developmental stages

A: Shoot tips with inflorescence primordia; B: Inflorescences (2-3 mm); C: Inflorescences (10-12 mm); D: Inflorescences (20-30 mm) with flower bud (1mm); E: Flower buds (2-3 mm); F: Flower buds (10-12 mm); G: Flower buds (18-20 mm) showing petal tip; H: Mature flower buds before open; I: Fully opened flower before senescence.

5.3.4.4 Expression of *Clianthus* floral genes at different developmental stages

Similar to that for *Sophora*, the expression levels of four floral identity genes at a range of different developmental stages were tested in this experiment. In order to minimize any environmental effect and that of the continual mortality of inflorescence occurring before April, only the samples taken on 1 May 2003 for early inflorescence developmental stages, on 11 June 2003 for early floral developmental stages, and on 26 September 2003 for late floral developmental stages were used in this analysis (Table 5.2).

Relative expression results using real-time PCR assay revealed distinct expression patterns for each of the four *Clianthus* floral identity genes and significant variation among stages of inflorescence and flower development (Fig. 5.28).

CMLFY expressed at 5.3% of its peak level in shoot tips bearing inflorescence primordia. Expression increased very rapidly as inflorescences developed, showing 45% activity in inflorescences 1-2 mm in length, and reaching its expression peak in inflorescences of 15-20 mm in length, and inflorescences of 25-35 mm in length bearing floral primordia. As floral buds further developed and bud size increased, *CMLFY* declined quickly to 49% of its highest expression level in flower buds of 2-3 mm in length. When floral buds reached their size of 10-12 mm in length with petal tips emerged from the calyx, its expression dropped to 7.7% of its peak level. In mature floral buds and opened flowers, only 0.59% and 0.18% of the highest *CMLFY* mRNA level were detected, respectively (Fig. 5.28).

No *CMAP1* mRNA was detected in shoot tips bearing inflorescence primordia, while *CMAP1* expression was 8.9% of its highest expression level in early stage inflorescences 1-2 mm in length. *CMAP1* activity increased quickly to over 50% of its peak level in 15-20 mm long inflorescences, and to its peak level after the initiation of floral primordia. Thereafter, high *CMAP1* expression (over 80% of its peak level) was maintained as flower buds developed, until the appearance of petal tips. Under 30% of its peak mRNA level was detected in mature flower buds before opening, while only 0.25% of that expression remained in opened flowers (Fig. 5.28).

No *CMPI* activity was detected in inflorescence primordia. And very low level of *CMPI* expression was detected in the early developmental stages of inflorescences before the initiation of floral primordia, representing only 1.5% and 4% of its peak level in 1-2 mm

and 15-20 mm inflorescences, respectively. Although significantly higher *CMPI* expression levels (18-25% of its peak level) were detected after the initiation of floral primordia and during the early developmental stages (1-2 mm in length) of flower buds, a sudden increase in its expression occurred during the mid- late stages of flower bud development. *CMPI* expressed at the highest level when floral buds reached 10-12 mm in length, with petal tips emerged of from the sepals. High expression level (83% of its peak expression) was also detected in mature flower buds before opening, while only c. 10% of its highest expression remained in fully opened flowers (Fig. 5.28).

Similar to that of *CMPI,* no *CMAG* activity was detected in shoot tips containing inflorescence primordia. *CMAG* expression level was around 2.4% in undeveloped inflorescences 1-2 mm in length, and increased progressively as inflorescences developed and floral primordia initiated. Around 20% of *CMAG* activity was detected in inflorescences 15-20 in length, and the expression was nearly doubled when floral buds reached 2-3 mm in length. *CMAG* activity rose to its peak level when floral buds reached 10-12 mm in length, with petal tips emerged from the calyx, and then declined drastically as flower buds further expanded. Only 5-9% of its peak expression level were detected in mature flower buds and opening flowers (Fig. 5.28).

For the four tested *Clianthus* floral identity genes, two relatively similar groups could be distinguished according to their expression patterns, with *CMLFY* and *CMAP1* in one group, and *CMPI* and *CMAG* in the other. Both genes in the first group expressed at high levels during most stages of inflorescence and flower development, and at a low level in inflorescence primordia and opening flowers. While *CMLFY* expressed, to some extent, before *STAP1* did, the latter remained at a higher expression level in the later stages of flower development.

CMPI and *CMAG* were expressed much later than the first group genes. Their high expression levels occurred mainly in a short period during the mid-stages of flower development, with no expression in inflorescence primordia, and low expression levels during inflorescence and early flower developmental stages. Furthermore, *CMAG* activity was 2-4 fold higher than that of *CMPI* in early developmental stages until flower buds reached 2-3 mm in length, while the expression level of *CMPI* remained over 18-fold higher than that of *CMAG* in mature flower buds before opening.

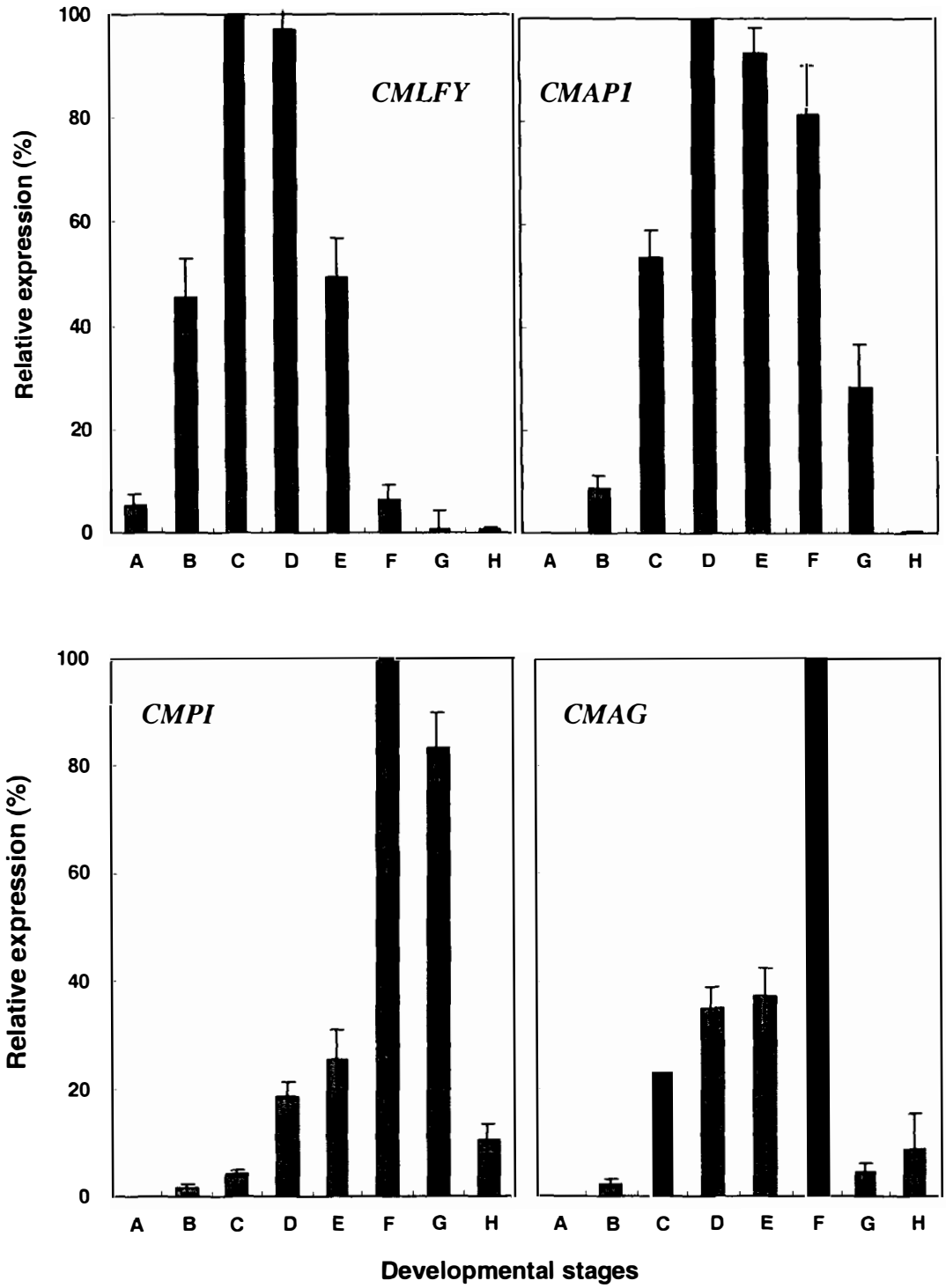

Figure 5.28 Floral identity gene expression in *Clianthus* tissues at different developmental stages

A: Shoot tips with inflorescence primordia; B: Inflorescences (2-3 mm); C: Inflorescences (15-20 mm); D: Inflorescences (25-35 mm) with flower bud (1mm); E: Flower buds (2-3 mm); F: Flower buds (10-12 mm) showing petal tip; G: Mature flower buds before open; H: Fully opened flower before senescence.

5.4 Discussion

5.4.1 Establishment and optimisation of real-time RT-PCR assay

Real-time quantitative RT-PCR is becoming the preferred method for high-sensitivity, rapid-throughput gene expression studies. However, it has also introduced new challenges that must be appreciated and dealt with if data are to be reported in a biologically relevant way (Bustin and Nolan, 2004). In the current studies, an efficient and comparatively inexpensive two-step real-time RT-PCR assay for the detailed expression analysis of large number of samples was developed, which employs the nonspecific double strand DNA dye SYBR Green® and the LightCycler™ instrument, and uses a relative quantification method accounting for differences in PCR amplification efficiency between the target and reference genes. The consistency of this protocol was optimized with regards to sample and template preparation, primer design, sample error control and experiment design.

The nonspecific DNA binding dye SYBR Green has been widely used in gene expression studies (Deprez et al., 2002; Ramos-Payan et al., 2003; Dorak, 2004; Bustin and Nolan, 2004). The advantages of SYBR Green over probe-based assays are that it can be incorporated into optimized and long-established protocols using any primer, and it is significantly cost effective. Therefore, despite the nonspecific nature of amplification detection, DNA-binding dye based assays need not be less reliable than probe-based assays (Bustin and Nolan, 2004). In the current study, the specific detection of SYBR Green was realized by using a specific and intron spanning primer designing strategy, apart from the general practice by melting curves analysis.

One of the distinguishing features of real-time PCR assays is their high running cost relative to conventional RT-PCR assay, and semi-quantitative methods such as RNA blotting. It was, therefore, prudent to expend extensive efforts on getting every stage of this process right, starting with consistency (Bustin and Nolan, 2004). Therefore, the following efforts were made to optimize the current real-time protocol before its application to expression study of *Sophora* and *Clianthus* floral identity genes.

RNA quality is one of the most important determinants of the successful quantitative RT-PCR assay. Many problems that affect reproducibility, and hence the relevance of results, are likely to have originated from low RNA quality. In the current study, over

40% of the final variation was contributed by RNA quality, when the same amount of total RNAs isolated from the same tissue sample was used for cDNA synthesis.

Although many isolation protocols gave satisfactory RNA yield and quality for conventional and real-time PCR assay, most of them required at least 0.5-1g tissue samples as starting material. In gene expression assays the availability of plant material is often limited. In the present study, a simple and efficient micro-preparation of the total RNA isolation protocol was established, which could yield 15-20 μg of high purity total RNA from only 5-10 mg fresh tissue sample, meeting the need for 10-20 cDNA synthesis and hundreds of real-time PCR reactions. This protocol could yield 1.5- to -2 fold of total RNA and was 20- to 30-fold more cost effective compared to the protocol using a commercial RNA isolation kit, with adequate RNA quality and integrity for cDNA synthesis and gene expression quantification using real-time PCR.

In the current study, four target genes (floral identity gene homologues) of unknown abundance were to be investigated, using *18S rRNA* as one of the internal controls. Therefore, neither oligo-dT nor the random priming method seemed to be the appropriate one to meet the needs of the current study. Hence, attempts to establish an appropriate cDNA synthesis method were made by combining the two priming strategies in the same reverse transcription reaction.

The obtained result showed that real-time PCR efficiency was drastically affected by primer type, and that a full strength mixture of oligo-dT primers and random primers in the same reverse transcription reaction yielded satisfactory cDNA for both target gene *STAP1* and 18S rRNA. Unexpectedly, cDNA was successfully synthesized from *18S rRNA* using oligo-$(dT)_{15}$ primers alone, although the resulted cDNA concentration was four to five-fold lower than that of hexa-random primers. This is contradictory to the general knowledge of no poly-A tails presented in rRNA populations. One possible explanation might be due to endogenous priming in which unintended endogenous priming can occur regardless of which primers are used to prime the RT reaction (Ambion technical tips, http://www.ambion.com).

For *STAP1*, mixed primers had a 2-fold higher expression level than that of the oligo-$(dT)_{15}$ primer alone. That was an interesting phenomenon and might be the result of the complemented effects of the specific binding of oligo-dT primers and the random binding of the random primers. Therefore, the results suggested that the mixture of

oligo-dT primers and random primers would be beneficial and appropriate for cDNA synthesis whether or not *18S rRNA* was used. This beneficial effect of combined oligo-dT and random primers in the same RT reaction has not been reported elsewhere.

The detrimental effects of DTT to RT-PCR using SYBR green I was reported by Deprez et al. (2002). Omitting DTT from the reverse transcription reaction resulted in a lower C_t value, or a higher level of the transcript in the cDNA preparation. The current study showed that omitting DTT did have some, but not significant, beneficial effects for both 18S rRNA and *STAP1*. However, all PCR reactions resulted in normal and typical accumulation curves, without showing significant inhibitory effects of DTT, indicating that the presence of DTT had less detrimental effects than that reported by other authors. The inhibitory effects of DTT might have been removed by the 30-fold dilution of the cDNA template applied in the current study.

The initial real-time PCR assays using 5-fold diluted cDNA as template, resulted in very unstable PCR results accompanying abnormal amplicon accumulation curves. Results from a series of cDNA dilutions (up to 1000-fold) showed that a 10-fold dilution of cDNA was not sufficient to eliminate the inhibitory effects of cDNA reactions in most cases, but a100-fold dilution was sufficient to do so. Orsel et al. (2002) also used a 100-fold dilution of cDNA to quantify an *AtNRT2* family gene. However, a cDNA dilution of 100-fold or higher resulted in a delayed C_t value, which would make the accurate quantification of low expressed target genes more difficult. In the experimental conditions employed in the current study, a 20-50-fold dilution was satisfactory to remove most of the inhibitory effects and to have the PCR result comparable to that of the 100-fold cDNA dilution in most cases.

$MgCl_2$ plays a very important role in the PCR amplification process and can greatly affect PCR efficiency. The current study showed that different genes had different optimum concentrations: 3-4 mM for most genes and 5 mM for a couple of other genes. This partially conflicts with the results of Deprez et al. (2002) in which 5 mM $MgCl_2$ was always better than 3 mM for the four different primer pairs they tested. This could be due to the nature of the genes and primer sequences. Considering the large number of reports that have successfully applied a wide range of $MgCl_2$ concentrations, it is suggested that the appropriate $MgCl_2$ concentration should be determined for each of the genes under investigation, and even for each primer combination of the same gene.

5.4.2 Determination of housekeeping genes as internal controls

For gene expression studies, it is now a common practice for the expression levels of target genes to be normalized by one or more carefully selected, stable internal control genes. With the increased sensitivity, reproducibility and large dynamic range of real-time RT-PCR methods, the requirements for a proper internal control gene have become increasingly stringent (Vandesompele et al., 2002). Housekeeping genes are believed to be constitutively expressed and minimally regulated, and have been widely used as internal controls for quantitative analysis in real-time RT-PCR assays (Bustin and Nolan, 2004). However, a great number of recent studies have reported that housekeeping gene expression can vary considerably (Tricarico et al., 2002; Aerts et al., 2004; Andersen et al., 2004; Dheda et al., 2004). Therefore, if housekeeping genes are to be used, they must be validated before each experiment (Andersen et al., 2004; Bustin and Nolan, 2004). However, to validate the presumed stable expression of a given control gene, prior knowledge of a reliable measure to normalize this gene is required in order to remove any nonspecific variation (Vandesompele et al., 2002). To address this circular problem, the expression stability of housekeeping genes was evaluated using cDNAs from the same tissue sample and target genes (*AG* and *LFY* homologues) as expression controls. In this case, variation of the target gene could be supposed to be totally introduced by experimental error. Three most widely used housekeeping genes, *18S rRNA*, *β-actin*, and *GAPDH*, were chosen in the current study based on an analysis of the literature (Suzuki et al., 2000; Aerts et al., 2004).

None of the housekeeping genes, *ST18S*, *CM18S*, *STACT*, *CMACT*, *STGAP*, and *CMGAP*, investigated in the current study expressed in a stable manner across tested floral developmental stages. The expression variation was also very remarkable between two species for the same housekeeping gene, being 16- to 25-fold for *Sophora* and 3- to 6-fold for *Clianthus*. This result is consistent with those of the most recently published reports, in which considerable expression variations were detected for the same housekeeping gene between experimental conditions in the same study and among different experiments (Warrington et al., 2000; Suzuki et al., 2000; Bustin, 2000; Tricarico et al., 2002; Aerts et al., 2004; Andersen et al., 2004; Bustin and Nolan, 2004; Dheda et al., 2004). Therefore, these housekeeping genes are not recommended to be used independently as internal controls for normalizing the expression of target genes during different developmental stages, at least in the case of *Sophora* and *Clianthus*.

Among the three tested housekeeping genes, *STGAP* and *CMGAP* were more stable than *18S* and *β-actin* homologues in this study, in terms of both variation among replications within the same developmental stage, and among different developmental stages. Recent articles have discussed the problem of housekeeping genes (Bustin, 2000; Tricarico et al., 2002; Aerts et al., 2004; Andersen et al., 2004; Dheda et al., 2004), and it would appear that the housekeeping genes most commonly used are subject to variation in numerous experimental conditions, bringing into question the reliability of the results obtained by these authors. In particular, the use of *GAPDH* and *β-actin* has been severely criticized. Aerts et al. (2004) observed as high as a 154-fold difference of *GAPDH* expression among human tumor cell lines using the real-time RT-PCR method, while those for *β-actin* and *18S rRNA* were 10- and 2-fold, respectively.

Using these highly variable housekeeping genes as the internal controls for gene expression studies would be problematic since they cannot completely remove nonspecific variation, and could even add more variation, resulting in larger so-called gene-specific variations for the tested control genes and in missed small differences between genes of interest (Vandesompele et al., 2002). In the current study, the variation of target gene expression within the same tissue sample would have been enlarged after normalization using a single housekeeping gene. A similar situation was also shown in a study of human asthma, where the variation in target gene expression between samples was contributed by the normalizer *β-actin* rather than the target gene (Glare et al., 2002).

Vandesompele et al. (2002) observed that normalization with a single control gene consistently resulted in significantly higher gene-specific variations of the other control genes, and suggested that normalization be carried out using multiple housekeeping genes instead of one in order to measure expression levels accurately. In the current study, the variation of housekeeping genes could be greatly minimized using their geometric mean instead of single gene for both *Sophora* and *Clianthus* housekeeping gene homologues. When the normalization factor based on the geometric mean described by Vandesompele et al. (2002) was used to normalize the expression of target genes for *Sophora* and *Clianthus*, the variation between replicates within the same developmental stage was remarkably reduced. However, the attempt to remove the variation between developmental stages was not satisfactory.

According to the strategy describes by Vandesompele et al. (2002), a relatively large number of housekeeping genes was required to be tested to select the 3-5 most stable ones

for normalization factor calculation. However, the considerable high cost of the real-time PCR reaction and the technical difficulty to develop a large number of housekeeping genes made this strategy unfeasible brcause of the large number of experimental samples and detailed expression profiles being investigated in the current study. This may also not be feasible in small studies or those with limited budgets (Dheda et al., 2004). Based on the results obtained in the current study and other published studies, it is proposed that using three or four carefully selected and widely used housekeeping genes as the internal controls for normalization would meet the needs of normal experiments and be practicable for many laboratories.

In summary, none of the tested housekeeping genes is suitable as an internal control gene for quantifying gene expression using real-time PCR analysis when used individually. However, the combination of multiple housekeeping genes could remarkably improve their stability and reduce the expression variation of the target genes to a certain extent.

5.4.3 Expression characteristics of *Sophora* and *Clianthus* floral identity genes homologues

Much progress has been made in the last decade in understanding the initiation and development of floral organs in plants, through the verification of many of the master regulatory genes that trigger the developmental programs required for organogenesis. Intensive studies and analyses of identity mutations in *Arabidopsis* and *Antirrhinum* have led to the ABC model of floral organ identity specification (Bowman et al., 1991; Coen and Meyerowitz, 1991;Weigel and Meyerowitz, 1994). Over the years, this ABC model of floral development has proven to be significant and useful in explaining the development of the reproductive organs of many plant species, especially facultative long-day, herbaceous, annual species (Ng and Yanofsky, 2001b; Boss et al., 2003).

However, in contrast to a large amount of information concerning the molecular mechanisms of floral development in herbaceous species, only limited molecular studies have been carried out on floral development in woody species, especially woody legumes. Although similar genes may be involved at various stages of the flowering process in both categories of species, it is highly probable that they are regulated differently because there exist quite different flowering processes in woody perennial species. For instance, some of floral identity genes are also expressed at low levels in the primary vegetative

meristems of *Populus* where they may have alternate functions (Cseke and Podila, 2004). Despite the importance of these processes for the management and improvement of woody species, very little is known about their underlying molecular mechanisms (Carmona et al., 2002). Therefore, to understand and to control flowering in woody species, we need to adapt the flowering models that are based on work carried out with annual model species to fit the differences in flowering process in woody perennial species (Boss et al., 2003).

Significantly, the ABC model hypothesis does not satisfactorily explain a system in which more than one type of organ is being initiated at the same time, such as that in papilionoid and caesalpinioid legumes that have overlapping whorls. The ABC model also does not explain the concurrent initiation of the carpels and petals or stamens, which is usual in legumes. In these respects, flowering in legumes fails to conform to the ABC model (Tucker, 2003a).

Genes that control floral transition from inflorescence to flower, as well as affecting floral organ expression in legumes have been characterised (Singer et al., 1999; Hirsch et al., 2002; Taylor et al., 2002). Ferrándiz et al. (1999) demonstrated that flower development in pea proceeds through a sequence of events very different from those of model herbaceous species. These authors examined floral identity genes in pea, and concluded that the identity and developmental pattern of the four organ types in pea flowers are governed by at least the same three developmental functions, A, B, and C, proposed for the two model systems in *Arabidopsis* and *Antirrhinum*.

However, most of these studies focused on the initiation of the floral meristem and floral organs. Little was known about the detailed expression profiles of these floral identity genes during different developmental stages, and in different floral organs. Study of the expression profile of all the three classes of floral organ identity genes and their upstream controller genes in the same species and in the same experiment has not been reported. Furthermore, isolation and expression study of floral identity and specific genes in woody leguminous species have not yet been reported. The vegetative and reproductive development of *Sophora* and *Clianthus,* two woody legumes has now been characterized in the current study. These two species have distinct flowering processes to annual species, to other woody perennial species, and even between themselves.

To understand the genetic and molecular mechanisms underlying the flowering processes

in these two species, the *LEAFY* and ABC class homologues, *STLFY, STAP1, STPI* and *STAG* from *Sophora*, and *CMLFY, CMAP1, CMPI* and *CMAG* from *Clianthus* were isolated and their sequences analyzed (see Chapter 4). The high sequence similarity of these genes to their *Arabidopsis* homologues lent support to the hypothesis that the *Arabidopsis* based floral gene model would be applicable to these two woody perennial leguminous species. In this chapter, expression details of these genes were investigated using real-time quantitative RT-PCR. Their expression profiles in different vegetative and reproductive tissue types, in different flower organs, during the whole reproductive growing cycle as well as in detailed development stages were elucidated.

STLFY has a bimodal pattern of expression during the annual reproductive cycle, which starts from mid-spring (early October) at the time when new vegetative growth and inflorescence initiation started, and ends the following spring at flowering time, during this period *STLFY* shows two distinct peaks of activity. Similar bimodal expression patterns were described for *LFY* homologues in *Metrosideros excelsa* (Sreekantan et al., 2004) and other woody perennial species including *Actinidia deliciosa* (Walton et al., 2001) and *Vitis vinifera* (Carmona et al., 2002). In all of these studies, the highest expression of *LFY* homologues was detected during floral initiation, consistent with the current study. However, the two expression peaks were separated by a winter dormancy period in the cases of these species, whereas in *Sophora* the peaks of expression were separated by a summer-autumn period of low expression.

STLFY expression declined progressively at the beginning of the period of bud dormancy, remained low, and increased again before the breaking of bud dormancy. As to the cause of the expression characteristics of *STLFY,* it is unlikely that temperature has an important role in this phenomenon because the local summer-autumn temperature (15-28°C) is not likely to be beyond the upper or lower limit for normal plant organ development. In this context, the daylength might play a more important role.

In contrast to *LFY* homologues of other woody perennial species in which the first expression peak occurred at the time of inflorescence initiation (Walton et al., 2001; Carmona et al., 2002; Sreekantan et al., 2004), *STFLY* expression was quite low (15% of its peak level) at the same developmental stage. This low expression of *STLFY* in inflorescences compared to its high expression during floral initiation and early development suggests that *STLFY* might play a more important role in the inflorescence to floral transition than in floral organ development. This supports the primary roles of

LFY/FLO in conferring flower-meristem identity and in activating ABC classes of floral homeotic gene in the annual herbaceous species *Arabidopsis* and *Antirrhinum* (Coen et al., 1990; Weigel et al., 1992; Parcy et al., 1998; Busch et al., 1999; Wagner et al., 1999).

The up-regulation of *STLFY* when flower buds resume their growth from the long dormant period, and its dramatic down-regulation soon after the early stage of floral organ development strongly suggests that the quantitative accumulation of *STLFY* mRNA (or probably *STLFY* protein) might play an important role in activating some other co-factors that control floral organ development at later stages, instead of regulating the floral organ development on its own.

In contrast to *STLFY*, the expression of the *Clianthus LFY* homologue *CMLFY* had a unique peak in June at the time of floral primordia initiation. The high expression level was detected only at that time and was not separated into bimodal peaks. Low *CMLFY* activity (<4% of its peak level) was detected in vegetative shoot tips, with or without inflorescence primordia, during the whole year, and in mid-stage inflorescences before mid-autumn (April), coinciding with the continual mortality of inflorescences during this period. Thus, in terms of the coincidence of expression with developmental stages, *CMLFY* expression was very similar to that of *STLFY*.

STLFY and *CMLFY* were expressed in all tested tissue types, including reproductive tissues such as inflorescences, and flower buds, and vegetative tissues such as adult leaves and shoot tips, although at a level as low as 0.1-2.0% of their highest expression level in floral primordia. This suggests that *Sophora* and *Clianthus LFY* homologues in these two species are not sufficient to confer reproductive fate. Thus, the specification of reproductive characteristics may require other unknown post-transcriptional regulatory mechanisms, or the activity of additional factors. Functional studies of the role of *STLFY* and *CMLFY* would be required to demonstrate their function in the development of vegetative and reproductive tissue.

Expression of *LFY* homologues in vegetative tissues, apart from their normal expression in floral tissue, has been described in a number of other species. *AFL2,* the *LFY* homologue in apple, was expressed in the vegetative shoot apex and root (Kotoda et al., 2000; Wada et al., 2002). The *Vitis vinifera LFY* homologue, *VFL,* was expressed in the vegetative shoot apical meristem, leaf primordia and the growing leaf until later stages of development (Carmona et al., 2002). Low expression of *LFY* homologues in the

vegetative shoot apex or leaves have also been reported in *Actinidia deliciosa* (Walton et al., 2001), *Populus trichocarpa* (Rottmann et al., 2000), *Eucalyptus* (Southerton et al., 1998a), violet cress (Shu et al., 2000), tobacco (Kelly et al., 1995), *Impatiens* (Pouteau et al., 1997), and tomato (Molinero-Rosales et al., 1999). Carmona et al. (2002) suggested that the *LFY* homologue in developing *Vitis vinifera* leaves could be involved in maintaining proliferation and helping generate the programmed shape of the leaves. However, its role in the vegetative meristematic region is still unknown.

LEAFY has been shown to be necessary for the transition from vegetative to reproductive development in *Arabidopsis*. It confers flower-meristem identity at an early stage of flower development, similar to *FLORICAULA* (*FLO*) in *Antirrhinum,* and has therefore been defined as a floral meristem identity gene (Coen et al., 1990; Parcy et al., 1998; Wada et al., 2002; Komeda, 2004). Apart from its role in conferring flower-meristem identity, *LFY* also plays a major role in activating all three classes (ABC) of floral homeotic genes with spatially restricted patterns, including *AP1*, *AG*, and *AP3* (Parcy et al., 1998). High expression levels of *STLFY* and *CMLFY* in floral primordia and during the early development of floral organs suggest that both *Sophora* and *Clianthus LFY* homologues support the primary roles of *LFY/FLO* genes suggested in the annual herbaceous model species. However, their temporal expression patterns and expression details varied to some extent from those of the model species, and even from those of the reported woody perennial species.

The current study showed that the expression territory of *AP1* homologues in *Sophora* and *Clianthus* was strictly limited to floral primordia and floral buds. Although a proven detection power of 5- to 6-orders was demonstrated using the real-time RT-PCR technique, no *STAP1* and *CMAP1* activity was detected in non-floral organs such as adult leaves, vegetative shoot tips and seed pods. This supports a role for both *STAP1* and *CMAP1* strictly in the function of floral commitment, as described for *AP1* in *Arabidopsis* (Mandel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994). Kyozuka et al. (1997) observed a similar expression pattern of the *AP1* homologue in *Eucalyptus,* in which the *EAP1* gene was expressed in floral tissues but not in non-floral tissues.

During floral commitment, *STAP1* and *CMAP1* were mainly expressed during floral initiation and, at a higher level, during floral organogenesis and floral organ development. These expression patterns of *STAP1* and *CMAP1* were very similar to those described for *AP1* and its *Antirrhinum* orthologue *SQUA* (Bowman et al., 1989; Mandel and Yanofsky,

1998; Gustafson-Brown et al., 1994; Huijser et al., 1992). In *Arabidopsis*, *AP1* acts as both a floral meristem identity gene and a floral organ identity gene (Bowman et al., 1993; Weigel and Meyerowitz, 1994), and the different aspects of *AP1* function are reflected in its expression pattern (Jack et al., 2004). The current study showed that both the *Sophora STAP1* gene and the *Clianthus CMAP1* gene play similar roles to *AP1* in floral meristem identity, floral organ specification and flower development. Their behaviour was also consistent with reports for other *AP1* homologues in woody perennial species, including *EAP1* in *Eucalyptus* (Kyozuka et al., 1997), *AAP1* in *Actinidia deliciosa* (Walton et al., 2001), and *MESAP1* in *Metrosideros* (Sreekantan et al., 2004).

After floral organ differentiation within the flower, expression of *STAP1* and *CMAP1* was limited to sepals and petals. While no *AP1* homologue activity was detected in stamens and carpels of *Sophora* flowers and in stamens of *Clianthus* flowers, a trace expression of *STAP1* was detected in carpels in one of four replicates. Expression of *AP1* homologues in floral organs other than sepals and petals has been reported for few other species. In *Eucalyptus,* weak expression of the *EAP1* gene was detected in anthers (Kyozuka et al., 1997). Similarly, *BoiAP1* from broccoli was expressed in the stamen primordia (Carr and Irish, 1997). The other A-class gene, *SQUA* from *Antirrhinum,* is often expressed in carpels and non-floral organs (Huijser et al., 1992; Jofuku et al., 1994). On the other hand, absence of *AP1* homologue expression in the outer whorls has also been described in apple, in which the *MdAP1* gene expressed exclusively in sepals during flower development (Kotoda et al., 2000). Given that the trace expression of *STAP1*was over 1000-fold lower than that in other expressed organs, and that the expression was detected in only one of the four replicates, it could be safely considered as arising from contamination during sample preparation or other RT-PCR steps. If this is the case, both *STAP1* and *CMAP1* expressed exclusively in sepals and petals. This suggests that *STAP1* and *CMAP1* expression was not only more similar to that of *AP1* than *SQUA*, but its expression was typical of *AP1*.

In *Arabidopsis*, the two functions of the *AP1* gene in flower meristem specification and floral organ specification are believed to be separate. During the very early floral stages, *AP1* activity is required to specify floral meristem identity. At later floral stages, *AP1* activity is required to specify the identity of floral organs (Mandel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994). These two separate roles of the *AP1* gene were very clearly demonstrated for its homologue in *Sophora*, in which the *STAP1* gene

was characterized by a bimodal expression pattern. This bimodal expression pattern of *STAP1* coincided with the reproductive growth cycle of *Sophora*. The floral initiation and floral organ development were temporally separated by a long period inactivity of floral buds in autumn and early winter. The first expression peak was detected during floral initiation and the second peak appeared during floral organ development after six months of its down regulation. Despite considerable temporal separation between floral commitment stages in *Sophora*, the present study strongly suggested that the *AP1* homoloque in this woody perennial legume has similar functions to *AP1* in both floral meristem specification and floral organ development.

The bimodal expression pattern of *AP1* homologues observed in this study has also been reported in two other woody species. In *Actinidia,* Walton et al. (2001) observed that the first peak of *AAP1* expression occurred in first-order bud development (spring of first year), and the second peak occurred ten months later. In *Metrosideros excelsa*, two expression peaks of *MESAP1* were detected in autumn and spring (Sreekantan et al., 2004), with 1-2 months down regulation during flower bud dormancy. Although the bimodal expression cycle was completed in two years time for *AAP1* in *Actinidia,* and within one year for *MESAP1* in *Metrosideros,* the two expression peaks in both cases were consistent with the floral meristem initiation and floral organ development stages, which were very similar to the *Sophora STAP1* gene observed in this study. However, in contrast to *STAP1,* for which a low expression level is maintained during late summer and autumn, a down regulation occurred during winter season for both *MESAP1* and *AAP1*. These are coincident with the flower bud dormancy period of each species.

The role of *AP1* in both floral meristem identity and floral development was clearly demonstrated by the temporal separation of *Sophora* floral organ initiation and development, and by the bimodal expression pattern of *STAP1*. In inflorescence primordia and early stage inflorescences (<2 mm), *STAP1* was significantly up-regulated before the immergence of floral meristem, and remained high during floral meristem and floral organ initiation. However, *STAP1* was down regulated and kept at low level during the long rest period of floral development and up-regulated again when floral organ resume their development. The dramatic up-regulation of *STAP1* during floral initiation and organ development strongly suggest that *STAP1* in *Sophora* plays similar role to *Arabidopsis AP1* gene in floral meristem identity and floral organ development (Coen et al., 1990;

Weigel et al., 1992; Huijser et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994; Parcy et al., 1998). This provides a further strong support for the functions of *AP1* homologues in floral meristem identity in annual herbaceous model species.

AP1 acts downstream of *LFY* to specify meristem identity, and can be induced very early by *LFY* during the formation of any primordia that could potentially become a flower (Weigel et al., 1992; Mandel et al., 1992). The role of activating *AP1* by *LFY* was supported by the current study. In *Sophora* and *Clianthus,* the expressions of *LFY* and *AP1* homologues followed a very similar temporally sequential pattern at early and mid-stages of floral development. Furthermore, *STLFY* and *CMLFY* expressions were always immediately followed by those of *STAP1* and *CMAP1,* respectively. This is consistent with the role of *LFY* in directly activating *AP1* after meristem identification. The closely related activation time of *LFY* and *AP1* in wild type *Arabidopsis* plants implied that regulation of *AP1* by *LFY* could be direct (Simon et al., 1996; Hempel et al., 1997). In *Arabidopsis,* the onset of *API* expression was delayed in *lfy* mutants, indicating that *LFY* is a positive regulator of *API,* which is consistent with molecular and genetic evidence provided by previous gain-of-function experiments (Liljegren et al., 1999). The direct binding of *LFY* protein to the *API* promoter observed by Parcy et al. (1998) provided further support for the role of *LFY* in activating *AP1* directly.

On the other hand, the role of *LFY* in identifying floral meristem and in activating *AP1* was obscured for its homologue in *Clianthus.* No *CMAP1* gene activity was detected in normally developing inflorescences initiated in late spring, summer and early autumn, although a low expression level of *CMLFY* was detected. This, together with the fact that *STLFY* and *CMLFY* also expressed at low level in all of the tested non-floral tissues in this study, suggests that roles of *LFY* in identifying floral meristem and in activating *AP1* might be restricted until its expression accumulates to a certain level, or might need other co-factors with which to work. This seems to conflict with the general understanding of the role of *LFY* in directly activating *AP1.* However, in the majority of experiments carried out so far to substantiate the direct role of *LFY* in activating *AP1,* high levels of *LFY* expression or over expression of transgenic *LFY* gene constructs were used (Simon et al., 1996; Parcy et al., 1998; Liljegren et al., 1999). There has been no direct evidence that low expression levels of *LFY* could directly activate *AP1.* In contrast, *AP1* might play

a more important role in defining and identifying the floral meristem, because significant expression of *STAP1* and *CMAP1* were detected only after floral initiation.

For both *Sophora* and *Clianthus*, low expression level of *AP1* homologues, *STAP1* and *CMAP1*, was detected in early inflorescence stages. Hempel et al. (1997) demonstrated the utility of *AP1* expression as an indicator of floral determination in *Arabidopsis*, as its expression could be detected in the meristem only after floral induction. This marker seemed to work well for the *AP1* homologue in *Actinidia deliciosa* (Walton et al., 2001). If this were also the case for *STAP1* and *CMAP1*, the commitment of floral meristem determination should be completed before any floral primordia become visible in the lateral axils of inflorescences. Yanofsky (1995) observed a similar phenomenon in *Arabidopsis thaliana* in which the expression *AP1* occurred prior to visible morphological changes, and before the establishment of the floral meristem.

Results from the current study showed that *AP1* homologues, *STAP1* and *CMAP1*, were required during the whole process of floral commitment, from before the floral meristem became visible until the opening of flower buds, although the highest expression was detected in early stage flower buds (1-2mm in length and earlier). During late stages of flower development, when the expression of *LFY* homologues almost disappeared from the flower organs, *STAP1* and *CMAP1* activity could still be detected. This suggests that the activity of the *AP1* homologue is not restricted to the early steps of determining meristem identity alone. The gene is also involved in later stages of flower development. A similar situation was described by Kyozuka et al. (1997) for *EAP1*, the *AP1* homologue in *Eucalyptus*, which was expressed at all stages of floral morphogenesis. It seems that *STAP1* plays an important role in organ development in mid- and late stages, not only because high expression levels were detected during these stages, but also because of the down-regulation of *STAP1* in floral buds with differentiated floral organs during the dormancy period, and its up-regulation again when flower buds resumed their development and during the rapid elongation of sepals and petals. It might be possible that the up-regulation of *STAP1* was again activated by *STLFY* because the expression of the latter increased temporarily, and sharply declined after *STAP1* expression dramatically increased and kept at a high level. Given the very similar expression characteristics of *CMAP1* to that of *STAP1* in late stage, the same important role of *CMAP1* in the maintenance of late-stage floral organ development could be proposed despite there being no apparent temporal separation of the floral organ developmental process.

Both *Sophora* and *Clianthus PI* homologues, *STPI* and *CMPI,* expressed exclusively within floral tissue, in petals and carpels throughout their development. Their activity was not detected in any tested vegetative tissues, the seed pod or the first- and fourth-whorl flower organs, even using the powerful real-time RT-PCR technique. This is consistent with the idea that *STPI* and *CMPI* are the *PI* homologues in *Sophora* and *Clianthus*, respectively. This result also supported current thinking that the B class genes, including homologues of the *Arabidopsis* loci *PI* and *AP3,* are the most conserved floral homeotic gene family for their function in the determination of petal and stamen identity across diverse angiosperm species of both eudicots (Goto and Meyerowitz, 1994; Theissen and Saedler, 1995; Irish and Kramer, 1998) and monocots (Whipple et al., 2004; Friedman et al., 2004), and even across some gymnosperm species (Theissen and Becker, 2004).

However, the expression patterns of *STPI* and *CMPI* conflicted with a considerable number of the studies of *PI/AP3* homologues. In some species of lower eudicots including those in the Fumariaceae, Ranunculaceae, and Papaveraceae, although expression of B class gene homologues in the stamens is conserved, their expression patterns in the petals differ from those found in the model species (Kramer and Irish, 1999; Jaramillo and Kramer, 2004). Furthermore, they are often found to be expressed in carpels and in sepals in a few cases, suggesting that the function of *AP3* and *PI* homologues as B-class organ-identity genes is not rigidly conserved among all angiosperms (Kramer and Irish, 2000). All these data together suggest that considerable variation in B-function regulation exists even among the eudicots (Vandenbussche et al., 2004).

The current result of *STPI* and *CMPI* also provided the most detailed expression profiles of *PI* homologues in a woody species in which the flowering characteristics are markedly different from those in annual herbaceous species. The only reports of quantitative expression pattern of *PI/AP3* homologues in angiosperm woody species to date are for *MdPI* in *Malus domestica* (Yao et al., 2001) and *BpMADS2* in *Betula pendula* (Jarvinen et al., 2003). Furthermore, the expression studies of these B class genes were carried out using only semi-quantitative RNA blotting. No detailed study for their precise expression in floral organ development has yet been reported.

STAG and *CMAG* activity was detected through all developmental stages of flower buds but was not detected in any tested vegetative tissues and inflorescence primordia. Within the flower bud, *STAG* and *CMAG* expressed exclusively in stamens and carpels. This is consistent with most *AG* homologues reported in a wide range of angiosperm species

including that of woody angiosperm species such as *BpMADS6* in *Betula pendula* (Lemmetyinen et al., 2004), *MdMADS15* in *Malus domestica* (van der Linden et al., 2002), *CaMADS1* in *Corylus avellana* (Rigola et al., 2001), and *LAG* in *Liquidambar styraciflua* (Liu et al., 1999). This highly conserved *AG* function can even extend to gymnosperm species, such as *Cycas edentata* (Zhang et al., 2004) and *Picea* spp. (Tandre et al., 1995; Rutledge et al., 1998). However, Brunner et al. (2000) detected expression of an *AG* homologue, *PTAG*, in vegetative buds, stems and leaves of *Populus trichocarpa*, in addition to its normal expression in stamens and carpels.

Moreover, both *STAG* and *CMAG* expressed in young seed pods. Although the *AG* gene has been suggested to function in the late patterning of carpel structures, apart from early specification of stamen and carpel identity (Lohmann and Weigel, 2002), extended expression of *AG* homologues to post-pollination has only been observed in strawberry (*Fragaria* x *ananassa*) developing fruit (Rosin et al., 2003). Together with the facts that no *STAG* and *CMAG* activity was detected in any tested vegetative tissues and inflorescence primordia, it could be suggested that *STAG* and *CMAG* are homologues of *Arabidopsis AG* gene in *Sophora* and *Clianthus,* respectively, and that their functions are consistent with that of *AG* in terminating floral meristem and specifying organ identification in whorls 3 and 4 (Bowman et al., 1989; Yanofsky et al., 1990; Mizukami and Ma, 1992), while with some variation in post-flowering development of seed pods.

The role of *STPI* and *STAG* in specifying floral organs was more clearly demonstrated than for their counterparts in *Arabidopsis* and other annual model species. *STPI* and *STAG* expression was detected as early as floral organogenesis, but kept at a low level before rapid elongation of floral organs. In *Sophora,* these two developmental stages were separated by five to six months dormancy, during which there was no or little activity in the advance of developmental stage. Although it would be reasonable that the B class genes continue to be expressed during stamen and petal development after their specification (Bowman et al., 1991; Jack, 2001), this evidently separate expression pattern of *PI* and *AG* homologues has not yet been observed in any other reports, including woody species that have a significant dormant period. In fact, little is known regarding the expression details of *PI* and *AG* homologues in eudicot woody species.

Apart from their role in specifying floral organs, *PI* and *AG* homologues in *Sophora* may presumably also act directly on further development and rapid elongation of floral organs, or they could be directly responsible for activating other co-factors and down-stream

genes with which to work to control organ development. In fact, Zik and Irish (2003) identified 47 genes likely to be regulated directly or indirectly by *PI/AP3* in petal and/or stamen development using microarray analysis, representing about 25% of the *Arabidopsis* genome. Therefore, there might be some 200 genes that are affected by *PI/AP3* activity (Scott et al., 2004).

The relative expression level of *CMAG* was markedly higher than that of *CMPI* in the early stage of floral organ development. This supports the previous microscopic observation of *Clianthus* floral ontogenesis where initiation and organogenesis of carpels were before that of stamens, and the petal development was much delayed compared to that of carpels and stamens (see Chapter 3). As a result, the proportion of tissue where *CMAG* expressed should have been higher than that where *CMPI* does. In this context, the expression levels of *CMPI* and *CMAG* relative to their highest expression levels seem to be comparable. However, the expression differences between *STPI* and *STAG* were less obvious although the similar developmental situation of floral organs was microscopically observed in *Sophora.*

The activation of B- and C-class genes is generally believed to be the direct result of *LFY* activity or the co-operation of *LFY* and *AP1* (Weigel and Meyerowitz, 1993; Parcy et al., 1998). *LFY* acts to regulate the expression of *AG* via direct transcriptional activation (Parcy et al., 1998; Busch et al., 1999). Furthermore, *LFY* acts both directly and indirectly, via *AP1*, to regulate B-class genes such as *AP3* (Ng and Yanofsky, 2001a; Lamb et al., 2002). However, the role of *LFY* and *AP1* homologues in activating *PI* and *AG* homologues in *Sophora* was obscured in the current study by the incomplete coincidence of the expression patterns between the former and the latter. Very low levels of *STPI* and *STAG* expression were detected during the first expression peak of *STAP1* and *STLFY*, while both *STPI* and *STAG* increased drastically during the second expression peak of *STAP1* and *STLFY,* and reached their expression peaks when *STLFY* expression dropped to very low level (less than 10% of its peak expression level). Although there was no obvious temporal separation of expression during early developmental stages, a similar situation existed for *Clianthus* homologues *CMPI* and *CMAG* during later stages of flower development. This result does support the positive role of *LFY* and/or *AP1* in activating B and C class genes in annual model species. On the other hand, it suggests that this role might not be direct. Consequently, there should be some other factors involved in the up-regulation of the *PI* and *AG* homologues.

UFO is believed to have a major role in promoting B function as evidenced by the lack of normal petals and stamens in *ufo* loss-of-function mutants and the supernumerary petals and stamens observed in lines over-expressing *UFO* (Levin and Meyerowitz, 1995; Lee et al., 1997). Unfortunately, it was beyond the scope of this study to determine if *WUS* or other genes play roles in stimulating the massive expression of *PI* and *AG* homologues in *Sophora* and *Clianthus*.

In the current study, a two-step quantitative real-time RT-PCR protocol was established. Using this protocol, the expression differences in the range of 6-8-orders between different genes, and 3-4-orders between different stages of flower development and different tissue types of the same gene were effectively detected, suggesting that this protocol is a very powerful tool for precise gene expression studies. Another important advantage of this protocol in comparison with the semi-quantitative RNA blotting technique was that as little as 3-5 mg of fresh tissue sample is needed to quantify dozens of target and reference genes with adequate replicates, if combining the RNA micro-preparation protocol developed in this study. This is particularly useful to quantify gene expression at very early developmental stages, or when the availability of tissue sample is in some way limited for RNA blotting. In comparison with *in situ* hybridization, this protocol can provide information of spatial profile and quantity of gene expression at the same time, apart from its ease of use and time effectiveness. Therefore, it could be a good alternative to *in situ* hybridization for spatial expression studies, on condition that the excision of different organs to be studied is practicable.

This study provided the first evidence of expression profile of all ABC class representative genes together with their upstream regulating gene *LFY* in a single study, including the annual model species such as *Arabidopsis*. It is also the first report of the detailed quantitative expression pattern of any floral homeotic genes in woody species using the real-time RT-PCR technique, and the first report of any floral homeotic gene information in woody leguminous species to date.

Chapter 6 Final discussion and conclusions

In the present study, a clear map of the current genetic status of *Clianthus* wild populations has been established using a combination of several of the most powerful and widely used molecular markers including AFLP, ISSR and RAPD. The genetic relationship was established not only for these populations and cultivars, but also for most individuals within each wild population. This is the first report regarding the detailed genetic relationship among all the remaining wild populations of this genus, and the results should provide useful information for species management and conservation strategy planning of this endangered plant. Results in this study also showed that the population in Kaipara Harbour that was recently recognized by Heenan (2000) as the only remaining wild population of *C. puniceus*, was not distinguishable, on a molecular basis, from *C. maximus* because it was consistently placed among *C. maximus* populations in the dendrograms based on the genetic distance revealed by all three marker systems.

The establishment of genetic relationships among New Zealand *Sophora* species seemed more problematic under the current conditions. Although overall a high level of genetic variation was detected and all species were distinguished at the species level, the genetic relationships among the six species in the *S. microphylla* complex remains to be illustrated. In this respect, the current result could not provide molecular support for the recent separation of five species from *S. microphylla*.

Results of postharvest treatments indicated that *Clianthus* has several features giving it potential as a commercial cut flower if appropriate postharvest treatment is applied. However, cut flowering shoots of *Sophora tetraptera* remained short lived in the vase despite using similar postharvest treatments as for *Clianthus*.

Detailed calendars of vegetative and reproductive growth, and of floral development were developed for *Clianthus* and *Sophora*, together with the detailed homologue gene expression patterns of the floral meristem identity gene *LFY*, and selected members of the ABC classes of floral organ identity gene, *AP1*, *PI* and *AG*. Contrasting behaviours of vegetative and reproductive growth, as well as gene expression patterns were observed between these two legumes, also contrasting with another native woody perennial plant studied in this laboratory, *Metrosideros excelsa* (Fig. 6.1).

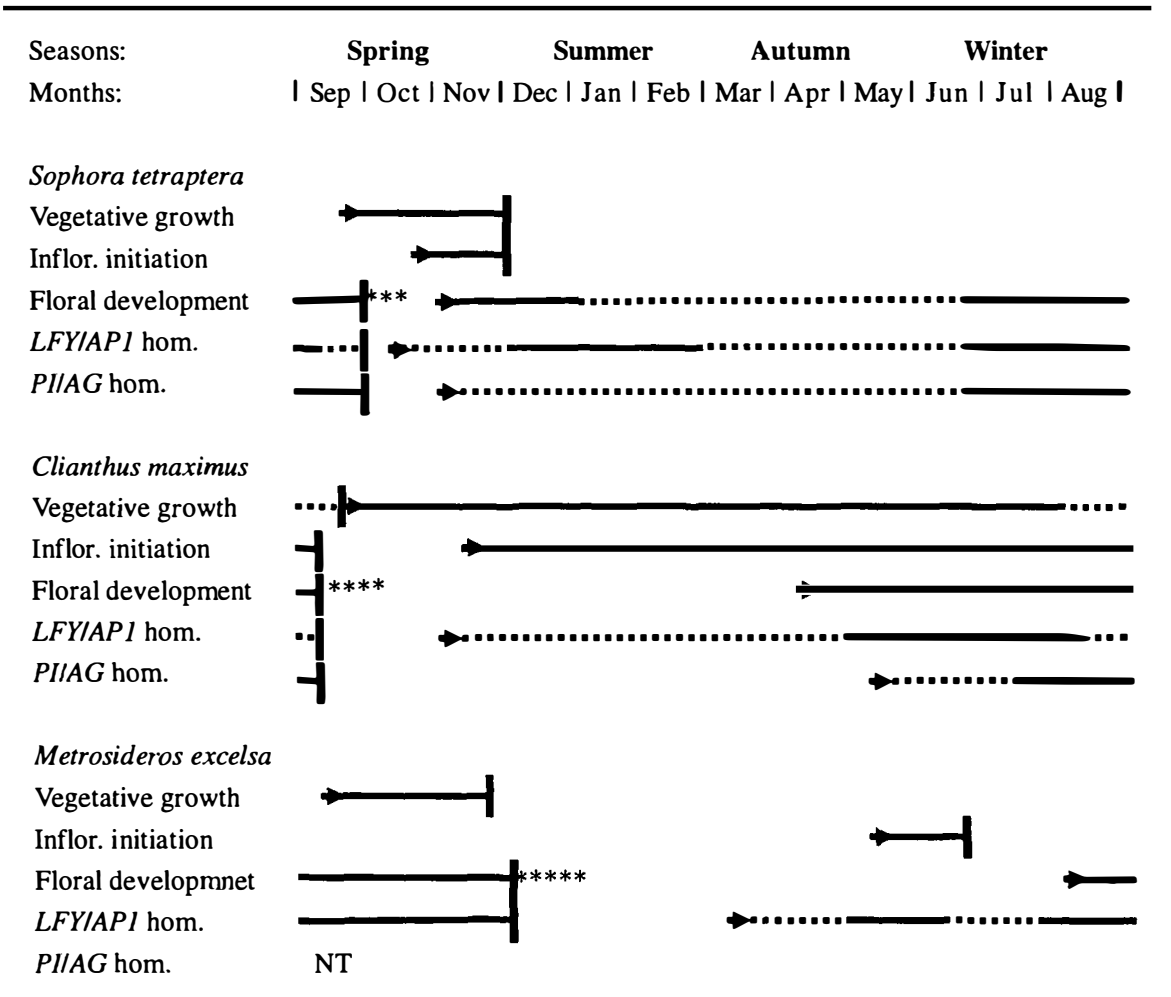

Figure 6.1 Summarised comparison of vegetative and reproductive development, and floral gene expression in *Sophora tetraptera*, *Clianthus maximus* and *Metrosideros excelsa*

→ starting point; | ending point; — high activity; ··· low activity; * flowering season; NT: not tested.

In *Metrosideros excelsa*, inflorescences are initiated during a short period in late autumn and early winter, followed by floral initiation and development from late winter, with a c. 1 month rest period between the two separate events (Sreekantan et al., 2001). Similarly, but in a variant of this sequence, *Sophora tetraptera* inflorescences are also initiated during a short period, in this case in late spring rather than in late autumn. Although inflorescence initiation is immediately followed by floral initiation and the onset of organogenesis, further development of floral organs in this species occurs only in early winter, after a 5-6 month rest period. There is, therefore, an interruption in the progress of

floral development in both species (*M. excelsa* and *S. tetraptera*): for c. 1 month in the former (between inflorescence and floral initiation), and for several months during floral organ development in the latter.

The bimodality of expression in the *LFY* and *AP1* homologues in *M. excelsa* (*MEL* and *MESAP1*) (Sreekantan et al., 2004) and in *STLFY* and *STAP1* in the current study, corresponds with peaks and troughs of inflorescence/floral initiation and floral development, respectively. This agreement between floral development events and *LFY* and *AP1* homologue expression suggests that both genes play important roles in floral meristem identity and floral organ identity as defined by the ABC model of floral development, despite the temporal separation of these two functions that necessarily cannot be observed in herbaceous model species, such as Arabidopsis. Based on this reasoning, one would expect to observe a contrasting expression pattern for *LFY* and *AP1* homologues in *Clianthus maximus*, because inflorescences are initiated from spring to winter without interruption, and floral organogenesis and floral organ development occurs as a continuous event, coincident with inflorescence initiation in mid-autumn. As one would predict, no bimodality of expression in *CMLFY* and *CMAP1* is observed. Moreover, low *CMLFY* expression occurs during the long period of inflorescence initiation, and *CMAP1* expression coincides with the onset of floral organ development. Where these two events occur synchronously, in late autumn and winter, both of these two genes achieve their highest expression (Fig. 6.1).

It is as yet unknown why floral development events are interrupted in *S. tetraptera*, and yet occur continuously in *C. maximus*. It was suggested that the temperature regime to which the plants are subjected during organogenesis could have an effect on the balance between floral and vegetative development in *Metrosideros* (Sreekantan et al., 2001). Henriod et al. (2000) also showed that a cool temperature regime (10-12°C) was required to avoid flower bud abscission and resumption of vegetative development of the terminal inflorescence bud in the same species. However, this does not appear to be the case for *Sophora* and *Clianthus*, because inflorescence initiation occurred in spring or during most of the year in these two species.

The interrupted organogenesis and floral organ development in *Sophora* and the continual inflorescence initiation in *Clianthus* were as also contrasting with most other reports in spring flowering woody species. For example, in *Prunus persica*, inflorescence and floral

initiation and development occurred continually during late autumn and winter (Reinoso et al., 2002). Lamp et al. (2001) also observed the flower initiation and organogenesis of *Prunus dulcis* in mid- to end of August, without obvious interruption of organogenesis and floral organ development.

Interestingly, low *STPI* and *STAG* expression was observed during the long period of summer and autumn arrest in floral development in *S. tetraptera*. Both of these genes and those of *CMPI* and *CMAG* were highly expressed during the period of rapid floral organ development in late winter-spring in both *Clianthus* and *Sophora* (Fig. 6.1).

The unusual order of floral organ initiation and development observed by microscopy was perfectly consistent with the expression pattern of *PI* and *AG* homologues in both species. In *Sophora*, the precocious carpel initiation and delayed petal development was reflected by the simultaneous expression of *PI* and *AG*, especially during the early stage of organogenesis (Fig. 6.1, Fig. 3.11, Fig. 5.25). This situation was even more clearly demonstrated in *Clianthus*, in which the precocious initiation of carpels and delayed petal initiation, and especially the early development of carpels compared to all other floral organs, was reflected by the higher *AG* than *PI* expression during early stages of organogenesis (Fig. 6.1, Fig. 3.8, Fig. 5.26). In the order of floral organ initiation and development defined by the ABC model, *AG* is expected to express later, and at a lower level than *PI* because the volume of petals plus stamens (*PI* expression territory) would always be higher than that of carpel plus stamens (*AG* expression territory), especially in the early stages of organogenesis and floral development. However, direct comparison of these results with other species is not possible because there has been no other report of a detailed and quantitative study of expression during floral development for homologues of these two genes to date, even not in the herbaceous model species.

The spatial expression patterns of all the tested floral gene homologues were consistent with those of the herbaceous model species *Arabidopsis*. Together with their high sequence similarities to those of *Arabidopsis* and most homologues in other species, it is strongly suggested that the ABC model of floral development is generally applicable to both species. Variations of their temporal expression patterns from those observed in herbaceous species, and from each other, are explicable on the basis of the timing and order of developmental events occurring through the annual calendar.

This is the first report of a study of floral identity gene expression in woody legumes, and

among the few studies reported within the whole family Fabaceae. It is also the first report of the detailed quantitative expression patterns of a whole set of *LFY* plus ABC class genes homologues in the same species using the real-time RT-PCR technique, including herbaceous model species, such as *Arabidopsis*.

An efficient two-step quantitative real-time RT-PCR protocol for detailed expression analysis of large number of samples was developed in combining the non-specific DNA dye SYBR Green® with the LightCycler® instrument. Advances were also made in the choice of appropriate internal controls for gene expression quantification.

It should be noted that the molecular work in this study was carried out without any previous molecular information available in the species to be investigated and closely related species, and under an unfavourable regulatory regime: cloning of genes from New Zealand native species requires extended consultation with Māori. This could have hampered the progress of the study by limiting the use of one Sturt's Desert pea in review of the most widely used experimental methods. This was the case not only for the isolation of floral identity gene homologues (Chapter 4), but also for the RNA *in situ* hybridization. A significant effort was made to investigate the spatial expression of the floral identity genes (data not shown in this thesis). However, particular difficulties were encountered regarding the choice of hybridization probes since the most efficient and widely used strategy of using RNA probes was limited by current regulations. Despite the progress made using several alternative strategies, such as end labelled short oligonucleotide probes, the adaptation and optimization of the basic hybridization protocol, and an encouraging preliminary hybridization result, the expression patterns of these genes using *in situ* hybridization was not obtained because of the time frame of this thesis.

Future work could be carried out to investigate the spatial expression patterns of these floral identity genes using *in situ* hybridization, following the basic protocol established in the present study. It would be interesting to clone full-length genes of *CMLFY, STLFY, CMAP1, STAP1, CMPI, STPI, CMAG,* and *STAG* from *Clianthus* and *Sophora*, and conduct complementation studies in *Arabidopsis* mutants to validate the functions of these genes, although this would also be difficult under the current regulatory regime relating to New Zealand native species. It would also be interesting to conduct more microscopic studies for detailed floral ontogeny in order to verify the unusual initiation

order and the delayed petal development observed in the present study. Scanning electron microscopy (SEM) could be used for this purpose in order to discriminate different types of floral organ primordia at early stages.

Another area of future research would be to investigate the molecular mechanisms of the interruption of the progress of floral organogenesis by the long period of summer-autumn dormancy in *Sophora*, and the mass abortion of *Clianthus* inflorescences during most of the year. The proposed strategy for this purpose would be the detection of relevant genes involved before and after the application of selected environmental treatments (e.g., photoperiod and low temperature regime) using a microarray technique, followed by the investigation of their precise expression patterns using the real-time RT-PCR technique.

In the present study, a comprehensive knowledge of vegetative and reproductive growth behaviours, genetic status, and the molecular mechanisms of flowering has been gained in both *Clianthus* and New Zealand *Sophora* species, using a wide range of techniques including microscopy, molecular markers and real-time RT-PCR. Efforts toward the exploitation of these species as potential new cut flowers have also been made, fulfilling the requirement of the contract under which much of the research was pursued. This is the most comprehensive study reported for these species to date. The results obtained in this study provide important information for species conservation and management, particularly for the endangered *Clianthus*, and provide a better understanding for further studies of these and closely related species.

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Appendix 1. Summary of postharvest treatments of *Sophora tetraptera* cut flowers

A. Effect of sucrose holding solution treatment on vase performance

Sucrose (%)	Vase life		Open flowers	
	Days	Significance	%	Significance
0	4.25	a	35.0	b
1	4.00	a	36.5	b
2	4.50	a	32.0	b
4	4.75	a	37.5	b
8	4.25	a	36.0	b

B. Effect of sucrose pulse treatment on on vase performance

Sucrose (%)	Time (h)	Vase life		Open flowers	
		Days	Significance	%	Significance
0	24	5.50	a	38.5	b
8	4	5.25	a	35.5	b
8	24	6.00	a	39.0	b
16	4	5.75	a	40.5	b
16	24	6.25	a	42.0	b

C. Effect of 2 mM STS pulse treatment on on vase performance

Time (min)	Sucrose* (%)	Vase life		Open flowers	
		Days	Significance	%	Significance
0	0	4.50	a	52.5	b
30	0	4.25	a	50.5	b
60	0	4.00	a	46.0	b
120	0	4.00	a	40.5	b
0	2	4.75	a	50.5	b
30	2	4.50	a	52.0	b
60	2	5.00	a	55.0	b
120	2	5.25	a	53.5	b

* Sucrose in holding solution after STS pulse treatment.

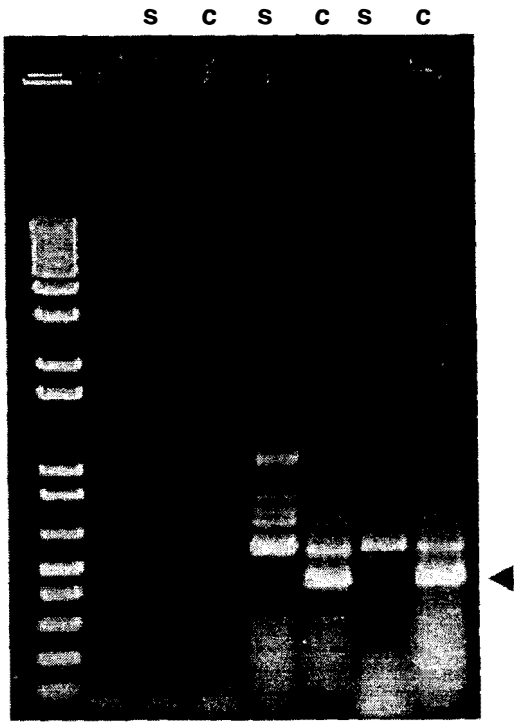
Appendix 2 Floral identity gene homologues used for sequence comparison and phylogenetic analysis of *Sophora* and *Clianthus* putative genes

Family	Gene	Species	Accession No.
LFY/ FLO	*AcLFY*	*Acacia caven*	AAS79826
	AFL1	*Malus domestica*	BAB83096,
	AFL2	*Malus domestica*	BAB83097
	ALF	*Actinidia deliciosa*	AAF01209
	BLFL	*Brownea leucantha*	AAS79851
	CFL	*Chrysanthemum lavandulifolium*	AAT84086
	ELF1	*Eucalyptus globulus*	AAC31359
	FLO	*Antirrhinum majus*	AAA62574
	GinLFY	*Ginkgo biloba*	AAF77075.
	LFL	*Lolium temulentum*	AAG41992
	LFY	*Arabidopsis thaliana*	NP200993
	MEL	*Metrosideros excelsa*	AF007869
	NEEDLY	*Pinus radiata*	AAB68601
	PTLF	*Populus trichocarpa*	AAB51533
	UNIFOLIATA	*Pisum sativum*	AAB88139
	ZFL1	*Zea mays*	AAO43175
A class	*AAP1*	*Actinidia deliciosa*	AF181664
	AP1	*Arabidopsis thaliana*	Z16421
	BOAP1	*Brassica oleracea*	CAA86024
	CsAP1	*Citrus sinensis*	AAR01228
	DcMADS1	*Daucus carota*	CAC81068
	EAP1	*Eucalyptus globulus*	AAG24909
	LpMADS1	*Lolium perenne*	AAO45873
	MdMADS2	*Malus domestica*	AAC83170
	MESAP1	*Metrosideros excelsa*	AAD01421
	NAP1	*Nicotiana tabacum*	AAD01421
	PEAM4	*Pisum sativum*	AJ291298
	PTAP1-1	*Populus trichocarpa*	AAT39554
	PTAP1-2	*Populus trichocarpa*	AAT39556
	RAP1B	*Oryza sativa*	BAA94342
	SQUA	*Antirrhinum majus*	CAA45228
	VvMADS6	*Vitis vinifera*	AAP32475

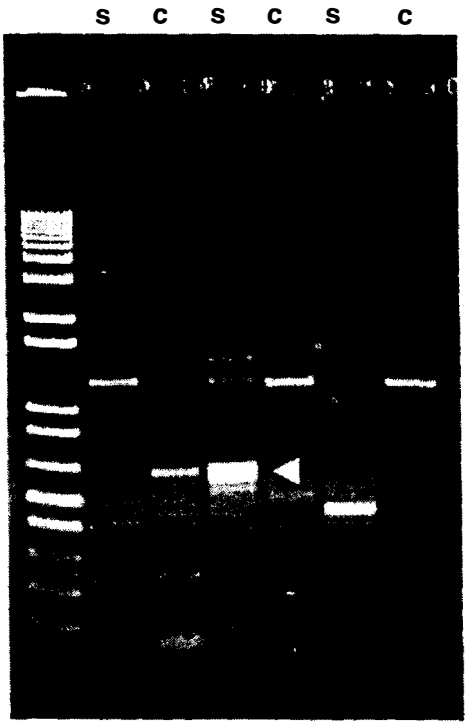
B class	*AP3*	*Arabidopsis thaliana*	NM115294
	DaPI	*Delphinium ajacis*	AF052862
	DEF	*Antirrhium majus*	X62810
	FBPI	*Petunia hybrida*	Q03488
	GLO	*Antirrhinum majus*	X68831
	HaPI	*Helianthus annuus*	AY157725
	LrGLO	*Lilium regale*	BAB91552
	MdPI	*Malus domestica*	AJ291490
	NGL9	*Medicago sativa*	AAK77938
	OrcPI	*Orchis italica*	AB094985
	OsMADS4	*Oryza sativa*	L37527
	PEAM1	*Pisum sativum*	AAW29099
	PhPI	*Peperomia hirta*	AF052865
	PI	*Arabidopsis thaliana*	D30807
	RbPI-1	*Ranunculus bulbosus*	AF052859
	SvPI	*Syringa vulgaris*	AF052861
C class	*AG*	*Arabidopsis thaliana*	X53579
	BAG1	*Brassica napus*	AAA32985
	HaAG	*Helianthus annuus*	AAN47198
	HvAG1	*Hordeum vulgare*	AAL93196
	LAG	*Liquidambar styraciflua*	AAD38119
	MADS13	*Oryza sativa*	AAF13594
	MADS15	*Malus domestica*	CAC80858
	NAG1	*Nicotiana tabacum*	AAA17033
	PLE	*Antirrhinum majus*	AAB25101
	PTAG1	*Populus trichocarpa*	AF052570
	PTAG2	*Populus trichocarpa*	AAC06238
	RAG1	*Rosa hybrida*	AAD00025
	TAG1	*Lycopersicon esculentum*	AAA34197
	WAG	*Triticum aestivum*	BAC22939
	ZAG1	*Zea mays*	AAA02933

Appendix 3 PCR products of the floral identity genes, as indicated, generated from *Sophora* (s) and *Clianthus* (c) cDNA samples using degenerated primers

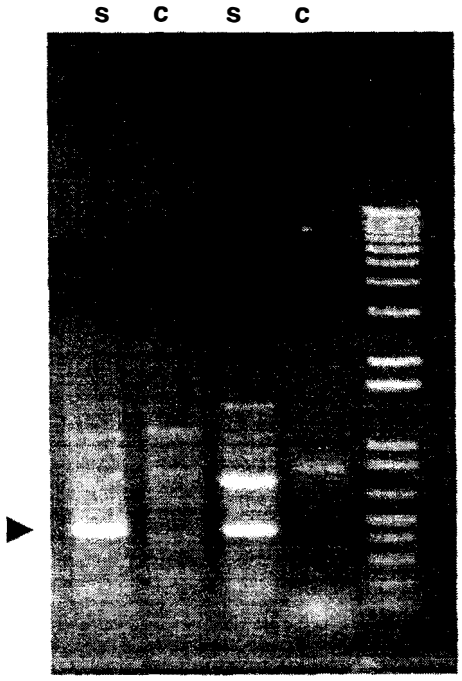
(Arrowheads show the bands to be isolated and sequenced)



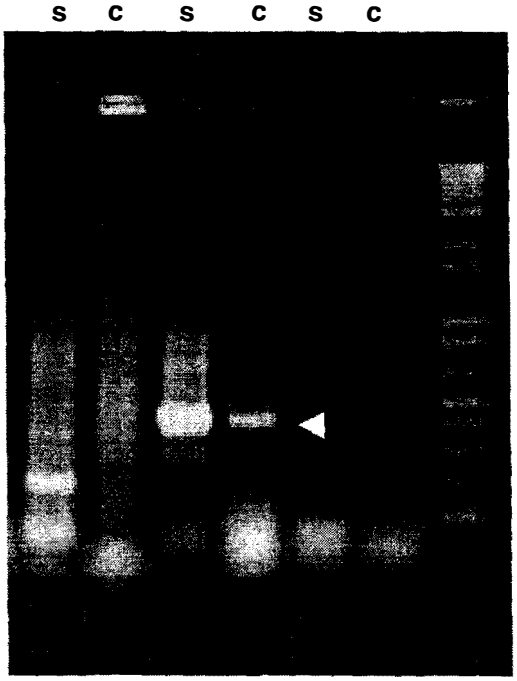
LFY



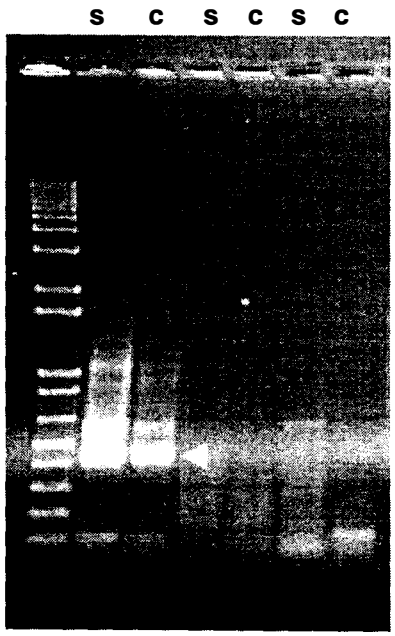
AP1



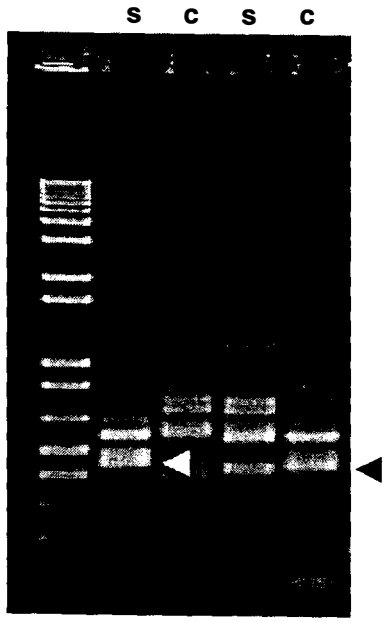
PI



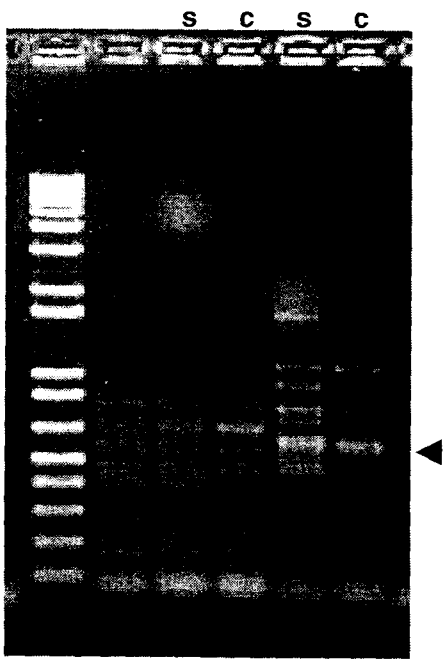
AG



18S



Actin



GAPDH

Appendix 4 Results of GenBank BLAST search using *CMLFY* aa sequence

Gene	Species	Identity (%)	Positive (%)	E value
UNIFOLIATA	*Pisum sativum*	97	98	3e-77
FLO/LFY-like	*Brownea leucantha*	96	98	3e-74
FLO/LFY-like	*Berlinia confusa*	96	98	3e-74
FLO/LFY-like	*Brownea ariza*	96	98	1e-73
FLO/LFY-like	*Cercis canadensis*	96	98	2e-73
FLO/LFY-like	*Ecuadendron acosta*	96	98	4e-73
FLO/LFY-like	*Elizabetha paraensis*	96	97	1e-72
FLO/LFY-like	*Cercis canadensis*	96	98	9e-71
FLO/LFY-like	*Bauhinia bohniana*	96	97	9e-71
FLO/LFY-like	*Lycopersicon esculentum*	95	99	2e-73
FLO/LFY-like	*Bauhinia syringifolia*	95	97	2e-72
FLO/LFY-like	*Salvia coccinea*	95	97	2e-73
FLO/LFY-like	*Citrus sinensis*	95	97	4e-73
FLO-like	*Mimulus ringens*	95	97	4e-73
FLO/LFY-like	*Apios americana*	95	97	5e-73
FLO/LFY-like	*Cercis canadensis*	95	97	6e-70
FLO/LFY-like	*Anthonotha macrophylla*	95	96	4e-70
FLO/LFY-like	*Macrolobium ischnocalyx*	95	96	4e-72
FLO/LFY-like	*Vitis vinifera*	94	98	5e-73
FLO/LFY-like	*Cedrela fissilis*	94	98	5e-73
NFL2	*Nicotiana tabacum*	94	97	9e-73
NFL1	*Nicotiana tabacum*	94	97	9e-73
FLO	*Antirrhinum majus*	94	97	1e-72
LFY-like	*Eriobotrya japonica*	94	97	2e-72
LFY-like	*Pseudocydonia sinensis*	94	97	2e-72
LFY-like	*Cydonia oblonga*	94	97	2e-72
LFY-like	*Pyrus pyrifolia*	94	97	2e-72
AFL2	*Malus domestica*	94	97	2e-72
FLO/LFY-like	*Eperua falcate*	94	97	3e-72
PTLF	*Populus balsamifera*	93	99	3e-72
FLO/LFY-like	*Hevea brasiliensis*	93	99	3e-72
LFY-like	*Populus tomentosa*	93	99	1e-69
FLO/LFY-like	*Salix discolor*	93	98	4e-72
ALF	*Petunia hybrida*	93	97	3e-72
FLO-like	*Paulownia tomentosa*	93	97	4e-72
LFY-like	*Chenopodium rubrum*	93	97	5e-72
LFY	*Arabidopsis thaliana*	93	96	4e-72
LFY-like	*Jonopsidium acaule*	93	96	4e-72

LFY-like	*Ionopsidium acaule*	93	96	4e-72
FLO-like	*Mazus reptans*	93	96	8e-72
AAL	*Acacia auriculiformis*	93	96	2e-71
TroLFY	*Trochodendron aralioides*	93	96	2e-71
FLO/LFY-like	*Apios americana*	93	96	4e-71
LFY-like	*Pyrus communis*	93	96	9e-71
FLO/LFY-like	*Dialium guianense*	93	96	6e-69
LFY-like	*Pyrus pyrifolia*	92	96	9e-71
FLO-like	*Eriobotrya japonica*	92	96	1e-70
FLO-like	*Pedicularis groenlandica*	92	96	5e-72
LEAFY	*Arabidopsis lyrata*	92	96	8e-72
LEAFY-like	*Leavenworthia crassa*	92	96	8e-72
FLO/LFY-like	*Senna alata*	92	95	7e-68
LFY-like	*Cucumis sativus*	92	94	9e-71
FLO-like	*Chelone glabra*	91	96	9e-71
FLO-like	*Syringa vulgaris*	91	96	7e-71
FLO-like	*Titanotrichum oldhamii*	91	95	1e-70
LFY-like	*Pseudocydonia sinensis*	91	95	3e-70
LFY-like	*Cydonia oblonga*	91	95	3e-70
LFY-like	*Eriobotrya japonica*	91	95	5e-69
FLO/LFY-like	*Delonix elata*	91	94	8e-67
FLO/LFY-like	*Umtiza listeriana*	91	94	1e-66
PlaraLFY	*Platanus racemosa*	90	97	2e-70
LFY-like	*Eudianthe coeli-rosa*	90	96	1e-70
LFY-like	*Eschscholzia californica*	90	96	1e-70
BOFH	*Brassica oleracea*	90	95	3e-70
LFY-like	Pyrus communis	90	95	1e-69
LFY-like	*Idahoa scapigera*	90	95	2e-69
AFL1	*Malus domestica*	90	95	3e-69
FLO/LFY-like	*Entada polyphylla*	90	95	2e-67
LFY-like	*Mangifera indica*	90	95	4e-62
LFY-like	*Idahoa scapigera*	90	94	6e-70
FLO-like	*Verbena officinalis*	90	94	1e-69
FLO/LFY-like	*Tamarindus indica*	90	93	5e-68
FLO/LFY-like	*Entada polyphylla*	90	94	3e-67
FLO/LFY-like	*Acacia caven*	89	95	5e-66
FLO/LFY-like	*Delonix elata*	89	95	1e-65
FLO/LFY-like	*Berlinia confusa*	89	93	2e-67
LEY/FLO-like	*Eucalyptus grandis*	89	94	2e-69
FLO/LFY-like	*Humboldtia laurifolia*	89	93	6e-62
LFY-like	*Syzygium samarangense*	88	94	5e-63
ELF1	*Eucalyptus globulus*	88	94	2e-68
FLO/LFY-like	*Scorodophloeus zenkeri*	88	93	9e-65
FLO/LFY-like	*Crudia gabonensis*	88	92	3e-64

FLO-like	*Leucocarpus perfoliatus*	87	92	3e-67
FLO/LFY-like	*Anthonotha macrophylla*	87	92	2e-66
PepspLFY	*Peperomia sp.*	86	93	2e-67
FLO/LFY-like	*Amherstia nobilis*	86	92	2e-63
MEL	*Metrosideros excelsa*	86	91	4e-36
OrcLFY	*Serapias lingua*	84	92	1e-63
FLO-like	*Mimulus lewisii*	84	91	2e-64
NymodLFY	*Nymphaea odorata*	84	90	3e-64
ZFL2	*Zea mays*	84	90	5e-63
FLO/LFY-like	*Schotia afra*	83	91	2e-63
FLO/LFY-like	*Schotia afra*	83	90	2e-63
ZFL1	*Zea mays*	83	89	1e-62
FLO/LFY-like	*Schotia afra*	83	90	3e-62
OrcLFY	*Orchis italica*	82	91	3e-62
Floral	*Oryza sativa*	82	90	7e-62
RFL	*Oryza sativa*	82	90	7e-62
LFY-like	*Hyacinthus orientalis*	81	91	4e-63
JunefLFY	*Juncus effusus*	81	90	9e-63
FLO/LFY-like	*Lolium temulentum*	80	88	2e-60
WelLFY	*Welwitschia mirabilis*	79	88	3e-61
GnegnLFY	*Gnetum gnemon*	78	87	5e-60
LFY-like	*Picea abies*	78	87	7e-60
FLO/LFY-like	*Pinus caribaea*	78	87	9e-60
PRFLL	*Pinus radiata*	78	87	9e-60
GpLFY	*Gnetum parvifolium*	78	86	3e-59
LFY-like	*Picea abies*	76	85	3e-58
NEEDLY	*Pinus radiata*	76	85	3e-58
ZamfuNdly	*Zamia furfuracea*	76	84	5e-58
GinNdly	*Ginkgo biloba*	76	84	4e-58
FLO/LFY-like	*Cryptomeria japonica*	75	85	3e-58
GinLFY	*Ginkgo biloba*	75	85	1e-58
ZamfuLFY	*Zamia furfuracea*	75	85	2e-58
BotdiFLO	*Botrychium dissectum*	74	85	7e-57
AtranFlo1	*Atrichum angustatum*	72	84	1e-54
WelNdly	*Welwitschia mirabilis*	72	82	8e-56

Appendix 5 Results of GenBank BLAST search using *STAP1* aa sequence

Gene	Species	Identity (%)	Positive (%)	E value
PEAM4	*Pisum sativum*	91	97	2e-56
APETALA1	*Arabidopsis thaliana*	80	91	7e-49
AP1-like	*Daucus carota*	78	93	3e-50
SQUA	*Antirrhinum majus*	78	92	7e-51
PTAP1-1a	*Populus balsamifera*	78	91	3e-44
AP1-like	*Vitis vinifera*	77	93	8e-48
MADS-MC	*Lycopersicon esculentum*	77	92	5e-46
MADS3	*Betula pendula*	77	91	5e-48
PTAP1-1	*Populus balsamifera*	77	91	1e-47
MADSC-2	*Sinapis alba*	77	91	5e-47
Boi2AP1	*Brassica oleracea*	77	91	8e-47
Saap1	*Sinapis alba*	77	91	3e-46
BOAP1	*Brassica oleracea*	77	89	2e-46
AP1-like	*Malus domestica*	76	90	9e-48
Boi1AP1	*Brassica oleracea*	76	90	4e-47
CDM111	*Chrysanthemum morifolium*	76	89	2e-47
euAP1	*Corylopsis sinensis*	75	92	5e-45
PTM2	*Populus tremuloides*	75	91	1e-46
euAP1	*Heuchera americana*	75	90	8e-46
PTAP1-2	*Populus balsamifera*	75	89	1e-46
SAP1	*Sali discolor*	75	89	1e-47
MADS5	*Betula pendula*	74	92	4e-47
MADS box	*Capsicum annuum*	74	91	6e-46
HAM75	*Helianthus annuus*	74	89	1e-46
APETALA1	*Citrus sinensis*	74	88	5e-46
MADS 2	*Malus domestica*	73	92	9e-46
NAP1-2	*Nicotiana tabacum*	73	91	1e-46
MADS5	*Nicotiana tabacum*	73	91	3e-46
PpMADS6	*Prunus persica*	73	91	1e-45
FBP29	*Petunia hybrida*	73	91	4e-46
PTM1	*Populus tremuloides*	73	90	1e-46
MADS1	*Nicotiana sylvestris*	73	89	2e-44

MADS11	*Nicotiana tabacum*	73	89	3e-44
HAM92	*Helianthus annuus*	73	88	3e-45
CDM41	*Chrysanthemum morifolium*	73	88	1e-40
euAP1	*Phytolacca americana*	72	91	1e-46
MADS26	*Petunia hybrida*	72	91	2e-44
MADS2	*Nicotiana sylvestris*	72	90	1e-44
EAP1	*Eucalyptus globulus*	71	91	6e-44
AP1-like	*Petunia hybrida*	71	90	2e-44
SLM5	*Silene latifolia*	71	88	5e-46
AP2L	*Eucalyptus globulus*	71	88	3e-45
NAP1-1	*Nicotiana tabacum*	71	88	3e-44
MADS 6	*Vitis vinifera*	71	88	4e-44
EAP2S	*Eucalyptus globulus*	71	88	5e-43
euAP1	*Paeonia suffruticosa*	71	87	5e-43
CDM8	*Chrysanthemum morifolium*	69	89	1e-41
POTM1-1	*Solanum tuberosum*	67	88	3e-42
MADS4	*Betula pendula*	68	87	5e-42
SLM4	*Silene latifolia*	67	90	7e-44
MADS1	*Lolium perenne*	67	86	9e-40
GSQUA1	*Gerbera hybrid*	67	84	1e-39
AP1-like	*Oryza sativa var japonica*	66	86	2e-40

Appendix 6 Results of GenBank BLAST search using *CMPI* aa sequence

Gene	Species	Identity (%)	Positive (%)	E value
PEAM1	*Pisum sativum*	93	99	2e-47
HaPI	*Helianthus annuus*	82	91	7e-41
GGLO1	*Gerbera hybrid*	82	91	7e-42
PMADS2	*Petunia hybrida*	81	93	2e-41
CDM86	*Chrysanthemum morifolium*	81	90	3e-41
MADS 1	*Litchi chinensis*	80	94	4e-41
PI-like	*Hydrangea macrophylla*	80	89	3e-30
MADS 26	*Cucumis sativus*	79	93	9e-42
NGL9	*Medicago sativa*	79	92	5e-40
GLOBOSA	*Antirrhinum majus*	78	91	1e-41
PI-like	*Meliosma dilleniifolia*	78	88	1e-30
DePI-1	*Dicentra eximia*	77	92	2e-34
PI-like	*Eupomatia bennettii*	77	92	2e-29
PI-1	*Calycanthus floridus*	77	91	1e-28
PI-1	*Lindera erythrocarpa*	77	91	3e-29
MdPI	*Malus domestica*	77	91	1e-38
PI-like	*Elaeis guineensis*	77	90	3e-39
FEGI	*Elaeis guineensis*	77	90	6e-39
MADS 9	*Phalaenopsis hybrid*	77	90	2e-3
MADS10	*Phalaenopsis hybrid*	77	90	9e-38
MADS 15	*Phalaenopsis hybrid*	77	90	3e-37
PI-like	*Betula pendula*	77	88	5e-37
PI-like	*Eucalyptus grandis*	76	91	2e-39
AtPI	*Akebia trifoliata*	76	89	2e-39
TdPI	*Thalictrum dioicum*	75	90	4e-38
PI-like	*Asimina triloba*	75	90	7e-29
PI-like	*Lilium regale*	75	89	2e-37
MADS	*Lilium regale*	75	89	8e-37
PI-like	*Ribes sanguineum*	75	89	7e-29
SLM2	*Silene latifolia*	75	88	4e-39
NTGLO	*Nicotiana tabacum*	74	91	2e-36
GLO1	*Petunia hybrida*	74	91	3e-36
SvPI-1	*Syringa vulgaris*	74	90	3e-34
PnPI-1	*Papaver nudicaule*	74	88	2e-36
BsPI	*Brasenia schreberi*	74	87	1e-37
PI-like	*Rosa rugosa*	74	85	3e-35
PI-like	*Alpinia hainanensis*	73	90	4e-37
FBP1	*Petunia*	73	90	9e-35
PI-like	*Chloranthus spicatus*	73	89	5e-34
PI-like	*Nuphar japonica*	73	88	8e-37

HAM31	*Helianthus annuus*	73	88	6e-38
PI-3	*Cimicifuga racemosa*	72	90	1e-30
PI-like	*Nymphaea tetragona*	72	89	2e-36
PI-like	*Nuphar japonica*	72	89	2e-36
MADS	*Tulipa gesneriana*	72	88	4e-36
PI-2	*Drimys winteri*	72	88	2e-28
PI-like	*Saruma henryi*	72	88	2e-28
PI-like	*Euryale ferox*	72	88	9e-36
PI/GL●-like	*Orchis italica*	72	87	7e-35
DaPI-1	*Delphinium ajacis*	71	91	2e-31
PI-like	*Triticum aestivum*	71	88	1e-36
PI-like	*Arabidopsis lyrata*	71	88	6e-36
PI-like	*Spinacia oleracea*	71	86	9e-36
PI-like	*Commelina communis*	71	87	1e-35
PI-like	*Arabidopsis thaliana*	71	87	9e-36
PI-like	*Musa acuminata*	70	89	6e-33
PI-like	*Amborella trichopoda*	70	88	9e-34
PI-like	*Tradescantia reflexa*	70	87	9e-34
RMADS219	*Oryza sativa*	69	89	5e-36
PI-like	*Cabomba caroliniana*	69	88	8e-35
PI-like	*Aristolochia manshuriensis*	69	88	4e-31
PI-like	*Agapanthus praecox*	69	88	4e-36
PI-like	*Brassica juncea*	69	88	1e-35
PI-like	*Tradescantia reflexa*	69	86	1e-33
PI-like	*Thalictrum dioicum*	69	84	3e-33
PI-like	*Zea mays*	68	88	1e-35
MADS2	*Hyacinthus*	67	88	2e-35
PI-like	*Eranthis hyemalis*	67	87	4e-29
HPI2	*Hyacinthus orientalis*	66	88	7e-34
MADS1	*Oryza sativa*	66	88	8e-31
PI-like	*Oryza sativa*	66	87	8e-34
PI-like	*Sagittaria montevidensis*	66	85	2e-31
PI-like	*Asparagus officinalis*	65	89	3e-34
PI-like	*Zea mays*	65	86	1e-33
PI-like	*Daucus carota*	65	87	4e-33
MADS-	*Houttuynia cordata*	65	79	4e-31
PI-like	*Oryza sativa*	64	83	3e-33
PI-like	*Hordeum vulgare*	63	88	3e-33
PI-like	*Zea mays*	63	85	2e-32
MpMADS7	*Magnolia praecocissima*	58	85	2e-29
GbMADS4	*Ginkgo biloba*	55	82	2e-29

Appendix 7 Results of GenBank BLAST search using *CMAG* aa sequence

Gene	Species	Identity (%)	Positive (%)	E value
CMB1	*Cucumis sativus*	79	84	6e-3
NAG1	*Nicotiana tabacum*	78	86	8e-31
HAM59	*Helianthus annuus*	77	84	1e-30
pMADS3	*Petunia x hybrida*	76	85	4e-30
TAG1	*Lycopersicon esculentum*	76	83	3e-29
GAGA2	*Gerbera hybrid*	76	82	2e-29
AG homlogue	*Asparagus virgatus*	74	86	2e-28
AG homlogue	*Populus balsamifera*	74	86	5e-28
AG homlogue	*Vitis vinifera*	74	84	4e-29
GAG2	*Panax ginseng*	74	83	8e-29
AG homlogue	*Gossypium hirsutum*	74	83	1e-26
AG homlogue	*Betula pendula*	73	85	2e-25
PpMADS4	*Prunus persica*	73	84	7e-27
SLM1	*Silene latifolia*	73	84	1e-26
PLE	*Antirrhinum majus*	72	86	8e-28
AG homlogue	*Populus balsamifera*	72	86	2e-26
MADS1	*Corylus avellana*	72	84	2e-25
AG homlogue	*Rosa rugosa*	72	84	4e-28
AG homlogue	*Liquidambar styraciflua*	72	84	2e-26
AG homlogue	*Malus x domestica*	71	82	6e-26
AG homlogue	*Spinacia oleracea*	71	79	2e-26
CsaAG	*Camelina sativa*	70	86	1e-27
MADS	*Malus x domestica*	70	85	8e-29
GfAG3	*Guillenia flavescens*	70	83	9e-27
MpMADS2	*Magnolia praecocissima*	70	82	3e-27
GfAG2	*Guillenia flavescens*	70	83	1e-26
AG homlogue	*Cucumis sativus*	70	82	4e-26
CUS1	*Cucumis sativus*	70	82	5e-26
Me341	*Beta vulgaris*	70	80	7e-26
GAGA1	*Gerbera hybrid*	70	80	5e-29
CrAG	*Capsella rubella*	69	86	1e-26
CbpAG3	*Capsella bursa-pastoris*	69	86	1e-26

AG	*Arabidopsis thaliana*	69	86	5e-29
CbpAG1	*Capsella bursa-pastoris*	69	86	2e-26
CbpAG2	*Capsella bursa-pastoris*	69	86	2e-26
LpAG	*Lepidium phlebopetalum*	69	85	1e-26
AG homlogue	*Nymphaea sp.*	69	84	3e-26
GfAG1	*Guillenia flavescens*	68	86	1e-26
TaAG2	*Thlaspi arvense*	68	86	2e-27
TaAG1	*Thlaspi arvense*	68	86	7e-27
TAGL1	*Lycopersicon esculentum*	68	83	1e-27
CDM37	*Chrysanthemum morifolium*	68	79	8e-27
HAM45	*Helianthus annuus*	68	78	4e-26
AG homlogue	*Helianthus annuus*	68	77	8e-26
MADS1b	*Crocus sativus*	67	86	4e-27
AG homlogue	*Hyacinthus orientalis*	67	86	1e-25
EsAG2	*Eruca sativa*	67	86	7e-27
EsAG1	*Eruca sativa*	67	86	9e-27
CsAG1	*Coronopus squamatus*	67	86	2e-26
CsAG2	*Coronopus squamatus*	67	86	2e-26
BAG1	*Brassica oleracea*	67	86	3e-26
MpMADS11	*Magnolia praecocissima*	66	82	4e-26
EsAG3	*Eruca sativa*	65	86	4e-26
AG homlogue	*Zea mays*	65	82	2e-26
GbMADS2	*Ginkgo biloba*	63	81	8e-26