DISCUSSIONS
CHAPTER 5
DISCUSSION

5.1. Overexpression of LEAFY gene from Arabidopsis thaliana in Brassica juncea causes early flowering

In an attempt to observe the effect of overexpression of the LEAFY gene from A. thaliana in B. juncea, the LFY gene under the control of CaMV35S promoter in sense (pSL) and antisense orientation (pASL) was transformed into B. juncea. It was observed that during the initial phase, regeneration of plantlets from hypocotyls transformed with the pSL construct was slower as compared to those transformed with the pASL construct. The frequency of transformation was also lower in hypocotyls transformed with the pSL construct as compared with the pASL construct. The impact of overexpression of the LFY gene was clearly distinguishable on flowering in various transgenic plants vs. untransformed control plants. In the T0 generation, untransformed control plantlets flowered even after 100-120 days in culture whereas those having the LFY gene in sense orientation flowered in 45-60 days. The transgenic plants with the antisense construct behaved similar to the untransformed control plants and flowered in 100-120 days. Flowering in tissue culture was also reported for 35S::LFY aspen transgenic plants and lines which expressed in lower levels could only be transferred to soil (Weigel and Nilsson, 1995). Amongst the transgenic plantlets grown in culture, some of those which did not flower showed early flowering by 7-10 days on transfer from laboratory to land conditions as compared to the untransformed control plants. From these experiments, it is clear that overexpression of LFY gene from A. thaliana leads to early flowering in a heterologous system, B. juncea.

These results are in conformation with earlier reports by Weigel and Nilsson (1995), He et al. (2000), Rottmann et al. (2000) and Pena et al. (2001) who also showed early flowering in tobacco, aspen, rice, poplar and citrus by overexpressing LFY gene from Arabidopsis thaliana.

All the transgenic plants showed the presence of the transgene in Southern blot analysis confirming stable transformation of B. juncea. The copy number of transgene varied from one to three copies in the sense plants while all the antisense transgenic lines.
had a single copy of the transgene. The northern blots showed a message size consistent with the fusion transcript. This was absent in the untransformed control plants. However, the endogenous *LFY* transcript could be detected in all the transgenic plants as well as the untransformed control plants. In the T1 generation, plants overexpressing the *LFY* gene, early flowering (7-10 days) were observed as compared to the control untransformed plants. This shows that the *LFY* gene is stably overexpressed leading to early flowering.

The yield of seeds from the pSL transgenic plants was similar to the untransformed control plants as ~8 seeds per siliqua were obtained. This is significant as the naturally occurring cultivars of *B. juncea* which flower earlier (eg. TN-1 which flowers 5-10 days earlier than Varuna) are accompanied by yield losses. In the present study, while all the other morphological, vegetative and reproductive characters remained unchanged in the transgenic plants of Varuna, there was early flowering with no yield penalty. This could result in ‘genetic enhancement’ of *B. juncea*.

As regards the morphology of T0 transgenic plants, barring three lines, there was no difference between the transgenic vs. untransformed control plants. In the transgenic lines M3 3/7, M1523/7, O617/7, some abnormal floral structures were formed (Fig.16) at the basal portion of the shoot. These structures were hard and lacked proper reproductive organs. This could probably be due to the insertion of the *LFY* gene at a location in the genome where some essential functions are disrupted. Further experiments are required to confirm this. In T1 generation transgenic plants also, a single plant showed an altered morphology as it was short and bushy with abnormal leaf structures. This could also result from insertion of the T-DNA at a location where essential genes are present. Developmental alterations have also been reported in transgenic *Arabidopsis*, *Citrus* and *Populus* constitutively expressing the *LFY* gene. In *Arabidopsis* complete conversion of all the lateral shoots into single flowers and ultimately transformation of the main shoot into an aberrant terminal flower has been reported. In some cases, terminal flower formation occurred only after three true leaves were formed but these plants were sterile (Nilsson and Weigel, 1997). In poplar smaller, deformed leaves and bushier growth with frequent branching and shorter internodes were observed in some lines constitutively expressing *LFY* gene (Rottmann et al., 2000) and in rice it was accompanied with panicle abnormality (He et al., 2000). The *Citrus* plants overexpressing the *LFY* gene had small

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and curled leaves and reduced number of thorns (Pena et al., 2001). Further investigations are required to find out the cause for the morphological abnormalities observed in the few \textit{LFY} \textit{B. juncea} transgenic plants.

5.2. \textit{LEAFY} is expressed in both vegetative and reproductive phase but expression increases before flowering

To determine the involvement of \textit{BjLEAFY} in the developmental programme of \textit{B. juncea}, temporal and spatial expression in different organs during the vegetative and reproductive phases was studied.

The \textit{LFY} gene expression was detected in all the four floral organs although there were differences in the level of expression. This is similar to the expression pattern observed in \textit{A. thaliana} and \textit{Antirrhinum majus} (Weigel et al., 1992; Coen et al., 1990). The expression of the \textit{LFY} gene in the floral organs of \textit{B. juncea} reflects their role in establishing floral organ identity like the other \textit{FLO/LFY} genes.

The \textit{LFY} transcript was detected in stem, leaf, bracts and siliques but not in root. The \textit{BOFH} gene from \textit{B. oleracea}, \textit{ELF} gene from \textit{Eucalyptus}, \textit{AFLI} gene from apple and \textit{NFL} genes from tobacco have not been reported to express in leaf, stem or root (Anthony et al., 1993; Southerton et al., 1998; Wada et al., 2002).

Most species for which expression of \textit{LFY} genes has been investigated have simple leaves and where \textit{LFY} expression occurs in leaves, it is low and restricted to early phases of leaf development (\textit{A. thaliana}; Blazquez et al., 1997, \textit{N. tabacum}; Kelly et al., 1995, \textit{Impatiens} species; Pouteau et al., 1997). An interesting observation by Lee et al. (1997) who reported the formation of lobed leaves in transgenic \textit{Arabidopsis} plants overexpressing \textit{UNUSUAL FLORAL ORGANS (UFO)} gene and this phenotype required the presence of functional \textit{LFY} gene. Therefore, \textit{LFY} could indirectly be involved in controlling the morphology of simple leaves. In the present investigation, the \textit{LFY} transcript in the leaves of \textit{B. juncea} was abundant during the initial one week of growth and decreased during the vegetative growth. It showed an increase during the transition phase (~26-34-day-old) from the vegetative to the reproductive phase and decreased slightly when reproductive development started. The modulation of \textit{LFY} gene expression
during the vegetative to reproductive development in *B. juncea* suggests that it has an important role to play. An in-depth study is needed to support this.

The *LFY* gene expression was detected very early in the shoot apices of *B. juncea* during the vegetative phase. The level of the transcript was more abundant in the early flowering cultivar, TN-1 followed by Varuna. The expression in PNMB and RNBL cultivars which are late flowering was very less in the early stages of growth (10 – 24 days after germination) but subsequently increased with age. The appearance of the *LFY* transcript much earlier and at higher levels in early flowering cultivates indicate that the threshold level required for floral transition is attained faster in these as compared to the late flowering ones. Though the *LFY* and *FLO* genes are expressed in the floral apices, the *NFL* transcript could be detected in the vegetative apices also (Kelly et al., 1995).

Although, the expression studies of *LFY* gene of *B. juncea* showed that it is expressed throughout the life cycle of the plant, there is a variation in the amount of transcript at different developmental stages and in different organs. Despite the differences in expression pattern, these expression studies indicate that *BjLFY* could be involved in regulating floral meristem identity and flowering time as in *LFY/FLO* homologs from other systems. Detailed *LFY* expression through *in situ* hybridizations in the leaf and floral primordia would give an idea about the specific regions of its activity and thus help in elucidating its functions. The expression pattern of *LFY* in *B. juncea* was different from its closely related species *A. thaliana* and *B. oleracea*, though there was high level of sequence similarity amongst them. Isolation and characterization of the promoter and cis regulatory elements of the *LFY* gene from *B. juncea* may provide explanation for the variation in transcript profiles. The higher level of transcripts could be due to the presence of multiple copies of *LFY* gene in *B. juncea*.

### 5.3. Flowering and *LEAFY* gene expression is controlled by various factors in *Brassica juncea*  
#### 5.3.1. Sucrose

Bernier (1988) proposed that floral evocation and morphogenesis can be caused by application of carbohydrates and different plant growth regulators. Sucrose has long been recognized as a floral promoting substance. Therefore, work done on *in vitro* flowering in
various species by different workers (Dickens and van Staden, 1988, Dielen et al., 2001) involved the use of higher concentrations of sucrose to produce flowering.

*In vitro* grown *B. juncea* seedlings flowered earlier when 4% - 5% sucrose was included in MS medium as compared to the seedlings grown on MS medium supplemented with 1%, 2% and 3% sucrose. No flowering occurred with 1% and 2% sucrose concentrations in MS medium although. The *LFY* gene expression was almost similar at all the concentrations. These experiments suggest that sucrose promotes flowering in *B. juncea* probably by acting as an inducer of *LFY* but a threshold concentration of sucrose is probably required for achieving this.

Studies by Blazquez et al. (1998) showed that sucrose may be involved in the transcriptional regulation of the *LFY* gene. Addition of 1% sucrose in the growing medium of transgenic *LFY::GUS* plants of *A. thaliana* enhanced the expression of the transgene in the vegetative plants. Studies with *phosphoglucomutase 1 (pgm1)* mutants of *A. thaliana* which are deficient in starch biosynthesis showed that sucrose plays an important role in floral initiation (Caspar et al., 1985; Yu et al., 2000). Addition of sucrose to the growing medium of *co3* mutants complemented the mutation but this treatment was not effective in *ft-l* mutants (Roldan et al., 1999; Ohto et al., 2001). This suggested that sucrose might act downstream of *CO* in photoperiodic pathway, but upstream or parallel to *FT*.

5.3.2. Hormones

5.3.2.1. Gibberellic acid

The role of gibberellins (GA) in flowering has been implicated since a long time (Langridge, 1957). Gibberellins are involved in regulation of flower development in *Arabidopsis* (Wilson et al., 1992; Putterill et al., 1995; Blazquez et al., 1998). Expression of *LFY::GUS* is reduced in mutants defective in GA biosynthesis and increased in mutants with constitutive GA signaling (Blazquez et al., 1998; Melzer et al., 1999). The activation of the *LFY* gene by GA appears to be sucrose dependent (Blazquez et al., 1998). A GA response element has been identified in *Arabidopsis LFY* promoter (Blazquez and Weigel, 2000). While GA generally induces flowering in many LD and cold requiring rosette plants, it inhibits flowering in many other plants (Bernier, 1988).
The effect of gibberellin on regulation of flowering time has already been discussed (see review of literature).

In this investigation the *LFY* gene expression in seedlings grown on different concentrations of gibberellic acid decreased with the increase in concentration. The role of GAs in flowering is complex and in case of *B. juncea* it inhibits the expression of the *LFY* transcript but whether it is a direct or indirect target cannot be said from these preliminary studies.

5.3.2.2. Cytokinins

In the present study, the *LFY* gene expression was found to increase with increasing concentrations of BAP (0.5 to 2.0 mg l⁻¹) and kinetin (0.5 to 2.0 mg l⁻¹). The present study with cytokinins indicates that it has a promotive effect on the expression of the *LFY* gene in *B. juncea*.

Exogenous cytokinins have been reported to cause promotion or inhibition of flowering in a variety of species, although promotive effects are much more frequent than inhibitory ones. It is also a requirement in many *in vitro* systems (reviewed in Bernier, 1988). The effect of cytokinin on flowering depends on the concentration and timing of the treatment. Role of cytokinins in the control of floral transition is suggested by the fact that changes in endogenous level were observed during floral transition in various species and increased concentration in apex appears to occur in all LDP, SDP and day-neutral plants (Kinet et al., 1993). Studies on *S. alba* indicate cytokinins as constituents of the floral signal transported in the phloem to the apex (Bernier et al., 1993). Different cytokinins promoted floral transition in *uniflora* mutant of tomato (Dielen et al., 2001). Cytokinins are also reported to increase in abundance in both mature leaves and phloem sap at the end of the inductive long days in *Arabidopsis* (Corbesier et al., 2003). Endogeneous cytokinins might play a role in the control of cell division during the floral transition in *Arabidopsis* (Corbesier and Coupland, 2005). However, little is known about their action on flowering gene expression in *Arabidopsis*, although in *S. alba*, cytokinins activate the expression of *SaMADSA*, the ortholog of *SOC1* (Bonhomme et al., 2000).

Further studies need to be done to establish the relationship between the putative floral signals such as sucrose, cytokinins and gibberellic acid. Effect of other factors like
Abscisic acid, auxins, ethylene and salicylic acid and the interplay among all of these will reveal the nature of the floral stimulus in *B. juncea*. The analysis of the *LFY* promoter from *B. juncea* will also help in deciphering whether the transcriptional control of these regulators is direct or indirect and which proteins might act as mediators between these and the *LFY* gene.

**5.4. The protein encoded by the *BjLEAFY* is significantly similar to other *FLO/LFY* homologs**

A cDNA (1261bp) was cloned from a cDNA library of *B. juncea* cv. Pusa Bold and was named as *BjLEAFY*-P. Another cDNA (1290 bp) was isolated from the cultivar, Varuna by RT-PCR and referred to as *BjLEAFY*-V. *BjLFY*-P encoded for a truncated protein due to a single base deletion at 762 bp while *BjLFY*-V encoded a full-length protein of 420 amino acids. The deletion of the base which created a premature stop codon might be due to discrepancies in the PCR reaction or a naturally occurring mutation. *lfy* mutants of *A. thaliana* have been reported which have new stop codons and missense mutations and the severity of the mutant phenotype depended on the molecular lesions in different alleles (Weigel et al., 1992). A strong allele, *lfy*-7 arose in a T-DNA insertion mutagenesis had a single base pair change which caused a premature stop codon (Feldmann, 1991). *Eucalyptus* has three homologous genes in the genome, but two of them have stop codons in their coding region and are therefore not expressed (Southerton et al., 1998).

The putative protein encoded by *BjLFY* in this study, shares a number of sequence motifs with a *LFY/FLO* like proline rich region near the amino terminus and a highly acidic region in the centre of the protein which are thought to be characteristic of plant transcription factors. These were present in the variable regions (low similarity) of the FLO/LFY proteins. The *B. juncea* LFY protein was evolutionary more closely related to the LFY proteins from *A. thaliana* and *B. oleracea* as was expected.

In this investigation, it was shown putative phosphorylation sites are present in the encoded protein and this suggests the possible involvement of phosphorylation/dephosphorylation events that might regulate the activity of LFY. The PTLF protein, a homolog of LFY from poplar species, showed altered migration during
gel electrophoresis. Post-translational modifications such as phosphorylation were thought to be responsible for it (Rottmann et al., 2000). Such post-translational modifications might affect LFY function. Presence of the binding site for SUMO (Small ubiquitin related modifier) protein - MKDE tetrapeptide motif in the sequence indicates that the BjLFY protein might be a SUMO modified protein. SUMOylation appears to influence the function of proteins in distinct ways—for example, by altering their cellular location, their activity, or their stability by antagonizing their degradation via ubiquitination and the 26S proteasome in mammals, yeast and Drosophila (Melchior, 2000; Muller et al., 2001). Similar roles for SUMO in plant protein modification have not been described. ESD4, which regulates the expression of the floral repressor FLC (Reeves et al., 2002) has been shown to processes the precursor of Arabidopsis SUMO (Murtas et al., 2003).

Southern blot analysis of B. juncea genomic DNA using the BjLFY-V cDNA as a probe gave multiple bands. B. juncea is an amphidiploid species derived from B. rapa and B. nigra (U, 1935). The multiple signals obtained in B. juncea may probably be due to the presence of multiple copies of LFY in B. juncea genome inherited from each progenitor species or through the presence of these sites within the introns of LFY gene. In most plants for which LFY homologs have been determined, these LFY-homologous genes had a single copy in each genome (A. thaliana, A. majus, pea, tomato, petunia and poplar; Weigel et al., 1992; Hofer et al., 1997 Souer et al., 1998; Molinero-Rosales et al., 1999; Rottmann et al., 2000). In apple genome there are two homologous copies of LFY genes (AFL1 and AFL2) (Wada et al., 2002) and so also in tobacco (NFL1 and NFL2). Eucalyptus has three homologous genes in the genome, but two of them were found not to be expressed and had stop codons in their coding region (Southerton et al., 1998). P. radiata has two homologous genes, NEEDLY and PRFLL which are expressed during vegetative development and in male cones during reproductive development respectively (Mouradov et al., 1998; Mellerowicz et al., 1998).

5.5.1. Expression of CONSTANS gene in B. juncea
The expression of CONSTANS gene was monitored in 5-day-old seedlings of B. juncea grown in continuous light and transferred to continuous darkness, using a radiolabeled
heterologous *A. thaliana* CO cDNA as probe. The CO mRNA level increased after shifting the plants to darkness with a peak at 20 hr after transfer and decreased after 36 hr. Night break reduced the CO expression compared to transcript level in complete darkness. Similar expression pattern has also been reported in the short day plant *Pharbitis nil* (Liu et al., 2001). A high level of CO expression was observed only in the seedlings which received night break and seedlings exposed to 8 hr darkness and 6 hr light. CO expression in *B. juncea* is thus circadian regulated similar to CO in *Arabidopsis* and *P. nil* (Valverde et al., 2000; Liu et al., 2001).

5.5.2. Isolation of a CONSTANS homolog, *BjCOL1* from *B. juncea*

A cDNA (1033 bp), BjCOL1, encoding CONSTANS like 1 protein was isolated from *Brassica juncea* cv. Varuna by RT-PCR. This has a molecular weight 39 kDa and pI 6.1. Multiple sequence alignment revealed the presence of two B-box zinc finger motifs and CCT motifs characteristic of CO and COL proteins within the conserved regions. PSORT analysis indicated that it is a nuclear protein. The presence of the zinc finger motifs and the fact that it is a nuclear protein indicates that it is a transcription factor. The BjCOL1 protein also has asparagine and cysteine rich regions. Post-translational modifications might be required for BjCOL1 to function as the presence of phosphorylation and glycosylation sites have been indicated by Expasy softwares.

*Arabidopsis* is reported to have 16 CO-like genes but the functions of only COL1 and COL2 have been analyzed. All of them are circadian regulated. Unlike CO, overexpression of the COL1 and COL2 genes has very little effect on flowering time. Overexpression of COL1 though shortens the circadian rhythm period (Ledger et al., 2001; Robson et al., 2001).

Phylogenetic analysis using Phylip software at Genebee showed that BjCOL1 protein has a common origin with *B. napus* protein and closer to *B. nigra* protein also. As both *B. napus* (AACC) and *B. juncea* (AABB) have a common parent *B. rapa* (AA), probably this gene derived from *B. rapa* in both the species.